Focuses on the issues that are unique for NK cells in their capacity to enhance antibody and T-cell responses and to potentiate the antitumor immune responses. More importantly, NK cells can target the more aggressive forms of highly resistant cancers by targeting cancer stem cells and poorly differentiated tumor cells. Indeed, a wide variety of therapeutic approaches using NK cells are currently being tested in clinical trials including haploidentical NK cells, umbilical cord blood-derived NK cells, stem cell-derived NK cells, NK cell lines, cytokine-induced memory-like NK cells, and chimeric antigen receptor NK (CAR-NK) cells. Since tumors develop several mechanisms to resist attack by T and NK cells, the above adaptations for ex vivo activation, expansion, and genetic modification of NK cells have resulted in the increase in their antitumor activity and overcoming resistance. This book discusses the various approaches by which NK cells are being exploited for cancer immunotherapy, alone and in combination with other therapeutics, for both hematological and solid tumors.

The review chapters in this book are written by leading experts on NK immunotherapy worldwide and include chapters on challenges of NK-based therapies, challenges of NKT cell immunotherapy, expansion of supercharged NK cells in vivo withstanding inactivation by tumors, pluripotent stem cell-derived NK cells, CAR-NK cells, NK cells and angiogenesis, targeting metabolism to augment NK activity, NK cells and pathogenesis of cancers, etc.

Key features

- Discusses the unique developmental applications of NK immunotherapy against highly resistant primary and metastatic cancers, alone or in combination with other therapeutics.
- Provides up-to-date, highly relevant new information on cellular level as well as underlying molecular mechanisms, which are reviewed by leading researchers in the field.
- Provides a significant number of schematic diagrams for easy comprehension of complex systems.
- Is considered a reference book on the rapidly evolving new field of NK immunotherapy.
SUCCESES AND CHALLENGES OF NK IMMUNOTHERAPY
SUCCESSSES AND CHALLENGES OF NK IMMUNOTHERAPY
Breaking Tolerance to Cancer Resistance

Edited by

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Academic Press
An imprint of Elsevier
Cover Image Legend

Top image: Diagram showing the possible mechanisms utilized by human iNKT cells to attack tumor cells, including direct killing (A), adjuvant effects (B), and TAM inhibition (C). APC, antigen presenting cell; CTL, cytotoxic T lymphocyte; DC, dendritic cell; iNKT, invariant natural killer T cell; NK, natural killer cell; TAM, tumor-associated macrophage; TCR, T-cell receptor.

Bottom image: Schematic representation of the four stages of NK cell maturation in the peripheral blood of cancer patients. Peripheral blood NK cells are identified as the CD16brightCD56dimCD-69− subset and they are capable of mediating cytotoxicity, thus they are capable of lysing cancer stem cells (CSCs) and poorly differentiated tumors. This constitutes stage 1 of NK cell maturation. NK cells with the CD16lowCD56brightCD-69bright phenotype have low to no cytotoxic ability but increased capacity to secrete cytokines. These cells, coined as "split anergized NK cells," constitute the second stage of NK cell maturation. These cells are able to regulate the function of other cells and differentiate tumor cells, thus playing a major role in halting the growth of the tumors. If NK cells lose both cytotoxic function and cytokine secretion capabilities (seen at the preneoplastic stages of pancreatic cancer and in cancer patients), they enter the third stage of NK cell maturation. In this stage, the cells may aid instead of eliminate tumors due to increased secretion of proinflammatory cytokines such as IL-6 and IL-10 in the absence of functioning IFN-γ secretion. Finally, the fourth stage of NK cell maturation is apoptotic/necrotic NK cells because the fate of functionally unresponsive NK cells may ultimately be clearance by cell death. IFN-γ, interferon γ; MIP1α, macrophage inflammatory protein 1α; NK, natural killer cell; TLR, toll-like receptor; TNF-α, tumor necrosis factor α.
Aims and Scope

We have witnessed during the last decade the emergence of immunotherapy as an advanced and effective new therapeutic strategy in the fight against a variety of deadly metastatic and unresponsive human cancers. Several of the T cell-mediated immunotherapeutic modalities are individualized and restricted to certain cancers and are in general very expensive to use. In addition, such therapies can also be accompanied by toxic side effects, such as cytokine release syndrome. Furthermore, patients who have undergone chemotherapy and radiation treatments will not have adequate peripheral blood T cells to donate in order to generate engineered T cells. It is well recognized that CAR-T cells, thus far, were not effective against solid tumors.

NK cells, however, are the first line of defense against cancer cells. A unique property of NK cells is their capacity to enhance antibody and T cell responses and potentiate the antitumor response. More importantly, NK cells were shown to target the more aggressive forms of cancer by targeting cancer stem cells (CSCs) and poorly differentiated tumors. For the last 20 years, researchers have been investigating how to utilize NK cells to fight against the majority of cancers. The success has been limited due to the use of autologous NK cells with dysfunctional properties, and the lack of adequate numbers of highly functional NK cells. These shortcomings were resolved through novel approaches.

Hence, a wide variety of therapeutic approaches of NK cells are currently being tested in clinical trials including haploidentical NK cells, umbilical cord blood-derived NK cells, stem cells-derived NK cells, NK cell lines, cytokine-induced memory-like NK cells, and chimeric antigen receptor NK cells (CAR-NK). Further, methods to augment cytotoxicity and survival in vivo include cytokine-based agents, immune-check point inhibitors, etc. Since tumors develop several mechanisms to resist attack by T and NK cells, the above adaptations for ex vivo activation, expansion, and genetic modification of NK cells have resulted in the increase in their antitumor activity and overcoming resistance.

This book discusses the various approaches by which NK cells are being exploited for cancer immunotherapy, alone and in combination with other therapeutics, for both hematological and solid tumors.
About the Editors

Dr. Benjamin Bonavida, PhD, (Series Editor), is currently Distinguished Research Professor at the University of California, Los Angeles (UCLA). He is affiliated with the Department of Microbiology, Immunology and Molecular Genetics, UCLA David Geffen School of Medicine. His research career, thus far, has focused on investigations in the fields of basic immunochemistry and cancer immunobiology. His research investigations have ranged from the biochemical, molecular, and genetic mechanisms of cell-mediated killing and tumor cell resistance to chemotherapeutic drugs. The reversal of tumor cell resistance was investigated by the use of various selected sensitizing agents based on molecular mechanisms of resistance. In these investigations, there was the newly characterized dysregulated NF-κB/Snail/YY1/RKIP/PTEN loop in many cancers that was reported to regulate cell survival, proliferation, invasion, metastasis, and resistance. Emphasis was focused on the roles of the tumor suppressor Raf Kinase Inhibitor Protein (RKIP), the tumor promoter Yin Yang 1 (YY1), and the role of nitric oxide as a chemotherapeutic sensitizing factor. Many of the earlier mentioned studies are centered on the clinical challenging features of cancer patients’ failure to respond to both conventional and targeted therapies.

The Editor has been active in the organization of regular sequential international mini-conferences that are highly focused on the roles of YY1, RKIP, and nitric oxide in cancer and their potential therapeutic applications. Several books edited or coedited by the Editor have been published. In addition, the Editor has been the Series Editor of books (over 23) published by Springer on Resistance to Anti-Cancer Targeted Therapeutics. In addition, the Editor is presently the Series Editor of three series published by Elsevier/Academic Press on Cancer Sensitizing Agents for Chemotherapy, Sensitizing Agents for Cancer Resistant to Cell Mediated Immunotherapy, and Breaking Tolerance to Anti-Cancer Immunotherapy. Lastly, the Editor is the Editor-in-Chief of the Journal Critical Reviews in Oncogenesis. The Editor has published over 500 research publications and reviews in various scientific journals of high impact.

Acknowledgments

The series editor acknowledges the Department of Microbiology, Immunology and Molecular Genetics and the UCLA David Geffen School of Medicine for their continuous support. The series editor also acknowledges the assistance of Mr. Rafael
Teixeira, acquisitions editor for Elsevier/Academic Press, and the excellent assistance and support of Ms. Samantha Allard, editorial project manager for Elsevier/Academic Press, for their continuous cooperation throughout the development of this book.

**Dr. Jewett** is a professor and director of tumor immunology laboratory in the Division of Oral Biology and Medicine, and Weintraub Center for Reconstructive Biotechnology at the UCLA School of Medicine and Dentistry. She has membership in Jonsson Comprehensive Cancer Center (JCCC) and is a member of UCLA Tumor Immunology subgroup. She is well known nationally and internationally for her contribution to the field of NK biology, tumor immunology, and cancer immunotherapy. She has received a large number of honors and awards and holds memberships in many professional organizations and societies. She has chaired a number of important senate and nonsenate committees at UCLA and University of California Regents, and has been instrumental in shaping the graduate studies for the health professionals at UCLA. She chairs and teaches several graduate level courses, and her laboratory is sought out by many foreign and domestic scholars who spend several years to receive training in NK studies. She serves on the editorial board of many prestigious journals, and has been a reviewer on the board of National Institute of Health study sections. She holds several patents, and has given more than 200 invited lectures and presentations nationally and internationally and has published more than 140 high-impact journal articles, reviews, commentaries, and book chapters in the field of cancer. She has research collaborations with investigators from China, Slovenia, Mexico, Poland, Germany, Thailand, Japan, Portugal, South Korea, and Sweden to name a few. She has organized a number of conferences on cancer immunity nationally and internationally. She has trained more than 150 graduate students and health professionals in her laboratory, many of whom are leaders in their respective institutions. She has served on review panels for grants from many countries including England, France, the Netherlands, Qatar, Poland, and Israel to name a few. She has received several grants from NIH as well as from other sources for her studies.

One of Dr. Jewett’s major contributions to science and NK cell biology was the identification, characterization, and the establishment of the concept of split anergy in NK cells. Equally important was Dr. Jewett’s discovery demonstrating that NK cells were important for the elimination, selection, and differentiation of cancer stem cells as well as healthy stem cells. Most recently she has identified, characterized, and patented a novel technology to expand large numbers of supercharged NK cells that are in use in clinical trials of cancer patients. In addition, she has developed disease-specific formulations of probiotic bacteria to prevent and treat cancer patients in combination with supercharged NK cells.
Immunotherapy is fast becoming the method of choice for cancer therapy. Although remarkable advances have been made in the field of immunotherapy, we still have significant challenges and difficulties since many of the current immunotherapeutic strategies are only effective in certain cancers, do not provide long-lasting remissions, and are effective in only a fraction of the cancer patients with significant collateral damage. The main objective of this book is to bring latest approaches and novel strategies to adopt NK cells for immunotherapies against a variety of cancers with minimal toxicities and with more clinical responses than current treatments.

Since the identification of NK cells in the 1970s, NK cell biologists have been studying these cells tirelessly. We have not only learned about the phenotypic and functional attributes of these important cells, but also we have learned about the nature of the cells with which they interact, and how their function is modulated in health and disease, and what consequences there are when their function is compromised. Although we have gained significant knowledge in the field, there still remains a lot to be discovered about these cells.

We have long known about the important roles of NK cells in cancer therapy; however, only recently have we started to appreciate the scope of the significance of these cells for tumor therapy. The identification of CSCs or poorly differentiated tumors as targets of NK cells has finally shown the indispensable role these effectors have in successful therapy. In addition, many previous studies have demonstrated the dysfunctional nature of NK cells in cancer patients, indicating that successful therapy requires restoration of NK cell function in cancer patients. NK cells mediate successful tumor cell control by their direct cytolytic activity and/or antibody-mediated ADCC or through differentiating and limiting tumor growth by the secretion of IFN-γ. As a consequence of optimal functioning NK cells in the tumor microenvironment, it is likely that other cancer therapeutic strategies such as chemotherapy and radiotherapy will be more effective in eliminating tumors in cancer patients. Due to suppressive nature of the tumor microenvironment and systemic immunosuppression, the selection of allogeneic or autologous NK cell immunotherapy must be carefully considered especially in patients for whom the autologous NK cells are significantly damaged, and are thus, inferior in both expansion and function to allogeneic NK cells. In addition, strategies should be designed to allow maintenance of good NK expansion and function in vivo in cancer patients since NK cells are important in expanding other important cells such as cancer-specific CD8+ T cells, while limiting the expansion of TAMs, Tregs, MDSCs, MSCs, and fibroblasts which are the suppressors of NK and CD8+ T cells and thus are hallmarks of aggressive tumors.

To be successful in eradicating cancer, NK cells have to withstand the suppressive nature of the tumor microenvironment; therefore, expanded NK cells should be able to maintain their viability and function when
interacting with the tumor cells and other suppressive cells in TME. Such assessments can be made along with the NK treatment strategies by using 3D tumor pieces from the patients to culture with the engineered NK cells to ensure that these tumors do not induce either functional inactivation or cell death in the expanded NK cells. The effectiveness of such tests was shown in recent work when functionally competent NK cells were cultured with tumorospheres, and were shown to penetrate and lyse the tumors by confocal microscopy. Therefore, a set of diagnostic and prognostic tests should be designed to test the expandability and function of NK cells along with the patient treatment to safeguard against unwanted outcomes. In addition, large numbers of functional autologous or allogeneic NK cells can be combined with other immunotherapeutic strategies such as oncolytic viruses (OVs), ADCC-inducing antibodies, checkpoint inhibitors, CAR-T cells, CAR-NK cells, chemotherapeutic and radiotherapeutic strategies for the ultimate goal of tumor eradication.

The book consists of contributions from established leaders in the fields of NK biology and immunotherapy. The reviews address the latest advances in cancer immunotherapy using NK cells and by addressing the challenges and difficulties in breaking the tolerance within the tumor stroma as well as circumventing the function of inhibitory immune factors that tolerize the cytotoxic effectors in the tumor microenvironment. The reviews also address whether NK cells can circumvent the resistance of cancer cells to current therapies. This book also reviews the current clinical applications of classical NK immunotherapies as well as the adoption of universal therapeutic NK cell preparations.

Part I: NK cells: General properties (four chapters)

In chapter titled “NK cells and CD8 T cells in cancer immunotherapy: Similar functions by different mechanisms,” Drs. Dunai, Collins, Barao, and Murphy review the similarities and differences between NK and T cells and their manipulations in clinical settings. They also discuss the challenges that would be encountered for individual patient’s responses.

In chapter titled “Challenges for NK cell-based therapies: What can we learn from lymph nodes?”, Drs. Rethacker, Dulphy, and Caignard review the role of NK cells in the lymph nodes where they terminate their maturation and interact with myeloid immune cells. The authors reviewed their own data on the mechanism by which NK cells in the lymph nodes regulate the immune response against tumors and suggested that these NK cells are the target for immunotherapy. They further discussed the role of NK cells in tumor therapy when combined with different treatment strategies.

In chapter titled “NK cells and oncolytic viruses in cancer therapy,” Drs. Choong and Fong review the interaction of NK cells with oncolytic viruses in both preclinical and clinical studies. They review the reported literature on the combination of NK cells and oncolytic viruses for their potential therapeutic applications. They also discuss the role of exosomes in potentiation of NK cell and OV therapy.

In chapter titled “Successes and challenges of NKT cell immunotherapy: Breaking tolerance to cancer resistance,” Li, Lee, Zeng, and Yang review the application of the subset of iNKT cells in immunotherapy. This subset with NK and T cell properties is unique in its functions and potential
adaptation for cancer immunotherapy. The biology and importance of iNKT cells in immunotherapy are reviewed. Also, the preclinical and clinical applications of iNKT cells in cancer immunotherapies are summarized and discussed. The authors also present the various challenges encountered with the application of iNKT cells in the clinic.

Part II: Activation of NK cells
(four chapters)

In chapter titled “The role of peptidases and their endogenous inhibitors in the regulation of NK cell cytotoxicity,” Drs. Kos, Jewett, Pišlar, Jakoš, Senjor, and Nanut review the role of cathepsins in the regulation of immune responses including NK cells. In NK cells cathepsins regulate the secretion of granzymes and perforin and, thus, regulate the cytotoxic activity of NK cells, a process mediated by cystatin F. This endogenous cysteine peptide inhibitor is secreted in the TME and upon its internalization will induce NK split anergy of NK cells and decrease their cytotoxic activity. They concluded that the precise regulation of cysteine cathepsins in NK cells, their target cells, and bystander cells in the TME can significantly improve cancer immunotherapy.

In chapter titled “Novel strategies to expand supercharged NK cells with augmented capacity to withstand inactivation by tumors,” Drs. Jewett, Kos, Turnsek, Chen, Breznik, Senjor, Chovatiya, Kaur, and Ko review their own findings and those of the others in the various functional characteristics of NK cell subsets and their roles in cancer as well as their therapeutic potentials. They emphasize the significance of primary NK cells isolated from peripheral blood in targeting cancer stem cells (CSCs) and poorly differentiated tumors, providing the rationale for the use of NK cell immunotherapy in combination with T cell therapy, since T cells rely on peptides presented in the context of MHC class I on tumor cells which are found to be low or decreased on the surface of CSCs/poorly differentiated tumors. They review the role of NK cells in the differentiation of stem-like tumors. Various analyses are discussed that include in vitro analyses, knockout and humanized mice, and means of expanding NK cells for therapeutic purposes. Of interest, they discuss the allogeneic supercharged NK cells and their superiority to autologous NK cells in the treatment of cancer patients. They also provide new unpublished data on a 3D model of tumorospheres to study NK functions. They propose the use of combinations of supercharged allogeneic NK cells with other therapeutics such as CAR-T, CAR-NK, checkpoint inhibitors, and conventional chemotherapy.

In chapter titled “NK cell-mediated immunotherapy: The exquisite role of PGC-1alpha in metabolic reprogramming,” Drs. Gerbec and Malarkannan review the role of the immunosuppressive microenvironment metabolism in the regulation of NK functions. The NK cells must adapt to the metabolic changes in the TME to perform their functions, namely, cytokine production and cytotoxic activity. Therefore, metabolic reprogramming is essential for NK cells to perform their functions and the authors discuss the pivotal role of PGC-1alpha in the metabolic reprogramming of NK cells.

In chapter titled “IL-15 and IL-15Ra: Something old, something new, and something blue,” Dr. Laouar reviews the studies of IL-15 or IL-15 superagonists, consisting of IL-15 linked to IL-15Ra portions in activation of NK cells and their use with adoptive transfer of NK cells in cancer patients. She
further describes the potential use of NK cells with IL-15 in survival and expansion of NK cells in vivo. IL-15 also has many other immunoactivating effects on other immune cells and its use in vivo in cancer patients have had positive therapeutic effects. Therefore, the combination of engineered NK cells and IL-15 had been initiated in many clinical trials in cancer patients.

Part III: Induction of NK cells from stem cells (two chapters)

In chapter titled “Advances in pluripotent stem cell-derived natural killer cells for cancer immunotherapy,” Drs. Coffey, Yamamoto, and Kaufman review the rapidly evolving therapy with the adoptive cell therapy of NK cells. The authors describe the derivation of NK cells from human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs). They report the advantages of iPSCs over other NK cells for adoptive therapy as they have minimal constraints that are associated with other NK therapies. Indeed, the adoption of iPSCs and genetic engineered iPSCs have entered clinical trials to determine their therapeutic potential.

In chapter titled “Phenotypic plasticity: The emergence of cancer stem cells, collective cell migration, and the impact on immune surveillance,” Dr. La Porta reviews the heterogeneity of tumors and the existence of a large number of subclones with different properties including the phenotypes and metastatic behaviors. In particular, Dr. La Porta discusses the phenotypic plasticity of the tumor cells that regulates the immune response. She proposes that targeting the plasticity and the TME may overcome resistance, leading to a beneficial and successful therapy.

Part IV: CAR-NK cells in immunotherapy (two chapters)

In chapter titled “Using CAR-NK cells to overcome the host resistance to antibody immunotherapy and immune checkpoint blockade therapy,” Dr. Hu reviews the use of CAR NK cells for the therapies of cancers that are resistant to both antibody and cell-mediated immunotherapies. The NK cell impairment in cancer patients contributes to the resistance observed in these patients. The author reports his own studies with the tissue factor or CD-142 that recognizes CAR-NK cells and mediates ADCC via CD16 and also kills cancer cells via CAR. In addition, since CAR-NK cells express PD-1, treatment with anti-PD-1 will increase PD-1 positive immune infiltration in the TME and benefit both responder and nonresponder patients. In summary, it is suggested that CAR-NK cells in combination with antibody and checkpoint inhibitors will achieve a significant therapeutic outcome in cancer patients.

In chapter titled “CAR-NK cell immunotherapy: Development and challenges toward an off-the-shelf product,” Drs. Mansour, Teng, Lu, Barr, and Yu review the recent advances made with CAR T cell immunotherapies and describe the inherently observed limitations. Hence, they describe the advantages of CAR NK therapies, namely, safer with limited cytokine release syndrome and neurotoxicity, cheaper to manufacture, and have high potential for an off-the-shelf product for all cancer patients.

Part V: Targeting NK cells (six chapters)

In chapter titled “Regulation of NKG2D by RKIP: Implications on NK-mediated
cytotoxicity and cytokine production,” Drs. Galal, Zaravinos, and Bonavida review the relationship between the expression of RKIP and NK cytotoxicity. The expression of RKIP in cancers is downregulated and its upregulation inhibits tumor cells proliferation, growth, and metastasis and reverses resistance to chemo-immunotherapeutic drugs. The dysregulated MAPK pathway by RKIP is also involved in the regulation of the NK-activating receptor, NKG2D. The presence of a cross talk between RKIP and NKG2D was investigated and the data indicated the existence of such a cross talk in an inverse relationship. Bioinformatic analyses corroborated these findings and demonstrated that NK cells have a lower expression of RKIP and lower expression of NKG2D. Thus, targeting RKIP may result in the overexpression of NKG2D and a better antitumor response.

In chapter titled "Checkpoint inhibition in the fight against cancer: NK cells have some to say in it,” Drs. Sordo-Bahamonde, Lorenzo-Herrero, González-Rodriguez, and González review the successful immunotherapeutic strategies in a subset of cancer patients that are due, in part, to the immune checkpoint blockade (ICB) treatments. While the majority of the findings with ICB treatments were due to the activation of the antitumor cytotoxic CD8 T lymphocytes, the authors discuss the role of NK cells in the ICB treatment in addition to the CD8 T lymphocytes.

In chapter titled “The dual role of Natural Killer cells during tumor progression and angiogenesis: Implications for tumor microenvironment-targeted immunotherapies,” Drs. Mortara, Baci, Coco, Poggi, and Bruno review the dysfunctional roles of NK cells in cancer patients as a result of many factors such as decreased expression of natural cytotoxicity receptors, reduced expression of the NKG2D-activating receptor, impairment of degranulation, and reduced release of IFN-γ and TNF-α. They discuss the dual role of NK cells during tumor progression and angiogenesis by focusing on the TME-related factors that regulate NK functions.

In chapter titled “Immunometabolic targeting of NK cells to solid tumors,” Drs. Lupo and Matosevic review the impairment of NK cells by metabolic adaptations leading to dysfunctional NK cells. The NK cells under these metabolic conditions undergo new phenotypic and functional signatures. The authors highlight the role of metabolism in supporting NK functions and propose novel targeting strategies to restore their antitumor functions.

In chapter titled “Targeting metabolism to potentiate NK cell-based therapies,” Drs. Dhar and Wu review one mechanism underlying the defective NK cell functions in the tumor microenvironment in cancer patients. They discuss the important role of cell metabolism in regulating the functions of NK cells in both normal and disease conditions. They also postulate that targeting cell metabolism in NK cells may restore their antitumor activities in cancer patients and promote tumor regression.

In chapter titled “New therapeutic modalities in breast cancer by targeting NK cell inhibitory and activating receptors,” Drs. Moyal, Navasardyan, and Bonavida review the role of NK cells in human breast cancer. They describe the various subsets of breast cancer and the status of the nonfunctional NK cells in these subsets. They also describe the status of the NK receptors in breast cancer and suggest the potential of targeting either the activating or inhibitory NK receptors to restore their cytotoxic and cytokine secretion functions that could lead to the inhibition of tumor growth. In addition, they suggest that the combination of targeting receptors and other current therapies against breast cancer may also enhance their therapeutic potential.
Part VI: NK immunotherapy in various cancers (four chapters)

In chapter titled “Adoptive NK cell therapies in children with cancer: Clinical challenges and future possibilities,” Drs. Nguyen and Furman review the role of the human leukocyte antigen (HLA)-killer immunoglobulin receptor (KIR) mismatch that regulates NK cytotoxicity. This initiated the KIR and HLA genotyping of donors and recipients to select appropriate donors. The mechanism of the adoptive HLA-KIR mismatched NK cells is not clear and is under investigation. In this chapter, the authors review the current state of the adoptive NK cell therapies in children with cancer and what can be done to improve such therapies.

In chapter titled “NK cells in prostate cancer,” Drs. Acikgoz, Sati, Soner, and Oktem review the interrelationship between the immune system and prostate cancer. They describe the important role of NK cells in the therapy of prostate cancer as a first line of defense. Various strategies are being discussed to amplify the NK activities by altering the phenotypic and functional properties of NK cells in both advanced naive prostate cancer and castration-resistant prostate cancer.

In chapter titled “Involvement of natural killer cells in the pathogenesis of lymphomas: Therapeutic implications,” Kosasih, Vivarelli, Libra, and Bonavida review the role of NK cells in the pathogenesis of lymphomas. Cancer patients harbor defective NK cell functions and NK cells with distinct phenotypic properties. The use of activating agents in vivo to boost NK functions and/or the use of ex vivo activated autologous NK cells or engineered allogeneic NK cells and CAR-NK cells are discussed for their potential roles in the treatment of resistant lymphomas. Preclinical findings and clinical trials are provided, and the latter are awaiting validation and approval.

In chapter titled “NK cells in brain tumors: From biology to treatment,” Drs. Fares, Gupta, Gopalakrishnan, and Khatua review the potential of NK therapy against brain cancers. Immunotherapy against brain cancers, such as GBM and DIPG, remains at best modest due to the immunosuppressive environment that interferes with the homing of NK cells and their functions. The authors propose a thorough understanding of the mechanisms underlying the resistance to NK therapy and the use of genetic engineering of various forms of NK cells. Such engineered NK cells in combination with other immune strategies may overcome immunosuppression and facilitate the antitumor response against brain tumors.

Clearly, from the above-listed reviews, an overall description of the current trend is being reviewed for the use of NK cells in immunotherapy against various cancers, particularly against cancers that are not responsive to conventional therapies. Several clinical studies are currently being considered to examine various NK cell-based strategies to treat cancers whether used alone or in combination with other therapies. It is important to consider this unique book of value for clinicians, scientists, students, pharmaceutical companies, and health providers.

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PART I

NK cells: General properties
NK cells and CD8 T cells in cancer immunotherapy: Similar functions by different mechanisms

Cordelia Dunai, Craig P. Collins, Isabel Barao, and William J. Murphy

Abstract

NK cells and CD8 T cells are cytotoxic lymphocytes that have critical protective roles against pathogens and cancers. NK cells are conventionally regarded as rapid-acting cells of the innate branch of the immune system, while CD8 T cells act later in infections and in long-term memory responses. Both cells have similar cytotoxic pathways and respond to the same cytokines. We discuss how NK cells and T cells break the mold of innate and adaptive immunity with evidence that NK cells exhibit memory-like responses and memory bystander T cells rapidly respond to cytokine signals in heterologous challenges. A great deal is still unknown about coregulation of NK cells and T cells and the factors that are necessary for preserving long-term memory responses; however, light is being shed on the importance of inhibitory receptors for preventing cells from activation-induced cell death (AICD). Above all, there is a fine balance between having a protective immune response at the time of challenge and preventing immune-mediated pathology, while also generating long-lived memory cell populations with enhanced functions for the future. There are many exciting new therapeutic approaches aimed at utilizing NK cells and T cells to their full advantage for potent and long-term antitumor responses.

Abbreviations

- ADCC: antibody-dependent cell-mediated cytotoxicity
- AICD: activation-induced cell death
- AML: acute myeloid leukemia
- APC: antigen presenting cell
- CML: chronic myeloid leukemia
| dt0035 | CMV | cytomegalovirus |
| dt0040 | GVHD | graft-versus-host-disease |
| dt0045 | GVT | graft-versus-tumor |
| dt0050 | HSCT | hematopoietic stem cell transplant |
| dt0055 | ILC | innate lymphoid cell |
| dt0060 | KIR | killer-cell immunoglobulin-like receptor |
| dt0065 | LCMV | lymphocytic choriomeningitis mammarenavirus |
| dt0070 | MAIT | mucosal-associated invariant T |
| dt0075 | MCMV | murine cytomegalovirus |
| dt0080 | MHC | major histocompatibility complex |
| dt0085 | NHL | non-Hodgkin’s lymphoma |
| dt0090 | NHP | nonhuman primate |
| dt0095 | NK | natural killer |
| dt0100 | PBMC | peripheral blood mononuclear cell |
| dt0105 | TiNK | tumor infiltrating NK cells |
| dt0110 | TME | tumor microenvironment |

### Conflict of interest

No potential conflicts of interest were disclosed.

### Introduction

NK cells and T cells are two key lymphocytes whose evolutionary lineages diverged with the emergence of somatic rearrangement of antigen receptors [1]. NK cells form a distinct lineage of the recently discovered family of innate lymphoid cells (ILCs, type 1), and are often described as the cytotoxic arm of the ILCs, or the innate immune counterpart to CD8 T cells [2]. The cytotoxic activity of NK cells was first observed in a phenomenon termed as hybrid resistance—this involves rejection of hematopoietic parental cells by F1 offspring which was different than the previously observed acceptance of parental tissue grafts. The results showing that hybrid resistance is mediated by a radiation-resistant cell type were published in 1971 [3]. The discovery of NK cells and the coining of their name were published in 1975 with evidence of their spontaneous (nonsensitized) killing of tumor cells [4,5]. Currently, our understanding of ILCs and memory T cells is expanding as these lymphocytes blur the line of the innate and adaptive immunity. ILCs elicit appreciation for the immune system’s ability to fill a wide range of functional and anatomical niches and to station fast-responding sentinels to protect against pathogens [1].

Both NK and CD8 T cells are cytotoxic lymphocytes armed to fight cancer and pathogens, but their recognition, specificity, sensitivity, education, and memory mechanisms have key differences. Currently, cancer immunotherapy is mainly focused on CD8 T cells (anti-PD-1, anti-CTLA-4, CARs), but issues of drug toxicity and efficacy occur [6,7]. Among other factors, cancer heterogeneity in the patient population and immune escape often result in lack of response to therapies and relapse [8]. NK cells have unique immunological features, and their clinical use is an attractive alternative to T cells. In contrast to the single dominant T cell receptor (TCR) on T cells, NK cells present a diverse repertoire of activating and inhibitory receptors [9] that act in balance to regulate functional activity. In addition, NK cells undergo an education process (or licensing) involving major histocompatibility complex (MHC) I molecules during development and maturation [10], which confers self-tolerance and also

## I. NK cells: General properties
impacts the strength of their responses against cancer. NK cells can also acquire target recognition and functional memory after encountering target cells (i.e., viral infection in preclinical and clinical models) [11–14]. A goal of the ongoing research is to develop patient-customized approaches that merge the power of NK cells (innate immunity) and T cells (adaptive immunity), along with refined targeting and successful delivery of activated NK cells and specific memory CD8 T cells for tumor eradication. The focus of this review is on the distinct but complementary roles of cytotoxic lymphocytes and applications in immunotherapy. We propose that developing clinical applications that play to the strengths of each cell type and use a combination of approaches (as happens in a natural immune response) is a promising strategy for cancer immunotherapy.

NK cells and cytotoxic CD8 T cells have many traits in common and optimizing their antitumor cytotoxicity has been a focus of cancer immunotherapy for decades. Both cell types respond to type I interferons, IL-2, IL-12, IL-15, IL-18, and IL-21, and kill target cells via natural killer group 2 member D (NKG2D)-mediated activation and degranulation of perforin and granzyme. One of the best-studied scenarios for NK cell and T cell interaction is during viral responses—both NK and CD8 T cells specialize in killing virally infected cells, complementing each other with NK cells being able to kill MHC I-negative cells which evade T cells. Another major distinction between the cell types is that NK cells are fast responders, whereas naïve CD8 T cells need at least 1–4 days to become primed by antigen-presenting cells (APCs) in lymphoid tissue, then expand, traffic to, and kill targets. In these ways, there is a division of labor in terms of timing and method of killing virally infected cells. However, bystander T cells (memory T cells responding to inflammatory signals, i.e., cytokine stimulation in the absence of TCR engagement) have been shown to facilitate cytotoxicity and play a role in heterologous protection in pathogen responses or collateral tissue damage in autoimmune diseases [15] (Fig. 1). Also acting outside of the classic innate immunology definition, NK cells can be adaptive—in the sense that they have receptors against specific pathogens and preserve a memory pool with enhanced secondary responses [i.e., cytomegalovirus (CMV)-specific receptors Ly49H and NKG2C in mice and humans, respectively] [11].

Adaptive NK cells have been described in chemical hypersensitivity and viral responses, as well as being generated by cytokine activation [16–19]. Mouse cytomegalovirus (MCMV)-specific Ly49H+ NK cells have been shown to exhibit the classical components of immune memory: antigen specificity, expansion, contraction, and secondary responses [17]. In DAP12-deficient neonatal mice (lacking Ly49H signaling) challenged with lethal dose MCMV, adaptive NK cells were more protective than naïve NK cells when given in equal numbers [17]. It took 10 times as many naïve NK cells to provide the same level of protection, which is evidence of a phenotypic advantage [17]. There is significant research on long-term adaptive NK cell responses in nonhuman primate (NHP) and humans with interest in utilizing their antigen-specificity and enhanced effector function in clinical settings [20–22]. Fascinatingly, NK cells mediated antigen-specific killing of dendritic cells up to 5 years after

I. NK cells: General properties
vaccine challenge in NHP [20]. In humans, an extremely diverse repertoire of NK cells has been discovered by examining subsets with mass cytometry. NKG2C+ NK cells are increased in CMV-seropositive healthy individuals and aviremic HIV-1 patients [23–46]. It has also been discovered that NKG2C+ NK cells expand during CMV reactivation posthematopoietic stem cell transplant (HSCT) and this repertoire change is long lasting (elevated frequency detected at 1 year) [22]. Activated NKG2C+ NK cells also contain a significant mature (CD57+) subset, which preferentially expands during acute CMV infection and can be detected years later [26]. It is not known if CD57 is associated with senescence in vivo or if it is just a marker of cells that have divided multiple times. This CD56dimCD57+NKG2C+ NK cell subset has been termed adaptive and its expansion has been associated with reduced leukemia relapse suggesting a cross-protective effect of these activated NK cells. However, the risks of CMV reactivation as a cause of nonrelapse mortality remain [21,27].

Bystander T cells have been shown to play beneficial roles in heterologous infections and antitumor responses by exhibiting NKG2D-mediated cytotoxicity, proliferation, and IFN-γ production [28–30]. It is postulated that because memory T cells express distinct chemokine receptors, they are poised to respond to inflammatory signals, unlike naïve cells which remain in circulation and pass through lymph nodes [31]. It has been shown that memory, but not naïve, T cells specific for ovalbumin peptide (therefore nonantigen-specific and acting as a bystander) can be found in virus-infected lungs [32]. The extent of cross-reactivity of TCRs for different MHC-presented peptides versus pure non-TCR-mediated responses is the subject of ongoing research. Surprisingly, virus-specific memory T cells have been shown to exhibit antitumor effects in response to checkpoint blockade [33]. This has interesting implications for the extent of potential protection provided by previously generated tissue-resident memory T cells. It is very informative to utilize preclinical models that include bystander T cells (e.g., previously infected and/or aged mice) to model the human scenario of having a large pool of tissue-resident memory T cells that are capable of rapid TCR-independent responses.

I. NK cells: General properties
NK cells and T cells have many differences across species. For example, NK cells have whole receptor family differences across species that carry out similar functions, such as Ly49s (C-type lectin-like receptors) in mice and killer-cell immunoglobulin-like receptors (KIRs) in humans, which are activating or inhibitory receptors that bind MHC I molecules and govern NK cell activity. A summary of select markers used to phenotype NK cells in mice and humans is provided in Table 1. The diversity of NK cells across species could be in part due to coevolution with species-specific viruses and the lack of MHC restriction. NK cells are defined by different markers in mice and humans, however, in both species, they are CD3− and NKp46+, so at minimum, they do not have TCR signaling and they express the activating receptor NKp46 which is conserved across mammalian species and has been shown to bind to hemagglutinin (HA) [34]. In C57BL/6 mice, NK1.1 is a dominant activating receptor and has been found to bind the MCMV-encoded protein m12 [35]. In humans, CD56 is present on all NK cells—being either highly or lowly expressed making up the bright and dim subsets discussed later. Interestingly, NK cells are found throughout tissues in humans, including lymph nodes and CD56bright NK cells are the predominat population there [36], while negligible numbers of NK cells are found in the lymph nodes of mice without infection, which could be due to their specific-pathogen free status contrasting with humans [37].

In humans only, there are two additional activating receptors in the Natural Cytotoxicity Receptor family with NKp46: Ncr2/NKp44 and Ncr3/NKp30. The Fc receptor, CD16, is

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Human</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>NK1.1</td>
<td>CD56bright and CD56dim</td>
<td>Expressed on NK cells</td>
</tr>
<tr>
<td>Ly49 Family</td>
<td>KIR Family</td>
<td>Activating or Inhibitory</td>
</tr>
<tr>
<td>Ly6C</td>
<td>CD57</td>
<td>Maturation</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mouse and human</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3−</td>
<td>Excludes T cells and NKT cells</td>
</tr>
<tr>
<td>NKp46</td>
<td>Activating, binds viral antigens</td>
</tr>
<tr>
<td>CD16</td>
<td>Fc receptor involved in ADCC</td>
</tr>
<tr>
<td>CD49a and CD49b</td>
<td>Integrins involved in migration and adhesion</td>
</tr>
<tr>
<td>CD69</td>
<td>Early activation, tissue retention</td>
</tr>
<tr>
<td>NKG2D</td>
<td>Activating, binds stress ligands</td>
</tr>
<tr>
<td>NKG2C:CD94</td>
<td>Activating, binds nonclassical MHC 1</td>
</tr>
<tr>
<td>NKG2A:CD94</td>
<td>Inhibitory, binds nonclassical MHC 1</td>
</tr>
<tr>
<td>KLRG1</td>
<td>Inhibitory, binds cadherins</td>
</tr>
<tr>
<td>TIGIT</td>
<td>Inhibitory, binds CD112 and CD155 (DNAM-1ligands)</td>
</tr>
</tbody>
</table>

I. NK cells: General properties
expressed by NK cells of both species and plays an important role in antibody-dependent cell-mediated cytotoxicity (ADCC). Certain Fc receptor polymorphisms have been associated with better responses to monoclonal antibody therapy—with the hypothesis that this depends on ADCC [38]. It is important to mention the concept of split anergy that is present in NK cells of both species and appears to be a trade-off in functions—they can specialize in cytotoxicity or cytokine production [39–43]. IL-12 and IL-18 stimulation leads to the loss of CD16 by cleavage by ADAM17 and a related increase in IFN-γ production and degranulation [44].

There is a difference in the family of molecules in NK cells related to education and licensing by receiving signals from MHC I molecules in mice and humans (discussed in Section “Phenotype and function of NK and T cells and models for study”). The Ly49 family of mice and the KIR family of humans bind MHC I molecules and can either be activating or inhibitory. Ly49H binds the MCMV-encoded glycoprotein m157 which is thought to have evolved originally as a way for the virus to mimic MHC I and inhibit NK cells, however, NK cells evolved in response to the complementary activating receptor. On human NK cells, NKG2C, also an activating receptor, has been shown to bind HLA-E stabilized by the CMV-encoded peptide UL-40 and in that way confers a CMV-specific response [45]. Human blood exhibits a broad range of NKG2C+ NK cells—with a positive correlation with seropositivity [46].

Roles of NK cells and CD8 T cells in infection

The immune system includes barriers and sensors to protect from pathogens. However, once a pathogen has invaded a cell, the best course of action is to kill that cell in a controlled manner to shut down pathogen replication, lysis, and spreading to neighboring cells. Cancer can be likened to a chronic infection where inflammation and suppressive factors persist for a long time and in a way, a new challenge emerges to keep a balance of immune invigoration without immune-mediated damage [47]. For example, the important role of NK cells in primary response to CMV has been highlighted in several case reports of immunodeficiency—in both cases, the lack of NK cells precipitated lethal CMV infections [48,49]. CMV is a highly prevalent, usually asymptomatic herpes infection, but NK cells play an important role in keeping the virus in check. The paradigm is that NK cells are able to respond faster than T cells and are most important in the early stages of viral infection, but we are currently learning more about bystander T cell rapid responses to TLR signaling, NKp30 and NKG2D signaling, and proinflammatory cytokine responses [50,51]. NK cells are also critical in antiviral responses in the lymphopenic post-HSCT environment in which exposure to CMV or viral reactivation results in the expansion of adaptive NK cells (CD56dimNKG2C+ CD57+) with potent ADCC and persistence long after resolution of the CMV infection [52].

There are numerous positive and negative feedback pathways during an antiviral response and the cellular interactions change over time as the immune system goes from early response through to resolve the inflammation and return to homeostasis. The paradigm is that the T cell and B cell receptor repertoire is altered upon exposure to pathogen as well as anatomical environment and these have long-lasting consequences. Unlike T and B cell diversity, NK cell
diversity increases with immune experience and reflects an individual’s history of pathogen encounters and memory capabilities which has been correlated with future susceptibility to disease [53,54]. The concept of trained immunity is continuously being investigated as even innate cells have long-lasting changes following pathogen exposure [55,56]. Future mechanistic studies will bring a new level of understanding of the dynamics of NK and T cell diversity and their functional responses, as well as insights to design therapeutic strategies in the settings of infection and cancer.

Phenotype and function of NK and T cells and models for study

NK cells and T cells develop from common lymphoid progenitors, with NK and CD8 T cells relying on many of the same transcription factors and signaling cues to differentiate with divergence of T cells undergoing education in the thymus. During thymic involution, which occurs with aging, the production of naïve T cells dramatically dwindles. NK cells develop in the bone marrow and appear to continuously develop throughout life. These cells are classically defined by their ability to lyse virally infected or transformed target cells in a non-MHC-restricted manner and without prior sensitization. However, there is some evidence that activation in the bone marrow environment has an effect similar to negative selection and deletes cells from the circulating NK cell repertoire and leads to tolerance to antigens that were encountered early in development [57,58]. By different mechanisms, both NK and T cells have inhibitory pathways rendering them tolerant to “self” cells in nondisease settings. NK cells are inhibited by self-MHC molecules, whereas T cells require priming via the TCR in the presence of costimulation which occurs in an inflammatory lymphoid tissue. In fact, the degree of MHC I inhibitory signals that NK cells receive determines whether they are licensed or not—a phenomenon similar to priming. NK cells that receive a strong inhibitory signal from MHC I because they express inhibitory receptors that bind MHC I (Ly49 family in mice or KIR family in human) are more cytotoxic when they encounter an MHC I-negative target cell [59]. Once activated, NK and T cells can migrate to target tissues following a chemokine gradient. Memory T cell populations reside in tissue, in addition to innate-like T lymphocytes including mucosal-associated invariant T (MAIT) cells, and tissue-resident NK cells. The NK cells in human lymphoid tissue are predominately CD56 bright, unlike in the peripheral blood where the CD56 bright NK cell population is only ~10% with the rest being CD56 dim NK cells that have been found to have higher cytotoxicity [60]. The activity of NK and T cells is governed by activating and inhibiting markers, as well as by cytokine signals and metabolic and innate receptors (Table 2). The essence of their role is to selectively kill virally infected cells without causing host damage. They can mediate target cell death via four main pathways: FasL, TNF, TRAIL, and perforin/granzyme B, all of which can be triggered by NKG2D signaling. Perforin-mediated lysis primarily induces necrosis in the target cells and it is this property that dominates short-term (4 h) in vitro cytotoxicity assays, which may lead to an underestimation of their true killing potential as long-term studies demonstrate involvement of these other pathways [61]. NK cells were originally described as large granular lymphocytes and this highlights their ability to rapidly respond to stimulation and degranulate, unlike T cells which require priming. Degranulation is the predominant pathway for killing target cells and
NK cells are ready to do this spontaneously. Cytotoxic cells can kill multiple targets sequentially and without toxicity to themselves through the creation of a unidirectional synapse that protects them from perforin and granzyme, a mechanism of interest in the ongoing research [62].

There have been many studies aiming to elucidate the lifespan of lymphocytes and it is difficult to separate a clonally expanded subset that persists over time versus the duration of a single cell’s survival just by assessing the phenotype. Further complicating studies of secondary responses, epigenetic changes can be passed on from parent cells which could bestow an enhanced protective phenotype [17,63]. Studies with deuterium-enriched glucose in humans have shown that NK cells in peripheral blood have a turnover rate of 14 days, while that of T cells in peripheral blood is ~100 days [64,65]. Bar-coded cell studies have revealed that NK cell clones persist for several months [66,67]. Interestingly, subpopulations of NK and T cells have been found to persist in tissue for more than a decade as discovered in the studies of liver transplants [68]. In addition to a difference in lifespan, the production of NK and T cells is very different—NK cells are continuously produced from bone marrow progenitor cells, however, as the thymus involutes with age, the production of naïve T cells is drastically reduced over time and the T cell repertoire shifts to predominantly memory cells.

The classic assays of cytotoxicity involve measuring death of target cells; in the case of NK cells: MHC I-negative targets (Yac-1 for mice, K562 for humans), while T cells can target tumor cells and cells with NKG2D ligands. NK and T cells are studied in vivo in the context of infections and cancer. Ex vivo functional assays can be used to measure their cytokine production and degranulation (CD107a marker). In the mouse model of hepatitis, infection with lymphocytic choriomeningitis virus (LCMV) has garnered much important information about the roles of NK and T cells in infection, their coregulation, and exhaustion over time [69,70]. The development of NKP46 (Ncr1) knockout mice has opened the field to cell-specific deletion and shed light on nonredundant roles of NK cells in viral infection [71].

### Table 2: NK and T cell activation comparison.

<table>
<thead>
<tr>
<th>Cell property</th>
<th>NK cells</th>
<th>Ag-specific CD8+ T cells</th>
<th>Bystander CD8+ T cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Development</td>
<td>Bone Marrow</td>
<td>Thymus</td>
<td>Thymus</td>
</tr>
<tr>
<td>Turnover</td>
<td>Rapid</td>
<td>Slow</td>
<td>?</td>
</tr>
<tr>
<td>Key receptors</td>
<td>NCRs, KIR, NKG2D NKG2A/C/E, cytokine receptors</td>
<td>TCR, CD8, CD28, CD40L, cytokine receptors</td>
<td>NKG2D, TLR, cytokine receptors</td>
</tr>
<tr>
<td>Signals for</td>
<td>MHC I deficiency, activating receptor engagement (e.g., stress ligands), cytokines</td>
<td>TCR:pMHC I, costimulation, cytokines, NKG2D</td>
<td>NKG2D</td>
</tr>
<tr>
<td>cytotoxicity</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Major site of</td>
<td>Hematologic</td>
<td>Tissue</td>
<td>Tissue</td>
</tr>
<tr>
<td>cytotoxicity</td>
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I. NK cells: General properties
Regulation of NK and T cells and modifying factors

A sometimes unappreciated aspect of NK cells is their role, not just as cytotoxic cells, but as regulatory helper cells—secretory cytokines that can support antigen presentation (i.e., GM-CSF increasing costimulatory markers on APCs and the T cell response)—this is fulfilled by unlicensed NK cells in mice and humans and CD56<sup>dim</sup> NK cells in humans [37]. There is evidence of NK cell and dendritic cell (DC) cross talk within lymph nodes in the T cell zone, which puts them in the right place at the right time to influence the adaptive immune response [37,72]. Depletion of the unlicensed NK cell subset, but not the licensed subset, resulted in fewer mature DCs, which led to fewer antigen-specific T cells and increased viral titers in mice influenza and CMV models [37]. There is a multitude of ways that NK and T cells interact and regulate each other. Not only do they compete for the same cytokines, but also produce cytokines that inhibit each other, namely IL-10 and TGF-β. Regulatory T cells (Tregs) are able to suppress both cell types, but there is evidence of direct competition between NK cells and CD8 T cells where depletion of one cell type lead to compensatory proliferation of the other cell type and preserved overall cytotoxic capability [73]. It has also been shown in multiple models that NK cells, in addition to being rapid first responders, play a role in culling T cells and preventing immune-mediated pathology, i.e., NK cells can kill activated CD4<sup>+</sup> T cells via FasL and reduce lung tissue damage in LCMV infection [69]. However, a fine balance needs to be achieved because, depending on the viral load, the presence of NK cells can exacerbate pathology by affecting the dynamics of the antigen-specific T cell response [69]. In several viral and tumor models, depletion of NK cells is counterintuitively beneficial to the adaptive immune response as evidenced by increased antigen-specific tetramer<sup>+</sup> T cells; although timing is critical—with early-stage depletion or complete lack of NK cells still being detrimental to the overall immune response [49,74,75]. This could be related to effects on the viral load and subsequent antigen presentation to T cells. Modifying factors such as age, obesity, and comorbidities affect the function of NK and T cells and it is important to include these factors to make preclinical mouse models more relevant and reflective of clinical scenarios. Aging has been associated with changes in the frequencies of NK and T cells and impaired function. Obesity also has been associated with detrimental effects on function [76]. Long-term chronic infection has been shown to drive exhaustion of T cells and can detrimentally skew the immune repertoire with inflammatory clones [77].

The role of IL-15 in lymphocyte development, activation, and maintenance

While initial studies used IL-2 to expand NK cells, studies with IL-2 knockout mice demonstrated normal NK cell numbers and function [78]. However, IL-15 is one of the more critical cytokines for development, activation, and maintenance of both NK cells and memory CD8 T cells. IL-15 is trans-presented by stromal cells and DCs on the IL-15α receptor to NK and T cells bearing the beta and common gamma cytokine chain (CD122 and CD132). Studies by Caligiuri and others using IL-15 and IL-15R knockout mice elegantly demonstrated that IL-15 was critical for not only NK cell development but also NK cell survival.

I. NK cells: General properties
Subsequently, there has been intense interest in the application of IL-15 as a means to augment NK cell recovery after HSCT and also with NK cell adoptive immunotherapy [79,80]. The trans-presentation aspect of this cytokine presents some hurdles to induce optimal efficacy but efforts using conjugation with soluble receptor [81] or antibody complexes [82] appear to circumvent many of these limitations. While increased activity is indeed shown with IL-15 stimulation, prolonged exposure has been demonstrated to result in NK cell anergy in vitro [83] and in vivo [84], although this has recently been brought into question with clinical results following IL-15 exposure [85]. This brings up the question: what is the optimum way to activate NK cells prior to adoptive transfer and is there a way to prolong their activity and survival?

**Inhibition of NK and T cells**

Major hurdles for harnessing NK and T cells for immunotherapy are increasing infiltration into tumors, optimizing sustained antitumor responses, and avoiding suppression in the tumor microenvironment. Immune cells can become dysfunctional by three main pathways: exhaustion, anergy, and senescence, reviewed previously [86]. Immune cells are held in check by inhibitory receptors, which are advantageous for self-tolerance and could have a role in long-term persistence and memory response preservation [70,87,88]. However, many immunotherapies aim to unleash NK and T cells by blocking these inhibitory pathways. The best-studied inhibitory receptors are: CTLA-4, PD-1, TIM-3, LAG-3, and more recently, TIGIT. These are all found on NK and T cells, with the exception of conflicting reports about whether NK cells express CTLA-4 and PD-1 [89,90]. There is scant data on CTLA-4 on NK cells, but it has been reported in mouse NK cells following in vitro IL-2 stimulation and also within mouse tumors [91].

The possibility of NK cells expressing PD-1, and therefore being a key effector/responder in checkpoint blockade, has received a lot of attention in recent years. The concept that NK cells survive long enough to become exhausted given their high turnover rate and rapid responses is controversial in itself. PD-1 expression on NK cells was first reported in humans with multiple myeloma in 2010 [89]. However, the antibody used, CT-011 (pidelizumab), has since been shown to also bind Delta-like ligand 1, which is found on NK cells [92]. PD-1 expression on NK cells has been reported in mice and humans—after stimulation with IL-2 or IL-12, IL-15, and IL-18 plus glucocorticoids [93]; in infection settings [94]; and predominately in tumors (solid and hematologic) [95]. Summaries and commentaries on these findings have been published previously [96,97]. However, there were many studies that did not report PD-1 expression on NK cells [7,98]. Some possible reasons for this discrepancy are: (1) the difficulty in staining immune cell populations from tumor cells that have been treated with enzymes (which can remove markers such as CD3 necessary to delineate NK cells from T cells), (2) the lack of uniform analysis and interpretation of flow cytometry data—with the possibility of not excluding dead and dying cells which have been shown to bind anti-PD-1 antibodies nonspecifically [99], and (3) the confounding factors of inflammatory environments where increased expression of Fc Receptors could nonspecifically bind to detection antibodies and also where cells could undergo higher levels of trogocytosis (displaying membrane...
proteins from cells with which they have interacted). NK cells have been shown to acquire markers from other cells via trogocytosis, particularly when they are activated [100,101]. Additionally, there is a possibility that human and mouse NK cells are regulated differently and express different inhibitory receptors over time. Human NK cells can live much longer than mouse NK cells in vitro—15 weeks versus 7–10 days, so there is a possibility that there is suite of factors associated with survival, proliferation, and associated regulation that differ across the species.

Modifying factors such as age, obesity, and CMV infection, which cause increased expression of PD-1 on T cells, did not induce significant levels of PD-1 expression on NK cells [96]. More recently, minimal PD-1 expression (<10% PD-1+) was detected during the following conditions for mouse and human NK cells: in vitro IL-2 and IL-15 activation, culture with MHC I-negative feeder cells, CMV infection, and in vivo solid and hematologic malignancies. In contrast, in all these scenarios of immune stimulation, TIGIT induction on NK cells was observed, highlighting its role in the inhibition of NK cells to counterbalance activation and the potential for therapeutic checkpoint blockade. The possibility of intracellular PD-1 expression was also investigated as PD-1 mRNA can be translated into protein and stored in the cytoplasm prior to surface expression. Using PD-1 reporter mice with a tumor model, NK cells once again showed minimal PD-1 expression levels compared to T cells (<10%) [96]. However, treatment with anti-PD-1 has indirect effects on NK cells by affecting several feedback pathways and has been shown to ameliorate the exhaustion phenotype of NK cells as characterized by the reversal of low IFN-γ, granzyme B, and NKG2D expression [83]. It is also likely that NK cell expression of PD-L1 allows for PD-1/PD-L1 blockade to have an effect on NK cells’ control of T cells [75,83]. In particular, the increase in NK cell antitumor efficacy by the blockade of the PD-1/PD-L1 interactions is of importance for the treatment of tumors deficient of MHC I or displaying low mutational loads, because T cells are often inactive in these situations. For example, lymphomas expressing low MHC I (or negative for this molecule), but upregulated PD-L1, responded well to immunotherapy PD-1/PD-L1 blockade, indicating the pivotal antitumor role of NK cells [95,102].

Sources of NK cells

HSCT is a treatment for patients with hematological malignancies who are not eligible to receive intensive cytoreductive therapy and patients experiencing relapse [103,104]. Lymphocyte reconstitution post-HSCT is a long process and leaves patients susceptible to life-threatening infections. NK cells are the first lymphocytes to recover following HSCT and provide critical protection prior to the recovery of T cells and B cells. NK cells can also kill leukemic cells and prevent relapse [105]. Although the rapid reconstitution of donor NK cells plays a critical role in their graft-versus-tumor (GVT) effect, they still take 6 months or more to acquire full phenotypic and functional maturation [106]. This immaturity and premature education status may result in impaired function of donor NK cells in the early stage post transplantation. Moreover, since the alloreactivity of donor cells accounts for clinical benefit, genotyping the polymorphism of KIRs, MHC haplotype, and Fc receptors are important for donor selection to maximize the GVT effect [107,108].
In addition to HSCT, adoptive transfer of purified NK cells is under clinical evaluation. NK cells can be isolated from peripheral blood, umbilical cord blood, placenta, and bone marrow (Fig. 2) [109,110]. NK cells have demonstrated adaptive phenotypes based on their environment and encounters with pathogens and tumors, though characterizing intrinsic functional differences from each source is challenging [110]. It is important to mention that there is ongoing research to optimize donor: recipient matches with evidence that KIR-ligand mismatch can augment GVT effects and avoid graft-versus-host disease (GVHD) which is a risk with allogeneic T cells [111,112]. The strategy of KIR-ligand mismatch in allogeneic transplants aims to take advantage of NK cells’ reactivity against recipient tumor and APCs that are not able to inhibit them via MHC I, thus facilitating antitumor effects and preventing GVHD [113]. However, the MHC haplotype, recipient conditioning, presence of T cells in the graft, type of cancer, and type of graft are all important factors in transplantation [114,115]. The presence of NK cells in HSCT that include T cells can also prevent GVHD via direct killing of allogeneic T cells [116].

Isolation of NK cells from peripheral blood mononuclear cells (PBMCs) in sufficient quantities for clinical investigation is another challenge due to NK cells making up a small percentage (approximately 10%) of blood lymphocytes [117]. This has led to the use of NK-92 cells, an immortalized cell line derived from the bone marrow of a non-Hodgkin’s lymphoma, as an alternative to primary NK cell isolation [117,118]. NK-92 cells are able to expanded indefinitely, and have a cytotoxic profile similar to primary NK cells, but have the drawback of needing irradiation prior to transplantation in order to prevent malignant proliferation, which reduces their persistence considerably [117].

**FIG. 2** NK cells in clinical therapy and treatments to augment NK cell antitumor effects.
Isolation of NK cells from PBMCs begins with density gradient centrifugation, a process that removes erythrocytes and granulocytes [119,120]. NK cells are then enriched by removing other leukocytes left over after centrifugation, utilizing magnetized antibodies specific for CD3+ and CD4+ T cells, CD19+ B cells, and CD33+ cells [119,120]. NK cells can be expanded through the use of cytokines such as IL-2, IL-12, IL-15, IL-18, and IL-21, but greater fold expansion and cytotoxic function depend on “feeder” cells, such as tumor cells or PBMCs [121-123]. Feeder cells interact with NK cells in a cell-cell dependent manner to signal to activating receptors such as NKP30, NKP44, and NKP46, while inhibitory receptors such as KIR and NKG2A become downregulated, enhancing proliferative and cytotoxic capabilities [124]. Infusion with NK cells expanded in this way has demonstrated promising results, such as reduced tumor growth and increased survivability in multiple mouse models [124,125].

Tumor-infiltrating NK cells (TiNKs) have a markedly different phenotype than peripheral NK cells due to certain factors in the tumor microenvironment (TME) [126]. Activating receptors, such as CD16 and NKG2D, are downregulated, while inhibitory receptors, such as TIGIT and KIRD2, are upregulated, which severely dampens the ability of NK cells to induce cytolytic activity [126]. TIGIT inhibits NK cell function by competing with costimulatory receptor CD226 (DNAM-1) for NFAT pathway-activating ligand CD155, for which TIGIT has higher binding affinity [127]. CD155 is highly upregulated on tumor cells, while TIGIT is highly upregulated on TiNKs, resulting in attenuated cytolytic and adhesive functions [127]. Blockade of TIGIT with monoclonal antibodies has been demonstrated to rescue NK dysfunction in a variety of mouse tumor models, such as CT26 carcinoma and B16 melanoma [128]. In this study, anti-TIGIT did not display antitumor effects in the absence of NK cells, and even though CD4 and CD8 T cells also expressed TIGIT, they still maintained an exhausted phenotype [128]. Coadministration of CD155 with anti-TIGIT has demonstrated a synergistic effect on NK cell function rescue, enhancing cytolytic degranulation and upregulation of adhesion molecules such as LFA-1 as the result of NFAT signaling through CD226/CD155 binding [127].

KIR inhibitory receptors bind HLA class I molecules, causing activation of intracellular protein tyrosine phosphatases SHP-1 and SHP-2, which counteract signals from activating receptors. A lack of MHC I signaling can trigger NK cell alloreactivity, a mechanism that allows for tumor cell killing [111]. Monoclonal antibody anti-KIRs, such as anti-KIR 1-7F9 (IPH2101) and lirilumab, have been investigated for their potential to bind KIR2D receptors, impairing HLA-C binding on tumor cells and enhancing NK cell-mediated cytolytic activity [111,129,130]. Investigation into 1-7F9, a monoclonal antibody that blocks KIR2 ligands, has demonstrated prolonged NK cell longevity and increased cytotoxicity against patient-derived AML tumors both in vivo and in vitro, greatly enhancing the survival of mice in comparison to those without treatment [131]. These therapeutics have proven beneficial in improving rejection of tumor lines and preventing relapse in human clinical trials, particularly in B-cell lymphomas and multiple myelomas, which are difficult to treat and have a high relapse rate [111,130]. Lirilumab has been combined with other B-cell lymphoma treatments, such as anti-CD20, to improve ADCC in vitro and in vivo in murine models [129].

I. NK cells: General properties
NKG2A is an inhibitory receptor that associates and dimerizes with CD94 [132]. NKG2A:CD94 recognizes peptide: HLA-E complexes, which, upon binding, send inhibitory signals through the SHP-1 pathway that prevent phosphorylation of Lck and ZAP70, dampening NK cell cytolytic activity [132,133]. HLA-E is overexpressed in the tumor environment, which suppresses the activity of TiNKs [133]. Anti-NKG2A antibodies, such as humanized monalizumab (IPH2201) and antimouse NKG2A/C/E 20d5, have shown efficacy in restoring function in NKG2A+ TiNKs [134]. Monalizumab has demonstrated the ability to inhibit the binding of NKG2A to HLA-E and has shown a synergistic effect when combined with αPD-1 in Rae-1β+ tumor suppression [134]. This has the effect of restoring cytolytic functionality to TiNKs as evidenced by increased expression of CD107a and CD137, as well as an increase in the production of IFN-γ and improved responsiveness to IL-15 in the form of enhanced proliferation and cell longevity [134]. This increased NK cell functionality has spurred investigation into combining anti-NKG2A with other checkpoint blockade therapies such as anti-PD-1 [134–136]. Preclinical investigation of monalizumab has shown efficacy in rescuing NK cells from dysfunction and restoring cytolytic activity against high HLA-E-expressing chronic lymphocytic leukemia in vivo [137], as well as acute lymphoblastic leukemia when combined with LIR-1 blockade [138]. A recent trial demonstrated that monalizumab has minimal toxicity in gynecologic solid tumor cancer patients, which is promising due to the high expression of HLA-E in these cancers [139]. The generation of NK cells lacking expression of NKG2A has also shown clinical potential recently [133]. Abrogation of the NKG2A receptor has been demonstrated to enhance the cytotoxic activity of NK cells against HLA-E expressing tumors both in vivo and in vitro while preserving proliferative capability [133]. Further investigation into the clinical application of NKG2A blockade is appealing due to the current difficulties faced in solid tumor treatment.

NKG2D is an activating receptor on NK and T cells, which is upregulated in response to IL-15 [140,141]. NKG2D recognizes and binds to MHC class I chain-related proteins A and B (MICA and MICB), UL-16-binding proteins (ULBPs), and RAE-1 proteins, stress ligands highly expressed on tumor cells [140–143]. After interactions with these proteins, NKG2D becomes internalized, forming a complex with DAP10 and activating PI3-kinase, which results in enhanced cytolytic function and survival [140,142]. Recent evidence suggests that internalization of NKG2D results in the NK cell having less response to further activation signals, desensitizing the cell in a NKG2D ligand-rich zone [142,143]. While NKG2D ligands expressed on tumor cells can cause immediate activation of NK cells, which can transiently prevent the metastasis of tumors, the overabundance of NKG2D ligands in the TME results in an attenuated NK cell response and eventual tumor progression [142,143]. While NKG2D plays an important role in immunosurveillance and NK activation, it paradoxically limits the effectiveness of NK cells in a prolonged solid tumor response and therapeutic modulation is under investigation.

BiKEs and TriKEs

Monoclonal antibody (mAb) therapies, such as anti-CD20 rituximab, have become a standard treatment option for hematologic malignancies such as B-cell non-Hodgkin’s lymphoma.
Cytokines: Preactivation vs administration

(NHL) and mixed lineage leukemias, primarily by opsonizing the cells for NK cell-mediated ADCC [144]. Limitations of mAb therapies include: having low affinity binding to targeted receptors, competition of host IgG binding to the Fc region, suboptimal triggering of ADCC, and having affinity to Fc motifs shared by both inhibitory and activating receptors [145,146]. The goal to overcome these limitations and design more optimized antibodies against specific tumor lines has resulted in the development and investigation of bispecific and trispecific antibodies (BiKEs and TriKEs), which contain multiple variable regions to engage NK cells with tumor cells [147–149].

BiKEs and TriKEs engage CD16 on NK cells, which acts as an excitatory signal for ADCC [147,148]. mAbs bind to CD16 via the Fc receptor, which puts them into competition with host IgG; without high serum levels of mAb sustained over the time course of the treatment, IgG will outcompete for binding and dampen therapeutic benefits [146,147]. BiKEs and TriKEs circumvent this competition by using anti-CD16 to directly bind to the receptor, resulting in a higher binding affinity interaction in comparison to mAbs [147,148]. BiKEs and TriKEs are formulated to have variable domains specific for tumor antigens that allow for guided targeting of NK cells, which form an immunological synapse upon binding, activating CD16 and ADCC [147,148]. Other benefits of BiKEs and TriKEs, as shown in multiple solid and hematologic malignancies in vitro and in vivo, include increased NK cell degranulation, increased production of cytokines such as IFN-γ and TNF, greater cytolytic activity, and prolonged stability in human serum [147], BiKEs and TriKEs have shown therapeutic efficacy, such as TriKE CD16xCD19xCD22 in the treatment of NHL and BiKE CD16xCD33 in the treatment of acute myelogenous leukemia [148,150].
by the FDA after demonstrating favorable efficacy in clinical trials, though this efficacy comes at the expense of considerable toxicity [155]. Low-dose and subcutaneous administration IL-2 avoids this toxicity to an extent, but only a moderate increase in T cell and NK cell activation is achievable [152,155]. IL-2 also has the potential of activating host Tregs, which outcompete other T cells and NK cells for IL-2 via CD25. This has led to investigation into alternatives to high dose IL-2 that achieve similar immunostimulatory effects while avoiding pitfalls like toxicity and Treg activation. CIML NK cells have shown promise in making low-dose IL-2 a more viable treatment, with NK cells showing significantly increased proliferative and cytotoxic activity while avoiding toxicity in NOD mouse models [152]. Clinical data is somewhat lacking in this regard, however.

IL-15 has been researched as an alternative or supplemental treatment to high-dose IL-2, as it interacts with the CD122 and CD132 cytokine receptor chains allowing for NK cell stimulation and expansion while potentially avoiding the level of toxicity associated with high dose IL-2 or undesired activation of Tregs [156]. However, clinical evaluations of recombinant human IL-15 (rhIL15) infusion in cancer patients have been underwhelming. Although significant NK cell expansion, increased cytolytic markers, and upregulated production of cytokines can be achieved, significant toxicity, lack of tumor regression, and the short half-life of rhIL-15 are critically limiting factors [157,158].

N-803 (formerly known as ALT-803), a recombinant IL-15:IL-15 receptor alpha superagonist complex, has overcome these limiting factors in clinical trials [159–161]. Toxicities shown in rhIL-15 trials have been minimized in N-803 trials, while the half-life of the compound is 25 h in comparison to rhIL-15’s half-life of 40 min [159–161]. NK cytolytic and proliferative markers have shown marked increases following N-803 stimulation in comparison to rhIL-15, with the added benefit of significant CD8 T expansion [159–161]. Tumor regression has been achieved in mouse and rat bladder cancer and mouse ovarian cancer models, and enhanced antitumor response has been recorded in human melanoma, renal, neck, lung, and ovarian cancers, which are notable due to the difficulties faced in treating solid tumors [82,160–163]. N-803 has also shown potential in combination therapy with anti-PD-1, in which NK effector function was increased more than either therapy alone, while still avoiding significant toxicity [160]. N-803 could potentially be combined with other immunotherapies, making a big impact on the clinical setting.

IL-21 has been reported to improve antitumor immunity but has also been identified as a potent mediator of autoimmunity [164]. IL-21, produced predominantly by CD4 T cells and natural killer T (NKT) cells, is a member of the common γ-chain family of cytokines (including IL-2, IL-4, IL-7, IL-9, and IL-15) [165,166]. Mice studies involving IL-21 constitute the majority of observations documenting the beneficial role of this cytokine on NK and T cell antitumor responses. IL-21 promotes the proliferation, survival, and differentiation of T helper 17 and T follicular helper cells, while enhancing the function of CD8 T cells [167–170]. In addition, IL-21 decreases the expansion of Tregs by suppressing Foxp3 expression and promotes the enrichment of antigen-stimulated CD8 T cells [171]. IL-21, which also enhances the differentiation, proliferation, function, and viability of NK cells [172,173], has been considered an attractive cytokine for clinical use. In regard to clinical applications, limitations associated with NK cells include their low expansion and short life span in vivo. High numbers of NK cells are crucial for effective immunotherapy [174,175], and IL-21 is an additional option to IL-2 and IL-15 stimulation for NK cell-based immunotherapy. One study showed that genetically
engineered APCs expressing membrane-bound IL-21 promoted sustained ex vivo proliferation of NK cells without evidence of senescence [175]. In addition, NK cells expanded with IL-21 express high levels of the activating receptor CD160 and are able to retain cytotoxicity (mainly through ADCC) against diverse cancers (e.g., sarcomas, melanomas, neuroblastomas) [175]. A second study reported that in vivo delivery of plasmid-mediated IL-21 enhanced NK cell activity, systemic IL-21 levels, and tumor control [176]. In vivo depletion of CD4 or CD8 T cells did not affect IL-21-mediated antitumor activity. However, depletion of NK cells completely abolished IL-21-induced tumor elimination. In clinical trials, IL-21 as a single agent or in combination with other drugs in different cancers (e.g., AML, melanoma, B cell lymphoma) was well tolerated and showed antitumor activity [177].

**CAR NK and T Cells**

Chimeric antigen receptors (CARs) are genetically engineered receptors that are transduced into immune cells to target specific tumor antigens. CAR T cell therapy targeting CD19 has greatly enhanced the remission rates of hematologic malignancies, such as B cell acute lymphocytic leukemia, multiple myeloma, and B cell lymphoma [178–180]. In solid tumors such as HER2-positive sarcoma, however, CAR T cells have shown limited efficacy, due to difficulties in trafficking to the tumor site, infiltrating into the tumor, and overcoming the immunosuppressive TME [180,181]. Tumor heterogeneity and heterogeneous antigen expression also pose a difficult challenge. While progress has been made in overcoming these obstacles, CAR T cell therapy remains an inefficient treatment against solid tumors.

Adoptive NK cell transfers of peripheral blood, umbilical blood, and culture grown NK-92 cells have demonstrated safety and efficacy against hematological malignancies, but success against solid tumors has been limited [182,183]. The use of CARs to enhance NK cell specificity and cytolytic activity against solid tumors has been an appealing idea given the success of CAR T cells and the advantages of NK cells in adoptive transfers, namely, in avoiding GVHD and mediating ADCC [109,117,184]. Genetically modifying primary NK cells through viral transduction, the methodology used to attach CARs, however, is a difficult and inefficient process due to the high activity of antiviral toll-like receptors present in NK cells [185]. This is exacerbated by the low counts of NK cells yielded from primary NK sources, while the need for irradiating NK-92 cells before adoptive transfer makes them less clinically viable due to limited persistence [109,117,184]. iPSC-derived NK cells, as recently demonstrated, remedy these issues by providing a clinical scale source of expandable, but not malignant, NK cells that can be genetically modified with CARs at a relatively high efficiency through both viral and nonviral methods [184,186]. An alternate method to viral transduction for CAR NK generation involves charge-altering releasable transporters [187]. This promising technique can be used to transfect primary human NK cells with CARs and preserve their cytokine production and cytotoxic capability [187].

NK cell CARs are designed with an extracellular antigen recognition domain linked to NK-activating intracellular signaling domains, such as CD16, NKp46, CD28, NKG2D, CD3ζ, DAP10, and CD137 [184,186,188]. CAR NK cells have demonstrated enhanced function in comparison to primary NK cells, having increased tumor-specific cytotoxicity and increased
secretion of cytokines such as IFN-\(\gamma\), TNF, and CCL5, and have exhibited reduction of tumor burden in A1847 ovarian cancer, a solid tumor, and shown increased effectiveness in hematological malignancies such as K562 myelogenous leukemia and acute lymphocytic leukemia, while still avoiding GVHD and other toxicities. There are currently 19 clinical trials investigating the safety and efficacy of CAR NK cells in both solid and hematological tumors; although CAR NK cells are a relatively new concept, they have demonstrated considerable therapeutic potential in research conducted thus far [184,188–190]. An ongoing question in the field is whether the limited lifespan of NK cells compared to T cells will hamper the success of CAR NK cells.

**Concluding remarks**

There are similarities and differences between NK cells and T cells and we have discussed many of their advantages and disadvantages relevant to manipulation and utilization in clinical settings. The common goal across cancer immunotherapies is to optimize antitumor cytotoxicity, minimize off-target killing, and sustain the response long term. However, the uniqueness of each patient’s cancer is an enormous challenge for medicine. In order to outpace tumor evolution and immune evasion, a combination of strategies should be used—perhaps an immediate early versus long-term sustained memory response, as modeled in natural infections, is an important strategy to follow. Although many immunotherapies are already routinely used in the clinic, we still have much to learn and discover through basic research. Studies of host-pathogen interactions continue to be a productive resource for cancer therapy as we can adopt the cooperative mechanisms that have evolved within the immune system.

**Acknowledgments**

Many thanks to Dr. Sean Judge, Michelle Bagood, Dr. Lam Khuat, Logan Vick, and the Murphy lab journal club group for helpful input and discussions. Work was supported by R01-HL-140921.

**References**


References


I. NK cells: General properties

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1. Comparison of NK cells and CD8 T cells in cancer immunotherapies


[37] Zamora AE, Aguilar EG, Sungur CM, Khuat LT, Venstrom JM, Baumgarth N, Murphy WJ. Licensing delineates helper and effector NK cell subsets during viral infection. JCI Insight 2017;2. https://doi.org/10.1172/jci.insight.87032, e87032.


References


I. NK cells: General properties
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[80] Miller JS, Vermeris MR, Curtsinger J, DeFor TE, McKenna D, Waldmann TA, Blazar BR, Weisdorf DJ, Cooley S. A phase I study of intravenous NCI IL-15 to enhance adoptively transferred NK cells uncovers defects in
References


1. Comparison of NK cells and CD8 T cells in cancer immunotherapies


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I. NK cells: General properties


Challenges for NK cell-based therapies: What can we learn from lymph nodes?

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Abstract

NK cells are innate lymphoid cells identified in the 1970s for their ability to kill various tumor cells. NK cells originate and differentiate in the bone marrow, migrate to secondary lymphoid organs, namely the lymph node, where they terminate their maturation, and interact with myeloid immune cells, including dendritic cells and macrophages. Developing programs on the NK cell immuno-surveillance of tumors (melanoma and breast cancers), we focus our interest on NK cells from the lymph nodes, a crucial relay for the immuno-surveillance of several solid tumors. We summarized our original data on lymph node NK cells and their involvement in the immune response in the tumor-draining lymph node, a first site of metastatic invasion. We discuss different options aiming at the manipulation of these immune populations in new immunotherapeutic strategies.

Abbreviations

- ACT: adoptive cell therapy
- ADCC: antibody-dependent cellular cytotoxicity
- ALL: acute lymphocytic leukemia
- AML: acute myeloid leukemia
- BM: bone marrow
- CAR: chimeric antigen receptor
- CTLA-4: cytotoxic T-lymphocyte-associated protein 4
- DC: dendritic cell
- HL: Hodgkin lymphoma
- NHL: non-Hodgkin lymphoma
- ICB: immune checkpoint blockers
- IFN: interferon
Introduction

Natural killer (NK) cells are innate lymphoid cells (ILCs) that play a major role in host defenses against pathogens and in the immuno-surveillance of tumors. ILCs are divided into five main subsets based on their developmental transcription factor (TF) requirements and cytokine production patterns: NK cells, ILC1, ILC2, ILC3, and lymphoid tissue inducers (LTi) [1]. NK cells and ILC1s produce IFN-γ and depend on TF T-bet and Eomes for NK cells. ILC2s produce IL-5 and IL-13 and depend on GATA3. ILC3s produce IL-17 and IL-22 and express the TF RORγt. LTis play a critical role in the development of secondary lymphoid organs and the formation of lymph nodes (LN) during fetal life.

The present review is focused on the cytotoxic ILCs, namely NK cells, important antitumor effectors that may be targeted to improve the efficiency of antitumor immunotherapies.

NK cells are identified by the absence of CD3 and the expression of the cell adhesion molecule CD56. They can be divided into two main subsets: CD56brightCD16− and CD56dimCD16+. NK cells are the major ILC population present in the blood, constituting 5%–15% of the PBMCs. The majority of NK cells in the blood is CD56dim. In particular, the growing number of studies using single-cell RNA sequencing (scRNA-seq) technologies helped to define a comprehensive analysis of the immune system diversity and brought new findings on the heterogeneity of NK cells. In unstimulated and IL-2-activated NK cells from healthy cytomegalovirus (CMV)-negative donors, three NK cell subsets resembling the well-described populations; CD56brightCD16−, CD56dimCD16+CD57−, and CD56dimCD16+CD57+ and an additional novel blood NK cell subset characterized by a distinct signature was identified. Analysis of CMV+ donors established that CMV altered the proportion of NK cells in each subset, increasing the adaptive NK cells, as well as gene regulation within each subset [2]. Another study reports the identification of distinct NK populations in the human bone marrow and blood, including one population expressing higher levels of immediate early genes indicative of a homeostatic activation and a transitional population between CD56bright and CD56dim NK cells [3]. Two major NK subsets were identified in the blood and spleen with similarity between the mouse and

I. NK cells: General properties
NK differentiation and migration involve lymph nodes

NK cells mediate their activity through two main functions. The first one is the ability to recognize and directly lyse cells that have undergone malignant transformation, such as tumor cells, or cells infected by virus or other pathogens. After recognition of the target cells, cytotoxicity is mediated by the release of the intracellular perforin and granzymes. The cytolytic function of NK cells is mainly driven by the CD56dim NK subset and can be either mediated naturally through the triggering of activating receptors expressed on the NK cell surface or by antibody-dependent cellular cytotoxicity (ADCC). The second function of NK cells, mainly driven by the CD56bright subpopulation, is the production of inflammatory cytokines (IFN-γ, TNF-α) and growth factors (GMCSF) in response to activating receptor stimulation or induced by cytokines within the environment. The cytokines secreted by NK cells play a role in the regulation of the immune response. In particular, in the lymph node, IFN-γ produced by NK cells promotes the T_{H1} polarization of CD4+ T cells [5]. NK cell functions are regulated by a balance between activating and inhibitory signals received by a broad panel of receptors. Activating receptors including the natural cytotoxicity receptors (NCR1-3) or NKG2D recognize stress-induced molecules, whereas inhibiting signals are mediated by receptors specific for classical or nonclassical HLA-I molecules.

Many studies have demonstrated that NK cells contribute to cancer immuno-surveillance, especially in the control of metastasis [6]. A first link between cancer development and NK cell activity was reported in 2000 when a Japanese study, which has followed a cohort of 3625 individuals for 11 years, showed a correlation between natural cytotoxic activity of the blood NK cells and a reduced incidence of cancer [7]. Functional defects of NK cells were associated with particular NKG2D haplotypes [8]. Other studies showed correlations between the ratio of NCR isoforms or the NCR transcript levels and the development of more aggressive tumors and/or resistance to treatment [9–11]. Numerous studies have shown that NK cell infiltration of tumor areas is associated with better survival or a lower risk of cancer [12–14]. A correlation between high numbers of blood or tumor-infiltrating NK cells and the absence of metastasis has been established in gastric [15] and colorectal cancer [12], prostate carcinoma [16], and renal cell carcinoma [17].

The present review is focused on NK cells from the LN, a privileged site for NK cells to terminate their maturation but also for tumor cells as a first step of the metastatic spread. The review first recapitulates the NK cell differentiation and maturation in LNs and the role of LN-NK cells in tumor immuno-surveillance, then it presents an overview of NK cells in immunotherapies, and finally, a revisited role of the tumor-draining (TD) LN NK cells in the schedule of immunotherapies for cancer patients.
experiments showed that NK cells can be derived from the CD34+ hematopoietic progenitor cells (HPCs) coming from the thymus, secondary lymphoid tissues, or blood [18–20]. In the BM, HSCs differentiated into the common lymphoid progenitor (CLP), which can produce either T and B cells or the NK precursor (NKP). The NKP is characterized by the expression of the IL-2Rβ, CD38, and IL-15Rα transcripts, and does not express CD7, CD16, CD56, or NKG2A [21]. The NKP differentiates into immature NK (iNK) cells by the acquisition of CD161. However, these cells do not present yet the capacity to lyse perforin-sensitive targets or to produce IFN-γ [22]. Then, iNK cells acquire the expression of CD56, natural cytotoxic receptors (NCR), and other activating NK receptors including NKG2D. The production of a functional mature NK cells requires further maturation through a process referred as “education” that results from the capacity to recognize self-MHC class I molecules mainly by the KIR receptors expressed on NK cells. This mechanism generates educated KIR+ NK cells fully functional and, in parallel, hyporesponsive NK cells that have not been armed by the HLA-I/KIR interactions [23].

NK cell precursors can be found in secondary lymphoid tissues (SLTs), especially in LNs, where the NKPs coming from the BM, stimulated with IL-15 and IL-2, become CD56bright NK cells [24]. The stromal microenvironment of the LN and the presence of membrane-bound IL-15 are essential for NK cell differentiation and maturation. In the LN, the CD56bright NK cells are the most abundant subsets, while in the blood NK cells are mostly CD56dim [25]. The precise developmental relationship between CD56bright and CD56dim is still unclear, however, the vast majority of studies suggests that the CD56bright NK cells are the precursors of the CD56dim NK cells [26]. Resident CD56bright NK may acquire CD16 in the LN and recirculate into the blood [27]. In LN, NK cells interact with DCs through a bidirectional cross talk leading to NK cell activation and priming of DCs [28]. NK cell migration to the LN is dependent on the receptor CCR7, through the binding of its ligands CCL21 and CCL19 produced by the fibroblastic reticular cells as well as the lymphatic endothelial cells [29]. CCR7 is mainly expressed on the CD56bright NK cells, which coexpress the adhesion molecule CD62L (L-selectin). In the mouse, the recruitment of NK cells to the LN is CD62L dependent [30].

Lymph node relay for antitumor response initiation

LNs are a major site of innate immuno-surveillance and adaptive immune responses because antigens and antigen-presenting cells (APCs) from distinct organs migrate through the lymph fluid via the afferent lymphatics. In cancer, the LNs may be invaded by tumor cells that modified the microenvironment of these lymphoid organs and may impact on the NK cell development and function (Fig. 1). In the LN, INFγ and TNFα produced by NK cells will stimulate antigen-presenting DCs to support the activation of T and B cells. The regulation of NK cells by DCs and reciprocally the consequences of NK cell activation on DC functions are important physiological features [31]. Prolonged NK/DC contacts in the TDLN may turn resident NK cells into antitumor effectors [32] and DC priming by activated NK cells induce a protective cancer-specific immune response. Consequently, the DC-NK cell interplay in TDLN will determine the outcome of the ongoing immune response.
FIG. 1  Tumor-draining lymph node in immuno-surveillance: the pivotal role of NK cells. Tumor cells, tumor antigens on antigen-presenting cells, or soluble tumor-derived factors reach the proximal lymph node via lymphatic vessels (or blood) early in cancer development and interfere with NK cell maturation and NK-DC cross talk, orchestrating the onset of the tumor-specific response. As immunotherapies have proven their efficiency on metastatic tumors, their utilization is widened for less advanced tumors and in adjuvant and neoadjuvant settings. These new developments place the tumor-draining lymph node (TDLN) as a crucial relay, involving modulation of NK cells by the different immunotherapeutic agents.

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Lymphatic fluids from the primary tumor are drained by “sentinel” lymph node. Tumor factors drained into the sentinel node can modify the microenvironment leading to the development of a premetastatic niche [33]. Cancer cells can express CCR7 and migrate to the LN [34] through the lymphatic system before systemic dissemination through the blood. Thus, the TDLN is the first site of metastatic dissemination in many types of cancers including melanoma and breast cancer, and nodal invasion reflects a poor prognosis [35].

While the vast majority of human NK cell studies focused on blood NK cells, NK cells infiltrating secondary lymphoid organs and tissues were also characterized and the studies indicated the presence of tissue-specific NK subsets. Our group and others have demonstrated that NK cells can infiltrate the TDLN where they can act in the immuno-surveillance of metastatic dissemination. However, these studies are limited and, according to the tumor model, reveal somewhat different data. In melanoma patients, we found that the NK cell numbers in the sentinel LN correlated with a higher rate of 5-year relapse [36]. In metastatic LNs, the percentages of invading melanoma cells decreased the activation of CD56bright/CD16+ TDLN-NK cells. This population of mature NK cells displayed lytic potential toward melanoma cells following in vitro activation by IL-15 or IL-2 [37,38]. A different population, the CD56dim CD57+ NK cells have been found to infiltrate the LNs from melanoma patients and were cytotoxic against autologous melanoma cells [39], while the CD56+ NK cells in the metastatic regional LN of melanoma patients showed a reduced IFNγ production [40]. In colorectal cancer, CD57+ NK cells were correlated with a diminution of LN metastasis [41]. In breast cancer, axillary LN metastases with high amount of CD56+ NK cells were associated with a pathological complete response [42]. We found an increased CD56+/CD16+ PD-1+ NK subset in BC patient LNs that may be targeted with cetuximab and anti-PD-1 therapy [43].

**Tumor immunotherapy, the emerging role of NK cells**

In recent years, immunotherapy has become a recognized therapy for a variety of cancer types. The development of immune checkpoint blockers (ICB) therapies, the adoptive transfer of tumor-specific T cells [adoptive cell therapy (ACT)] or the generation of T cells engineered with chimeric antigen receptors (CAR) have been successfully applied to elicit durable immunological responses and sustained control of the disease in cancer patients. However, not all the patients respond to these therapies and therapeutic improvement is still required. One obvious but yet disregarded option is the NK cells. Beside their direct role on transformed cells, NK cells play a pivotal role in the activation and modulation of the adaptive immune response against the tumor. Recently, several efforts were made to develop strategies aimed to harness these cytotoxic effectors in the context of cancer immunotherapy. New therapeutic approaches were implemented to broaden the efficacy of the antitumor functions of NK cells in cancer patients.

**Promoting NK cell-mediated ADCC**

Therapeutic monoclonal antibodies (mAbs) targeting tumor-specific antigens are actively developed in the clinic to treat hematologic and solid tumors. One of the mechanisms of
action of certain of these mAbs involved ADCC mediated by NK cells, as the CD16 (FcγRIII)
can bind the Fc portion of the therapeutic mAbs, which have recognized their specific tumor
antigen.

Demonstrating the implication of ADCC in the antitumoral response, it has been shown
that polymorphisms in the Fc receptor CD16 [V158V (V/V) and V158F (V/F) polymorphism]
modulated the clinical response to mAbs [44]. Consequently, modified mAbs mediating
stron ADCC were developed to overcome these limitations. For instance, obinutuzumab,
a new mAb targeting CD20, has been glyco-modified to reduce Fc fucosylation [45] and in-
duces higher ADCC than rituximab against B-CLL cells [46].

In metastatic colorectal cancer (CRC), cetuximab mediated NK cell activity on EGFR+ cells
in a CD16-dependent manner [47]. In a phase II clinical trial, ex vivo ADCC and NK cell
response were correlated with clinical response in head and neck carcinomas [48]. For
HER2-positive metastatic breast cancer patients, trastuzumab an anti-HER2 mAb was shown
to activate ADCC of NK cells in vitro [49] and high levels of NK activity and ADCC were
correlated with clinical response in metastatic breast cancer [50]. To face patient’s resistance
to trastuzumab, a second mAb anti-HER2 has been developed, pertuzumab, binding a differ-
ent epitope. A recent in vitro study demonstrated that the effect of the combination
trastuzumab plus pertuzumab is increased with IL-2-activated NK cells [51].

Another field of therapeutic mAbs concerns plurispecific killer cells engagers which asso-
ciate several specificities and, therefore, interact with two or more surface antigens, building a
bridge between the tumor cell and the NK effector cell. BiKEs are bi-specific antibodies
targeting the effector cells and the tumor to improve the immune response against tumor
cells. Yet, BiKEs are mainly used in liquid tumors [Hodgkin lymphomas (HL), non-Hodgkin
lymphomas (NHL), mixed lineage leukemia, AML]. Usually, BiKEs target CD16 on the sur-
face of NK cells and an antigen on the tumor cell, for instance, CD33 at the surface of AML
cells, or CD30 in HL [52].

A further generation of therapeutic mAbs based on BiKE technology called TriKEs or
trispecific killer engager is under development. This technology is based on mAbs targeting
simultaneously tumor cells and NK cells (through CD16), and also associates to this structure
an IL-15 moiety to trigger NK cell activation and proliferation. In a study comparing BiKE and
Trike in myelodysplastic (MDS) patients, the authors showed that BiKEs (CD16/CD33) failed
to induce the proliferation of NK cells. Moreover, NK cells were still inhibited by MDSCs
through CD155-TIGIT engagement. Addition of IL-15 to generate a TriKE CD16/IL15/
CD33 allowed the proliferation of NK cells without any increase of TIGIT expression and
led to the elimination of MDSs, avoiding inhibition by MDSCs in the bone-marrow microen-
vironment [53]. Moreover, CD33 expressed by tumor cells as well as MDSCs, this TriKE could
eliminate both cell subsets decreasing the immnosuppression in the bone-marrow microen-
vironment [54]. TriKE combining anti-CD16, IL-15, and anti-CD33 was evaluated in AML and
high-risk MDS. In chronic lymphocytic leukemia (CLL) patients, TriKEs that combine anti-
CD16 for NK cells recruitment, IL-15 to provide an activation signal, and anti-CD19 to target
leukemic cells were developed [55].

To promote more efficient NK cell activation, a new family of trifunctional NK cell
engagers (NKCEs) is tested to target NK cell activation with two binding domains: one
targeting NKP46 and the second one targeting CD16. The development of such antibodies
is in line with studies demonstrating that a coengagement of NK receptors is needed for a

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full activation [56]. These NKCEs contain a third domain, binding to a tumor antigen, here CD20 [57]. Preclinical tests in mice showed better results than the anti-CD20 already in the clinic (rituximab, obinutuzumab) supporting the development of NKCEs as emerging immunotherapies. Of note, BiKE and TriKE strategies were mainly evaluated in hematological malignancies. However, some molecules were developed against solid tumors to target EpCAM [58] or more recently B7-H3 [59].

Immune checkpoint inhibitors

Tumor immunotherapy has been revolutionized by the discovery of immune checkpoint inhibitors. Immune checkpoint (IC) receptors are inhibitory receptors that decrease the antitumoral activity of the cytotoxic tumor-induced T cells. Blocking CTLA-4 and PD-1 pathways with mAbs (e.g., ipilimumab, nivolumab) reinvigorated the antitumor cytotoxic T cells and induced durable clinical responses and prolonged survival in some patients.

Importantly, NK cell activation can be regulated by PD-1 and CTLA4 following similar signaling pathways of the T cells. PD-1 is expressed by KIR+ CD57+CD56dim NK cells by contrast to less mature CD56bright NK cells by contrast. In multiple myeloma (MM) patients, NK cells express PD-1 and blockade of PD-1 on NK cells enhanced antitumoral NK cell function against autologous MM cells [61]. In ovarian carcinoma, PD-1+ NK cells are increased and present an impaired antitumoral function which can be restored by anti-PD-1 [60]. In different mouse models, activated NK cells expressed PD-1, the engagement with its ligand PD-L1 reduced NK cell activity, and the use of a PD-1/PD-L1 blocker restored NK cell response [62]. Immune deficient melanomas benefit from PD-1 blockade after targeted activation of type-I IFN that involved coordinated interactions between DC, NK, and T cells [63]. NK cell numbers were associated with clinical response to anti-PD-1 in metastatic melanoma patients including a subset of patients with downregulated MHC-I-expressing tumors [64]. An NK stimulatory DC axis involving the cytokine FLT3L enhanced checkpoint therapy response mediated by T cells in the tumor microenvironment [65].

The role of anti-CTLA-4 on NK cells is less clear. A study in a murine model showed that anti-CTLA-4 mAb increased NK cell antitumor activity, in a more important way than with IL15/IL15Ra [66]. They also looked at the CD56 expression in patients with cutaneous melanoma receiving ipilimumab and found that patients who responded expressed more CD56. Similarly, we found a correlation between soluble NKG2D ligands and response to Ipilimumab in melanoma patients [67]. Altogether, these observations suggest a close link between NK cells and response to Ipilimumab treatment in melanoma.

KIR and NKG2A inhibitors

Knowing the crucial role of HLA-I-specific receptors in NK cell regulation, mAbs targeting these inhibitory HLA-specific receptors, namely KIR and NKG2A receptors, were developed. Blocking the KIR/HLA-I interaction switches the balance toward NK cell activation. These mAbs were evaluated in patients with AML as it was shown that KIR2DL/HLA-C mismatch resulted in improved survival of AML patients transplanted with allogeneic stem cells [68]. Preclinical studies with a first generation of anti-KIR2DL1-3 (IPH2101) demonstrated that KIR
blockade increased NK cell-mediated cytolytic activity against AML and MM cells [69,70]. First clinical trials in AML and MM demonstrated no severe side effect but no efficacy in phase II [71,72]. The second generation of anti-KIR2DL1-3 (lirilumab/IPH2102/BMS-986015) has been developed and is currently being tested in combination with other drugs (rituximab, azacitidine) in lymphoma and MDS [73].

CD94/NKG2A heterodimer that binds to HLA-E molecules is a major inhibitory checkpoint of NK cells activation. HLA-E molecules are overexpressed in several solid tumors and hematological malignancies [74–76]. NKG2A is expressed by a large fraction of mature NK cells and by antigen experienced CD8+ T cells. A mAb blocking NKG2A (monalizumab) has recently been described and evaluated for its antitumoral activity in vitro and in vivo as single agent or in combination with anti-PDL1 (durvalumab) or anti-EGFR (cetuximab) [77].

**Other NK- and T-cell checkpoints**

While DNAM-1 is a potent coactivating NK cell receptor, its ligands are shared with two inhibitory receptors TIGIT and CD96, binding to CD155 (PVR) and CD112 (PVRL2) [78]. The expression of CD155 and CD112 was detected on tumors from epithelial origin such as breast cancer, colon cancer, and metastatic neuroblastoma as well as malignant hemopathies (MM and AML). Serum levels of both CD155 [79] and CD112 were associated with the prognostic of colorectal cancer and lymphoma [80]. Blockade of CD96 in a mouse model increased the antimetastatic activity of NK cells by an increased production of IFN-γ [81]. This increase is more effective in combination with anti-CTLA4 or anti-PD-1, and in the absence of TIGIT expression in mice. TIGIT has been associated with NK cell exhaustion in colorectal cancer and in several mice models, and anti-TIGIT therapy reversed NK cell exhaustion in mice [82].

TIM-3 is an immune checkpoint strongly upregulated, in parallel to PD-1, by exhausted T cells [83] and combined blockade of TIM-3 and PD-1 may restore the antitumor activity of exhausted T cells more effectively than the PD-1 blockade alone [84]. Similarly, TIM-3 is expressed on resting NK cells and is upregulated upon activation [85,86] and in cancer [87,88]. Importantly, NK cells from metastatic melanoma patients expressing TIM-3 are exhausted and can be reactivated by TIM-3 blockade [89].

**Adoptive cell transfer of NK cells**

Adoptive NK cell transfer is a cell therapy approach first developed for leukemic patients treated with allogeneic stem cell transplantation to consolidate a graft vs leukemia (GvL) effect [90]. Adoptive transfer of autologous NK cells in patients with solid tumors was also evaluated. Autologous transfer of NK cells consists in sorting of NK cells from the peripheral blood of patients, often exhausted in a tumor microenvironment, stimulate those cells with a cocktail of cytokines (IL-2, IL-15, or IL-21), and reInjected in patients with or without an addition of IL-2 injection to maintain NK cell activation. However, the efficiency of this immunotherapy was very limited when used to treat in renal cancer, metastatic melanoma [91], lymphoma, and breast cancer [92]. The expansion of Tregs, due to the injection of IL-2, and the expression of self-HLA-I molecules by the tumor cells may explain the weak efficiency of this strategy. Such therapy was combined with other immunotherapy and chemotherapy to be effective. A phase I clinical trial studied the safety and efficacy of infusion of NK cells.
cells activated in vitro in combination with antimyeloma chemotherapy. Four of five MM patients showed a disease stabilization, and two showed a reduction in BM infiltration and a long-term (>1 year) response [93].

To potentiate an antitumor effect, adoptive transfer of allogeneic NK cells (or the NK cell line NK92/NeukoplastTM) was performed. The benefit of using allogeneic NK cells is the KIR/KIR ligand mismatch setting allowing the presence of donor-derived alloreactive NK cells endowed with the cytolytic potential of tumor cells not engaging inhibitory KIR. Another main advantage is that donor-derived allogeneic NK cells have not been altered by the immunosuppressive environment of the patient. Several clinical trials of allogeneic NK cells transfer were attempted or completed. At the time of redaction of this review, more than 160 “allogeneic NK cell” trials were referenced in the ClinicalTrials.gov website (https://clinicaltrials.gov/ct2/home) using NK cells alone or in combination in different indications (AML, non-Hodgkin lymphoma, or HER2 + breast cancer). A phase I clinical trial (NCT02845999) showed the safety and efficacy of in situ delivery of allogeneic NK cells combined with cetuximab in patients with either colorectal or prostatic cancer-bearing liver metastases and a higher efficiency with cell products harboring KIR ligand mismatches. This trial demonstrated that combining allogeneic NK cells transfer via intrahepatic artery, cetuximab, and IL-2 is feasible, well tolerated, and may result in clinical responses [94].

Adoptive transfer in immunotherapy of cytotoxic NK cell lines especially NK-92 was also evaluated. This cell line is particularly interesting because it is easy to amplify in vitro and the cells lack KIR expression avoiding inhibition by HLA recognition [95]. Several studies have been done in renal cell carcinoma [96].

**CAR-NK cells**

The new development in the field of adoptive cell transfer of antitumor immune effectors involved genetically engineered T or NK cells using chimeric antigen receptors (CAR) technologies. The vast majority of the works on this approach concerns CAR-T cells and hematologic diseases. Promising results have thus been obtained with CD19-CAR-T cells to treat lymphoma patients. However, loss of antigen expression and the cytokine storm induced by high numbers of infused of reacting CAR-T cells are serious side effects of this approach [97].

In that context, CAR-NK cells may present several advantages. NK cell transfer does not induce high cytokine release syndrome or graft vs host effect so that fully functional donor allogeneic NK cells can be engineered. Of note, NK cells derived from induced progenitor stem cells (IPSC) can be engineered providing off-the-shelf resource for cancer therapy [98]. New CAR with accurate signaling dedicated to activate NK cells may be designed and known NK ligands expressed by solid tumors may be targeted. First data emerging with CAR-NK cells are indeed promising.

CAR-NK cells have been successfully genetically modified from primary NK cells, and from the NK-92 cell line with a vector to express specific receptor for recognition of tumor cells. CAR-NK cells are being evaluated in several preclinical trials in different hematological tumors such as B-ALL, targeting CD19 [99], MM, targeting CD138 [100], and solid tumor such as breast cancer [101]. Some CAR-NK studies are currently in phase I and phase II clinical trials in AML (NCT02944162) and B-ALL (NCT01974479). A recent study described the encouraging results obtained with CAR-NK cells engineered to recognize CD19 with the...
absence of deleterious cytokine release and a response to treatment to CAR-NK cells of relapsed refractory CD19+ cancer patients [102].

**LN NK cells and immunotherapy**

Considering the role of NK cells in the control of metastasis and the frequent involvement of the LN in the metastatic spreading of solid tumors, it is of interest to question the impact of immunotherapies on the lymph node that drains many tumors (Fig. 2). The data presented above raise several questions and suggest some strategies for scheduled NK-based immuno-interventions.

As immunotherapies are developed with success for various solid tumors, if surgery remains a recommended act in the treatment of primary tumors, practice is changing. For tumor prone to invade the TDLN such as melanoma and breast cancers, the resection of the sentinel LN was recommended for many years. However, it was shown that for stage III melanoma patients, there is no improvement of completion of LN dissection for most patients [103,104]. Furthermore, whether the TDLN can favor clinical response to immunotherapies is an important issue to determine the utility of the sentinel lymph node procedure.

A better understanding of the involvement of the LN in adjuvant neoadjuvant treatment setting is required. After surgical primary tumor resection, patients can receive an adjuvant therapy to kill the remaining metastatic cells as half of the patients can relapse after surgery due to metastatic development. In melanoma patients, clinical trials have evaluated the efficacy of several immunotherapies in adjuvant treatment. It was hypothesized that immunotherapies may impact the TDLN and promote an antitumor response. Several studies have shown that the ICB, specific of PD-1 and CTLA-4 in adjuvant therapies can prevent relapse.

**FIG. 2** Proposed strategies for scheduled NK-based immuno-interventions in cancer patients.

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For breast cancer patients, in several studies, the monoclonal antibody trastuzumab (anti-HER2) has been evaluated in adjuvant setting after chemotherapy and leads to increased survival [107].

Breast cancer patients may receive neoadjuvant treatment before surgery. This therapy can lead to the decrease of the tumor size, release of tumor antigens, and increased immune-surveillance. Chemotherapy was first tested but one clinical study showed a reduced activity of NK cells associated with a high risk of developing metastasis [108].

In metastatic melanoma patients, combined immunotherapies are being evaluated in a neoadjuvant setting. The rational is to promote a better activation of the immune system compared to the adjuvant therapies. Numerous studies have been launched, testing ICBs alone or in combination. In melanoma, anti-PD-1 or anti-CTLA-4 in neoadjuvant setting increases lymphocytes infiltration in the tumor [109]. Thus, the LN is considered as an important relay in the responses to neoadjuvant immunotherapies [110]. Neoadjuvant treatment can increase the antigenic presentation by APCs to the T cells in the LN and can interfere with NK cell differentiation and activation, which can be important for the response. Taking in fact that the LNs are the first site of metastasis and that NK cells play a role in the responses to adjuvant/neoadjuvant therapies, the study of LN NK cells is required to better understand the response and to increase the efficacy of this treatment.

Cancer-derived factors may render the TDLN permissive to tumor cell invasion and TDLNs have been involved in tumor-induced immune escape [111]. Thus, direct targeting of the TDLN with local delivery of adjuvanted nanoparticles to reshape the antitumor immune response was investigated in an experimental model [112].

**Conclusions**

NK cells exert antitumor cytotoxic function as well as a regulatory function. In the TDLN, these effectors require further characterization of their role in immune regulation. Such studies may open new avenues for adjuvant/neoadjuvant treatments for cancer patients.

**Acknowledgment**

*Financial support:* This work was supported by Institut National du Cancer (PAIR Melanoma 2013) and Société Française de Dermatologie (Grant to AC).

**References**


I. NK cells: General properties
References


2. Challenges for NK cell-based therapies


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References


[56] B978-0-12-824375-6.00002-3, 00002

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I. NK cells: General properties
NK cells and oncolytic viruses in cancer therapy

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Abstract

NK cell and oncolytic virus-based therapies have each shown effectiveness in preclinical and clinical studies. Each of these therapies has multiple complex interactions with the host immune system. Recently, data are emerging that oncolytic viruses and NK cells have interacting biologic effects. Further, host NK cells have been shown to be effectors in oncolytic viral efficacy. This chapter reviews the current data available on the combination of NK cell therapy and oncolytic viral therapy and discusses additional potential future applications.

Abbreviations

- CAR: chimeric antigen receptor
- CCL5: chemokine (c-c motif) ligand 5
- CCR5: chemokine receptor type 5
- CD19: cluster of differentiation 19
- CTLA-4: cytotoxic T-lymphocyte-associated protein 4
- EGFR: epidermal growth factor receptor
- GM-CSF: granulocyte-macrophage colony-stimulating factor
- HER2: human epidermal growth factor receptor 2
-IDO: indoleamine 2,3-dioxygenase
- IFN-γ: interferon gamma
- IL-2: interleukin 2
- IL-12: interleukin 12
- IL-15: interleukin 15
- IL-18: interleukin 18
- KIR: killer cell immunoglobulin-like receptor
- MHC: major histocompatibility complex
- NK: natural killer
- NKG2D: natural killer group 2d
Conflict of interest

Dr. Yuman Fong received royalties related to inventions in oncolytic viruses from Merck and Imugene.

Introduction

The existence of immune cells that mediate cellular cytotoxicity without prior activation has been known since the 1970s [1]. These cells were subsequently identified as natural killer (NK) cells. NK cells account for 5%-10% of the peripheral blood. There are many distinct subsets of NK cells and they have a complex role in tumor immunity and cancer immunotherapy. NK cells possess receptors that allow them to differentiate between “normal” and malignant cells. Initial studies have suggested that the lack of MHC I molecules activated NK cells due to “missing self-recognition.” As such, they can target cancer stem cells as well as undifferentiated or poorly differentiated tumors. Subsequent studies have shown that additional activating signals to tumor ligands, such as the natural killer group 2D (NKG2D) receptor, were needed to fully activate NK cells. Furthermore, cancer cells can express several additional ligands on their cell membranes and there are even soluble ligands that activate NK cell receptors. Another mechanism for NK cell activation is antibody-dependent cell cytotoxicity mediated by CD16 receptor, which binds the constant region of immunoglobulins on opsonized cancer cells. Finally, exposure to cytokines such as IL-2, IL-12, IL-15, and IL-18 all have stimulatory effects on NK cells and can induce a strong interferon-gamma production.

In addition to the activating signals, there are a host of inhibitory receptors such as killer cell immunoglobulin-like receptor (KIR) that prevent NK cell autoimmunity. Through a process called NK cell education, a combination of activating and inhibitory signals is used to generate a balanced NK cell response to future stimuli. Given that tumor cells often acquire NK cell-activating ligands and lose MHC expression, NK cells are an attractive target for cancer immunotherapy.

NK cells can cause tumor cell death via several mechanisms including direct interaction with tumor cells via the release of lytic granules, which lead to tumor cell apoptosis. NK cells can also secrete cytokines and chemokines such as IFNγ, TNF, GM-CSF, and CCL5, which can activate and attract other immune cells such as T-helper cells and dendritic cells.

NK cells have been used in cancer patients since the 1980s when IL-2-activated lymphokine-activated killer cells were infused, but the clinical utility was limited by the IL-2-related toxicity. Subsequent studies utilizing infusions of KIR-mismatched NK cells for acute myeloid leukemia in the early 2000s showed modest benefit.

In addition to their direct use in adoptive immunotherapy, NK cells have also been shown to be implicated in the efficacy of many current therapeutics. The use of checkpoint inhibition

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is spreading rapidly to multiple tumor types. Even though many antitumor mechanisms of checkpoint inhibition have not been completely elucidated, it is notable that NK cell subsets have been shown to be end effectors of anti-PD-L1 checkpoint inhibition. Furthermore, the presence of tumor-infiltrating NK cells has been shown to predict the response of HER2-positive breast cancers to anti-HER2-based neoadjuvant therapy with the presence of pretreatment tumor-infiltrating NK cells correlating with higher pathologic complete response rates as well as greater disease-free survival.

There are numerous complex interactions between NK cells and other immune cell lineages with regard to antitumor activity [2]. NK cells can modulate dendritic cell function and conversely, dendritic cells can promote NK cell activity via secretion of activating cytokines. The relationship between NK cells and dendritic cells has secondary influences in T cell differentiation and activation and it has been shown that the presence of NK cells is integral to an optimal antitumor response from CD8+ T cells.

The recent advances in oncolytic virus (OV) therapy are quite promising with the FDA approval of talimogene laherparepvec (T-Vec) in the treatment of malignant melanoma as well as the novel transduction of CD19 onto tumor cells to allow for chimeric antigen receptor (CAR)-T cell-targeted therapy [3,4]. In addition, there is an increasing evidence that the efficacy of oncolytic viral therapy is closely tied to the host immune response and in particular, NK cells have a complex relationship with oncolytic viral therapy. As such, there has been an interest in combining oncolytic viral therapy with NK cell therapy. In this chapter, we aim to discuss the relationship between oncolytic viral therapy and NK cells, summarize the available data utilizing NK cell and OV combination therapy, and discuss the future applications of the therapy.

How NKs can miss cancer/how the immunosuppression of NK function occurs

Despite the prior successes of NK cell adoptive immunotherapy, the use of NK cells has been limited by the vast array of immune escape mechanisms that are employed by tumors. The secretion of immunosuppressive cytokines by tumor cells such as PGE2, IDO, adenosine, and TGFβ causes downregulation of NK cell function. Shedding of ligands such as NKG2DL that can bind to the NKG2D receptor is a physiologic pathway that prevents autoimmunity and is another mechanism of NK cell downregulation. Modulation of the tumor microenvironment (TME) with the upregulation of regulatory T cells or myeloid-derived suppressor cells creates an immunosuppressive milieu to further reduce NK cell function. The downregulation of neoantigens that are targeted by NK cells also decreases their ability to detect cancer cells.

Efforts to overcome these challenges have been and are currently being explored including alternate in vitro and in vivo activating cytokine cocktails to improve NK cell survival after adoptive transfer, the engineering of chimeric antigen receptor NK cells, and the use of bi-specific proteins to cross-link NK cells to tumor cells and trigger tumor lysis.

The complex relationship between NK cells and multiple immunotherapeutics demonstrates how integral they are to the overall antitumor response and illustrates potential avenues for NK cell activation outside of direct NK cell modification.

I. NK cells: General properties
Oncolytic virus activation of any immune cell

OVs are replication-competent viruses that propagate in tumor cells and/or in the TME. OVs have been shown to have effects on numerous immunologic cell lines. The lysis of tumor cells releases tumor-associated and tumor-specific antigens which can, in turn, activate antigen-presenting cells, such as macrophages and dendritic cells. These, in turn, activate and not only increase the number of CD8+ cytotoxic T cells but also prolong the life of T cells and promote the development of immunologic memory. Furthermore, OVs can also reduce the population of regulatory and suppressor T cells as well as myeloid-derived suppressor cells in the TME.

Interaction of NK cells with OVs

Host NK cells have a complex relationship with oncolytic viral therapies [5]. On the one hand, they can adversely affect oncolytic viral therapy by limiting the extent of virus-mediated oncolysis or by preferentially killing virus-infected tumor cells (Fig. 1) [6,7]. Conversely, many studies using different OVs in the treatment of numerous tumor types have also shown that oncolytic viral therapy induces the recruitment of NK cells which in turn mediate additional antitumor effects [8–11]. Furthermore, certain oncolytic viral therapies have been shown to directly infect and mature dendritic cells, which in turn activate NK cells leading to oncosuppressive effects (Fig. 2) [12]. As such the general thought is that a lower number...
of NK cells at the time of initial oncolytic viral therapy is needed to allow for viral infection of tumor cells and propagation of the virus, but a significant NK cell population is needed after the initial phase of oncolytic viral therapy to allow for the activation of tumoricidal host immune responses.

Resistance and overcoming resistance to OV therapy

Despite the advances in oncolytic viral therapy, several limitations significantly reduce their effectiveness. These include impaired viral infection (viral tropism and tumor heterogeneity), viral clearance by the host’s innate immune system, and effects of the immunosuppressive TME [13]. Efforts to overcome these hurdles include further engineering of OVs with additional fusion proteins and tumor-associated antigens to facilitate cell entry and viral spread. Checkpoint inhibitors can be used to reduce the immunosuppressive effect of the TME. In particular, the combination of T-Vec with the anti-CTLA-4 monoclonal antibody, ipilimumab, as well as the anti-PD1 antibody, pembrolizumab, have shown promising results in separate phase I studies with phase III studies ongoing [14].

The administration of stimulatory chemokines/cytokines or the engineering of oncolytic viral genomes to enable them to generate their stimulatory signal could further modify the TME and leverage the host immune response to facilitate improved tumor lysis. An example of this is the administration of OVs expressing granulocyte-macrophage colony-stimulating factor (GM-CSF) leading to the development of immunologic memory cells, which then potentiate long-term survival.

I. NK cells: General properties
The combination of oncolytic viral therapy with other immunotherapeutic modalities has shown promise in overcoming the limitations of each therapy alone [3]. Specifically, the ability to deliver specific antigens such as CD19 onto tumor cells which normally do not uniformly express target antigens can allow for subsequent targeting of the previously heterogeneous tumor cell population with CAR-T cells. In addition to the abovementioned examples, the combination of OVs with additional immunotherapies has been quite promising with many additional studies ongoing.

Resistance and overcoming resistance in NK therapy

Despite numerous studies demonstrating the effects of NK cell-based therapies, there remain several barriers to the optimization of their use in cancer therapy. One challenge to the widespread use of NK cells has been the low percentage of NK cells in the peripheral blood, requiring significant ex vivo expansion to obtain clinical quantities. Furthermore, the need for cryopreservation in the setting of multiple-dose adoptive transfers can also potentially affect the cytotoxicity of the NK cells. Recent advances in the generation of stem cell-derived NK cells and the improved expansion, activation, and longevity of NK cells using IL-15 as well as a combination of IL-12, IL-15, and IL-18 are important steps in addressing these issues and have boosted interest in NK cell adoptive immunotherapy. NK cell therapy also suffers from some of the same limitations as other adoptive immunotherapies including immune escape mechanisms, tumor adaptation to inhibit NK cell activity, or modulation of the TME with the upregulation of regulatory T cells or myeloid-derived suppressor cells. The blockade of inhibitory receptors, eliminating immunosuppressive regulatory T cells and myeloid-derived suppressor cells, improving NK cell trafficking into solid tumors, and providing cytokines and growth factors to improve NK cell activation, proliferation, and persistence are all methods that are needed to augment NK cell-based therapies [15]. Efforts to augment NK cell activity on many of these fronts are underway. In addition, there have been recent studies evaluating the efficacy of CAR-NK cells in combination with NK cell-targeted bi-specific antibodies in efforts to improve the efficacy of NK cell therapy.

The combined use of NK and OV therapies

The combination of NK cell therapy and oncolytic viral therapy is intriguing for many reasons. As mentioned above, there is a close relationship between host NK cells and oncolytic viral therapy with NK cells at times hindering as well as promoting the effects of oncolytic viral therapy. Beyond needing NK cells as end effectors, oncolytic viral therapy can in turn allow for improved tumoral infiltration of NK cells and allow for immune-mediated tumor clearance. To date, there have been three studies published that have evaluated the combination of NK cell therapy with oncolytic viral therapy. These studies are summarized in Table 1. Chen et al. have shown that intra-tumoral epidermal growth factor receptor (EGFR)-CAR NK cell therapy in combination with intra-tumoral oncolytic herpes simplex virus (oHSV) therapy had a significantly improved tumor eradication and overall survival compared to each therapy in isolation in a mouse model of breast cancer brain metastases [16]. Yoo et al. have shown that bortezomib, a peptide-based proteasome inhibitor, sensitized oHSV-
constructed tumor cells to NK cell therapy in a mouse glioblastoma model and that the addition of NK cell therapy to bortezomib and oHSV significantly improved survival [17]. Recently, Feng et al. utilized a combination of NK cells transduced to express the chemokine receptor type 5 (CCR5) with an oncolytic vaccinia virus engineered to express chemokine ligand 5 (CCL5) in the treatment of a mouse cervical cancer model and showed improved survival and in some cases complete responses [18].

These early studies show the potential of combination therapy with NK cell and oncolytic viral therapy. Additional modifications to either therapy can continue to help overcome the limitations of the other. In particular, the use of OVs to modulate the TME could greatly improve the efficacy of NK cells (Fig. 3). This could be done by driving infected tumor cells to secrete growth factors, cytokines, and/or chemokines to further attract and activate tumor-infiltrating NK cells. OVs could also be used to alter the TME to decrease the population of regulatory T cells and myeloid-derived suppressor cells, resulting in the reduced immunosuppression of NK cells and potentially allow for a more durable response. Furthermore, the transduction of novel antigens on the surface of tumor cells could allow for CAR-NK cells to better target previously difficult to target tumors. Further studies including eventual human trials will be needed to evaluate the efficacy of these approaches.

How exosomes can be used to further understand and potentiate NK cell and OV therapy

While the combination of NK cell therapy and oncolytic viral therapy shows promise, the complexity of immune, biochemical, and physical barriers is likely to continue to inhibit tumor clearance. It is our hypothesis that further evaluation of the roles of exosomes will be pivotal in the understanding of these limitations as well as ultimately the way to overcome them.

### TABLE 1 Animal studies looking at NK cells and OVs.

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>Tumor</th>
<th>NK cell therapy</th>
<th>OV therapy</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chen [16]</td>
<td>2016</td>
<td>MDA-MB-231 (Breast cancer) brain metastases</td>
<td>Intra-tumoral EGFR-CAR NK-92 Cells</td>
<td>Intra-tumoral oHSV</td>
<td>Improved tumor eradication and overall survival with CAR-NK + oHSV combination therapy.</td>
</tr>
<tr>
<td>Yoo [17]</td>
<td>2016</td>
<td>GBM30 (glioblastoma) intracranial</td>
<td>Intra-tumoral NK cells</td>
<td>Intraperitoneal bortezomib + Intra-tumoral oHSV</td>
<td>Improved survival with addition of NK cells to mice treated with bortezomib and oHSV</td>
</tr>
<tr>
<td>Feng [18]</td>
<td>2020</td>
<td>HCT-116 (cervical cancer) subcutaneous</td>
<td>Intravenous NK-CCR5 cells</td>
<td>Intra-tumoral OV-ffluc-CCL5</td>
<td>Improved survival and observed complete response rates with NK-CCR5 and OV-ffluc-CCL5 combination therapy</td>
</tr>
</tbody>
</table>

EGFR-CAR, epidermal growth factor receptor-chimeric antigen receptor; oHSV, oncolytic herpes simplex virus; NK-CCR5 cells, NK cells transduced to express CCR5; OV-ffluc-CCL5, Oncolytic vaccinia virus engineered to express CCL5.
Investigators have attempted to use exosomes as a means of viral delivery to evade immune neutralization including immune neutralization by NKs. This mode of cloaked viral delivery is feasible in preclinical studies and has the potential to overcome the limitations of viral delivery to solid tumors, immune-related viral clearance, and viral tropism [19]. The role of tumor-derived exosomes in oncolytic viral propagation after initial tumor cell infection remains to be fully elucidated. Once better understood, the ability to harness exosomes could substantially increase the effectiveness of OVs and by extension combination NK cell therapies.

Tumor cell-derived exosomes have been implicated as mediators of treatment resistance to chemotherapy and immunotherapy in the TME [20]. A better understanding of exosomes in both circulation, as well as the TME, will generate future targets to overcome their immunosuppressive effects. The immunosuppressive exosomes could potentially be blocked by the addition of monoclonal antibodies. NK cells could be modified to evade their immunosuppression. OVs could be modified to adjust the characteristics of the exosomes themselves, leading to a less immunosuppressive TME.

**Conclusion**

The combination of NK cell therapy and OV therapy has the potential to overcome the limitations of each therapy in isolation. Early studies have shown promise, but further studies evaluating the effects of combination therapy are needed. A better understanding of exosomes would allow for modifications in NK cell therapy and oncolytic viral therapy.
to evade their immunosuppressive effects. Additionally, the potential to harness exosomes to further augment the activity of NK cell and oncolytic viral therapy remains to be determined.

Acknowledgments
We acknowledge the expert assistance of Supriya Deshpande in the preparation of this chapter.

References

I. NK cells: General properties

3. NK and OV therapy

I. NK cells: General properties
Successes and challenges of NKT cell immunotherapy: Breaking tolerance to cancer resistance

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Abstract

Natural killer T (NKT) cells comprise a small population of αβ T lymphocytes. They bridge the innate and adaptive immune systems and mediate strong and rapid responses to many diseases, including cancer. This has made NKT cells attractive agents for cell-based cancer therapies. Various approaches have been applied in clinical trials to target NKT cells, and the recent advances in chimeric antigen receptor (CAR) have also shown great potential in increasing the efficacy of NKT cells. In this chapter, we first introduce the biology and importance of NKT cells in antitumor immunity. Then, we summarize the preclinical data and clinical trials utilizing NKT cell-based immunotherapies. Finally, we discuss the challenges and future work that could be done to unleash the full potential of NKT cells for cancer immunotherapy.

Abbreviations

\begin{itemize}
\item NKT: natural killer T
\item CAR: chimeric antigen receptor
\item TCR: T-cell receptor
\end{itemize}

\* Contributed equally to this manuscript.
Biology of NKT cells

NKT cells are a unique subset of αβ T lymphocytes characterized by their expression of both a αβ T-cell receptor (TCR) and NK lineage markers. They derive from the same lymphoid precursor pool as T cells and mature in the thymus [1,2]. However, unlike conventional T cells, which recognize peptide antigens, NKT cells respond to lipid antigens presented by the nonclassical monomorphic major histocompatibility complex (MHC) molecule CD1d. Despite their small numbers in vivo (~0.1%–1% in mouse blood and ~0.01%–1% in human blood), NKT cells are some of the first cells to be activated during an immune response and can rapidly produce copious amounts of cytokines and chemokines, thereby functioning as a “bridge” linking the innate and adaptive immune responses [3].

Subtypes of NKT cells

NKT cells are generally divided into two major subsets based on their TCR expression. Type I NKT cells, or invariant NKT (iNKT) cells, are the most prevalent type of NKT cells and express a restricted TCR recombinant comprising a semi-invariant TCR α chain
(\(\text{V} \alpha 14-\text{J} \alpha 18\) in the mouse and \(\text{V} \alpha 24-\text{J} \alpha 18\) in humans) paired to a limited repertoire of \(\text{V} \beta\) chains (\(\text{V} \beta 2, 7,\) or \(8.2\) in mice and \(\text{V} \beta 11\) in humans) [2,3]. iNKT cells can be further divided based on their CD4 and CD8 expressions. Human iNKT cells include CD4\(^+\)CD8\(^-\) (CD4\(^+\)), CD4\(^-\)CD8\(^+\) (CD8\(^+\)), and CD4\(^-\)CD8\(^-\) (DN) populations, whereas mice only have CD4\(^+\) and DN populations [2]. For both mice and humans, iNKT cells are also functionally defined by their ability to recognize the prototypic antigen \(\alpha\)-galactosylceramide (\(\alpha\)-GalCer) [4,5]. \(\alpha\)-GalCer is a strong activator of iNKT cells and has been widely used to study iNKT cell biology. In contrast to type I NKT cells, type II NKT cells express more diverse TCRs and are therefore also called variant NKT cells [6–8]. Importantly, type II NKT cells do not respond to \(\alpha\)-GalCer. Given this and the lack of specific markers, study of this subpopulation has been more challenging. Currently, no experimental tools are available to identify and analyze the entire population of type II NKT cells.

From studies of animal models and humans, both subsets of NKT cells have been shown to play a role in diverse immune settings, particularly tumor immunity [9,10]. Although their functions sometimes coordinate, in most cases, they counteract each other. Compared to type II NKT cells, type I NKT cells are better characterized and known to possess a superior antitumor activity. Thus, hereafter this chapter will focus on discussing the role and current status of type I NKT cells in cancer immunotherapy.

Activation of iNKT cells

There are three main mechanisms to activate iNKT cells: (1) via TCR/CD1d/lipid stimulation, (2) via cytokine stimulation, and (3) via activating NK receptor stimulation [11–14]. Potent glycolipid antigens (e.g., \(\alpha\)-GalCer) presented by CD1d can directly activate iNKT cells in a TCR-dependent manner without the need for costimulation or cytokine receptor engagement [3]. However, in a more physiological context, iNKT cells are stimulated by microbial or self-lipid antigens that are weakly immunogenic [15]. In this case, iNKT cell activation requires a second activation signal from proinflammatory cytokines. Interestingly, cytokines alone (e.g., IL-12, IL-18, IL-23, and IL-25) are sometimes sufficient to activate iNKT cells even in the absence of TCR engagement. Several studies have found that iNKT cells release IFN-\(\gamma\) when stimulated by IL-12 during viral infections [16,17]. The remarkable capacity of iNKT cells to respond to cytokines is due to their elevated expression of functional receptors at baseline. iNKT cells also express activating and inhibitory NK receptors (NKR) and killer cell immunoglobulin-like receptors (KIR) on their cell surface, so they can become activated when the summation of stimulatory signals overcomes the inhibitory signals. Activating NKRs recognize a variety of MHC-like molecules and cellular targets frequently referred to as “stress proteins.” For example, DNAM-1 recognizes the poliovirus receptor and Nectin-2; the NKG2D receptor recognizes MHC class I-like molecules (MIC) A and B and unique long-binding proteins. Inhibitory NKRs and KIRs often bind to HLA molecules [14].

Effector functions of iNKT cells

Unlike conventional T cells, which emerge from the thymus “naïve,” iNKT cells leave the thymus fully mature and able to perform their effector functions immediately without priming [2,18,19]. The most significant effector function of activated NKT cells is secreting...
cytokines, including T helper (Th1-like (IFN-γ), Th2-like (IL-4, IL-13), Th17-like (IL-17, IL-22), and regulatory (IL-10) cytokines [2,20]. Which cytokines are produced is dependent on the mechanisms of cell activation, the location, and the iNKT cell subsets. For example, the activation of iNKT cells with the potent agonist α-GalCer leads to the production of both Th1- and Th2-like cytokines, whereas activation involving both recognition of the endogenous ligand/CD1d complexes and cytokine costimulation leads to the polarized production of Th1-like IFN-γ, but not Th2-like cytokines [15,21]. In addition, both human CD8+ and DN iNKT cells have been found to predominately express Th1 cytokines, whereas the human CD4+ iNKT cells predominately express Th2 cytokines, although this distinction is less apparent with the mouse iNKT cells [22,23]. Interestingly, subsets expressing different cytokines, transcription factors, and surface markers appear to be acquired during thymic development rather than as a result of peripheral experience [2,24].

In addition to cytokines, iNKT cells also produce cytolytic proteins such as perforin and granzyme B, and surface molecules involved in cytotoxicity such as Fas ligand (FasL) and tumor necrosis factor α (TNFα)-related apoptosis-inducing ligand (TRAIL) [25,26]. Given their ability to produce an array of effector molecules, iNKT cells can profoundly influence many other cell types, including dendritic cells (DCs), macrophages, neutrophils, NK cells, and T and B cells, thereby orchestrating immune responses during infection, autoimmune disease, allergy, and cancer [2].

**Mechanisms of iNKT cell-based cancer immunotherapy**

Recent progress in understanding the activation and effector functions of iNKT cells has led to an increased appreciation of their roles in health and disease. In particular, the antitumor potential of iNKT cells has made them extremely attractive agents for developing cancer immunotherapy [10,12,19,27]. There are compelling evidences suggesting the significant role of iNKT cells in tumor surveillance. In humans, the frequency of iNKT cells is decreased in patients with solid tumors (including melanoma, colon, lung, breast, and head-and-neck cancers) and hematologic malignancies (including leukemia, multiple myeloma, and myelodysplastic syndromes), and increased iNKT cell numbers are associated with a better prognosis [14,28,29]. In addition to correlative human data, the role of iNKT cells in exerting antitumor activity has been well characterized using murine models of tumors [28,30]. Direct tumor killing, cytokine-mediated regulation of effector cells, and the modulation of immunosuppressive tumor microenvironment (TME) are currently considered as the three major mechanisms of action (MOAs) of iNKT cells in combating cancer [10].

**Direct cytotoxicity against tumor cells (Fig. 1A)**

iNKT cells are known to exert a direct tumor-killing effect against CD1d+ tumors, mainly through the production of cytolytic molecules such as perforin and granzyme B [25,31,32], and the interaction of death-inducing receptors including Fas and TRAIL receptors [26,33,34]. The CD1d expression level in tumor cells has been demonstrated as an important determinant for iNKT cell-mediated cytotoxicity. The correlation between reduced CD1d
expression and enhanced tumor progression has been reported in a variety of tumor models [35–41], indicating that CD1d downregulation may be an important cause of tumor evasion from iNKT cell-mediated immunosurveillance.

Aside from recognition of CD1d, more than half of all iNKT cells also express NK-activating receptors, such as NKG2D, enabling direct cytotoxicity against tumors expressing corresponding ligands [22,42].

Regulation of antitumor effector cells (Fig. 1B)

In addition to their direct killing capability, iNKT cells are known to potentiate their antitumor effects by enhancing the immunogenic activities of a variety of immune cell subsets [43]. As mentioned above, iNKT cells can rapidly produce various Th1 and Th2 cytokines upon activation, leading to reciprocal activation and modulation of both innate and adaptive immune cells [2,44–47]. Notably, one of the major contributions of iNKT cells to immune surveillance is linked to DC maturation. Most DCs found in the TME are immature, and maturation of DCs is essential to initiate a sufficient T cell-mediated response [48]. Indeed, activation of iNKT cells causes the upregulation of the IL-12 receptor and CD40L and induces the maturation of DCs via a CD40-CD40L interaction. Mature DCs then express higher costimulatory molecules CD40, CD80, CD86, and CD70 and release more IL-12, which in turn further activate iNKT cells and amplify IFN-γ responses, leading to a positive feedback loop for Th1 immunity [49–51]. Furthermore, this maturation of DCs induces the transactivation of NK cells and contributes to the priming of CD8 T cells [52,53], allowing the establishment of both innate and adaptive immune responses to eliminate MHC-negative and -positive tumor cells, respectively.

Modulation of immunosuppressive TME (Fig. 1C)

iNKT cells can also augment their antitumor efficacy by counteracting immunosuppressive cells in the TME. The immunosuppressive microenvironment not only promotes tumor growth and migration, but also helps the tumor cells evade the surveillance of the host
immunity and resists immunotherapy [54,55]. Tumor-associated macrophages (TAMs) constitute a significant portion of cell populations in the TME and serve as major tumor-promoting immune cells [56]. It has been shown that iNKT cells can kill CD1d-expressing TAMs in primary human neuroblastoma samples, in part by relieving the immunosuppressive TME and limiting metastases [57]. In addition to TAMs, iNKT cells can also alter the numbers and effects of myeloid-derived suppressor cells (MDSCs), also known to be a population of myeloid cells with immunosuppressive properties [58]. In a model of influenza A virus infection, adoptive transfer of iNKT cells reduced the immunosuppressive activity of MDSCs [59]. In another study using murine tumor models, iNKT cells facilitated the conversion of immunosuppressive MDSCs into immunogenic antigen-presenting cells (APCs), eliciting successful antitumor immunity and providing the basis for alternative cell-based vaccines [60].

Advantages of iNKT cell-based immunotherapy

T cells engineered with chimeric antigen receptors (CARs) represent a novel class of immunotherapeutics that have shown promising clinical results and have obtained the FDA approval for treating blood cancers such as acute lymphoblastic leukemia and B cell lymphoma [61]. These CAR-T cells can respond quickly to CAR-specific tumor antigenic stimulation and rapidly produce cytokines to enable an effective antitumor response. However, there are several major drawbacks of CAR-T cell therapy, including serious side effects such as the cytokine release syndrome (CRS), lethal neurotoxicity, and graft-versus-host disease (GvHD) caused by allogeneic CAR-T cells [62,63]. In addition, the therapy is also less effective against solid tumors than against blood cancers [64].

In contrast, a major benefit of iNKT cell therapy is that iNKT cells have limited capacity to cause GvHD, and instead, is associated with reduced GvHD in clinical trials [65–68]. The presence of iNKT cells after hematopoietic stem cell transfer is predictive for survival with a reduction in GvHD [65–68]. Additionally, iNKT cells interact with the monomorphic CD1d [69–73] and are, therefore, not MHC restricted, making them highly suitable for “off-the-shelf” therapy [4,74,75].

The use of iNKT cells has mechanistic benefits beyond safety. iNKT cells can attack tumor cells through multiple mechanisms. Their TCRs can directly recognize CD1d+ tumors, using either perforin, granzyme B, Fas ligand, or TNF-α-mediated cytotoxic pathways. iNKT cells can also have indirect antitumoral effects by targeting CD1d+ tumor-associated macrophages and tumor-promoting myeloid cells [41,57,76]. In addition, iNKT cells express NK receptors such as NKG2D enabling direct cytotoxicity against tumors expressing NKG2D ligands [22,42]. Another advantage of iNKT cells is that they are an ideal vector to target nonlymphoid tumors: their interaction with tissue chemokines CCL2 and CCL20 allows them to migrate into nonlymphoid tissues [77,78]. In the tumor microenvironment, most DCs are immature. It has been shown that iNKT cells can promote the maturation of DCs through CD40-CD40L and CD1d/lipid antigen-TCR interactions [79]. Mature DCs can activate NK cells and iNKT cells by expressing costimulatory molecules (CD40, CD80, CD86) and cytokines, further enhancing the antitumor effects of these cells [80].

I. NK cells: General properties
Several preclinical and clinical studies have reported using ex vivo stimulation and loading of autologous DCs with α-GalCer prior to administration of DC vaccines [81–92]. A detailed analysis showed that injecting these vaccines into mice can augment the frequency of iNKT cells and circulating IFN-γ-producing cells in a solid tumor preclinical model [84]. In humans, α-GalCer-loaded DCs can increase the infiltration of lymphocytes into the tumor microenvironment and promote iNKT cell-induced immune memory upon secondary administration [87]. In a phase I/II trial, Chang et al. intravenously injected monocyte-derived mature DCs loaded with α-GalCer into five patients with advanced myeloma [89]. In all patients, the frequency of iNKT cells increased over a 100-fold and the number of iNKT cells stayed above baseline for 3 months. Elevated IFN-γ levels were also detected. These data demonstrate the feasibility of using α-GalCer-loaded DCs to improve iNKT-based therapies, including iNKT cells, in clinical settings.

Current status of iNKT cell-based immunotherapy

iNKT cells can induce strong antitumor immune responses in preclinical mouse models and in clinical trials via in vivo stimulation or adoptive transfer approach. These cells can also be activated by administering α-GalCer-loaded DCs. Activated iNKT cells increase IFN-γ production and cytokine secretion, which further enhances activation of other immune cells such as NK cells, T cells, and B cells. Multiple clinical trials based on α-GalCer-DC transfer have demonstrated the clinically relevant antitumor effects and the safety of this approach. In addition to α-GalCer-based therapy, there are other approaches to utilize iNKT cells for cancer treatments. These include using autologous and allogeneic transfer of iNKT cells, CAR-iNKT cells, hematopoietic stem cell (HSC)-derived iNKT cells, and induced pluripotent stem cell (iPSC)-derived iNKT cells. Numerous clinical trials utilizing these methods are summarized in Table 1.

Autologous transfer approach

The autologous adoptive transfer approach seeks to increase iNKT cell numbers in cancer patients by harvesting patients’ own PBMCs, expanding the PBMC iNKT cells by stimulation with α-GalCer and/or cytokines, and then infusing the enriched iNKT cell population back into the patient. This approach has been shown to be more effective in expanding iNKT cells than I.V. administration of α-GalCer [93–96]. In a preclinical study, adoptive transfer of IL-12-activated iNKT cells prevented hepatic metastasis of melanoma in mice [84]. This study also suggested the involvement of direct cytotoxic mechanisms rather than cytokine-mediated immune responses at the effector phase of the iNKT cell-mediated antitumor activity.

Motohashi et al. performed a phase I study with autologous activated iNKT cell therapy for non-small cell lung cancer patients [93]. No severe side effects were observed during this study and the number of IFN-γ-producing cells in the blood increased in some patients after the administration of activated iNKT cells. This study demonstrated the safety and feasibility of this approach, although no patient developed a partial or complete response. Subsequent adoptive iNKT cell-based clinical trials began treating patients with α-GalCer-loaded DCs in addition to iNKT cells [94–96]. In these studies, the treatments did not cause major adverse
<table>
<thead>
<tr>
<th>Year</th>
<th>Treatment</th>
<th>Tumor type</th>
<th>Safety</th>
<th>Clinical outcome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>2002</td>
<td>α-GalCer</td>
<td>Solid tumor</td>
<td>No severe side effects</td>
<td>Stable disease (7/24)</td>
<td>[86]</td>
</tr>
</tbody>
</table>
| 2004 | α-GalCer-loaded CD1d + immature DCs | Metastatic malignancy | No severe side effects | 1. Decreased serum tumor markers (2/12)  
2. Developed necrosis of tumor-infiltrating bone marrow (1/12)  
3. Inflammatory response at tumor sites | [87] |
| 2005 | α-GalCer-loaded IL-2/GM-CSF-cultured PBMCs | Non-small cell lung cancer | No severe side effects | Stable disease (3/11) | [88] |
| 2005 | α-GalCer-loaded mature DCs | Advanced cancer | No severe side effects | Decreased M spike levels in serum and urine (3/5) | [89] |
| 2008 | α-GalCer-loaded antigen-presenting cell (APCs) | Head-and-neck squamous cell carcinoma | No severe side effects | 1. Partial response (1/9)  
2. Stable disease (7/9) | [90] |
| 2009 | α-GalCer-loaded immature DCs | Non-small cell lung cancer | No severe side effects | Stable disease (5/17) | [91] |
| 2011 | α-GalCer-loaded immature DCs | Metastatic solid tumor | No severe side effects | 1. Stable disease (6/10)  
2. Reduction in tumor volume (3/10)  
3. Tumor inflammation (9/12) | [131] |
| 2012 | α-GalCer-loaded IL-2/GM-CSF-cultured PBMCs | Non-small cell lung cancer | No severe side effects | N/A | [92] |

Adoptive transfer of autologous ex vivo-expanded iNKT cells

<table>
<thead>
<tr>
<th>Year</th>
<th>Treatment</th>
<th>Tumor type</th>
<th>Safety</th>
<th>Clinical outcome</th>
<th>Reference</th>
</tr>
</thead>
</table>
| 2006 | Ex vivo-expanded iNKT cells with autologous α-GalCer-loaded PBMCs | Non-small cell lung cancer | No severe side effects | 1. No tumor regression  
2. Stable disease (2/9) | [93] |
| 2009 | Ex vivo-expanded iNKT cells with autologous α-GalCer-loaded PBMCs | Head-and-neck squamous cell carcinoma | Severe side effects (1); mild symptoms (7) | 1. Partial response (3/8)  
2. Stable disease (4/8)  
3. Progressive disease (1/8) | [94] |
| 2011 | Ex vivo-expanded iNKT cells (intra-arterial) and autologous α-GalCer-pulsed PBMCs (via nasal submucosal) | Head-and-neck squamous cell carcinoma | No severe side effects | 1. Objective tumor regression (5/10)  
2. Stable disease (5/10)  
3. Antitumor effects (8/10) | [95] |
TABLE 1  Clinical studies utilizing iNKT cell-based immunotherapies—cont’d

<table>
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<tr>
<th>Year</th>
<th>Treatment</th>
<th>Tumor type</th>
<th>Safety</th>
<th>Clinical outcome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>2017</td>
<td>Ex vivo-expanded iNKT cells</td>
<td>Advanced melanoma</td>
<td>No severe side effects</td>
<td>1. Patients deceased (3/9)</td>
<td>[96]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2. Patients progressed (3/9)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CAR-iNKT cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2020</td>
<td>Autologous GD2 CAR-iNKT cells overexpressed IL-15</td>
<td>Neuroblastoma</td>
<td>No severe side effects</td>
<td>1. Reduction of tumor volume (2/5)</td>
<td>[97] (Kuur Therapeutics)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2. Near complete response (1/5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3. Plan to increase dosage level</td>
<td></td>
</tr>
<tr>
<td>2020</td>
<td>Allogeneic CD19 CAR-iNKT cells overexpressed IL-15</td>
<td>B-cell malignancies</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A (Kuur Therapeutics)</td>
</tr>
<tr>
<td></td>
<td>Stem cell-derived iNKT cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2020</td>
<td>In vitro-differentiated iPSC-derived iNKT cells</td>
<td>Head-and-neck cancers</td>
<td>N/A</td>
<td>N/A</td>
<td>[98] (BrightPath Bio)</td>
</tr>
</tbody>
</table>

A variation on this approach, the Yang laboratory sought to increase circulating iNKT cells through infusion of TCR-engineered HSCs [99,100]. Smith et al. demonstrated the feasibility of dramatically increasing iNKT cells in mice through TCR gene engineering of autologous HSCs [99]; Zhu et al. utilized a BLT (human bone marrow-liver-thymus engrafted NOD/SCID/γc<sup>−/−</sup> mice) humanized mouse model to support the engraftment of engineered human HSCs and give rise to human iNKT cells [100]. iNKT TCR-engineered HSCs could generate a clonal population of iNKT cells. These HSC-iNKT cells closely resembled endogenous iNKT cells, could deploy multiple mechanisms to attack tumor cells, and effectively suppressed tumor growth in vivo in multiple tumor mouse models [100]. Preclinical safety studies showed no toxicity or tumorigenicity of the HSC-iNKT cell therapy [100].

Allogeneic transfer approach

Allogeneic hematopoietic cell transplantation (allo-HCT) is another powerful tool for treating hematological malignancies, but the development of GvHD remains a significant
clinical challenge. Preclinical studies have demonstrated iNKT cells can simultaneously prevent GvHD while retaining the graft-versus-tumor (GvT) effect following allo-HCT. Murine studies demonstrated that selective depletion of host conventional T cells through fractionated total lymphoid irradiation (TLI) and antithymocyte globulin (ATG), and thereby selectively enriching host iNKT cells, prior to allo-HCT, attenuated GvHD through host iNKT cell elevation of IL-4 secretion and polarization of donor T cells toward a Th2 cytokine pattern. This, in turn, inhibited donor T-cell early expansion and infiltration of GvHD target organs [101]. Likewise, the addition of donor or third-party CD4+ iNKT cells to the allograft was found to prevent GvHD by inhibiting T-cell proliferation, promoting Th2-biased cytokine response, and expanding donor MDSCs [102,103]. Notably, enrichment of the iNKT cells, whether host, donor, or third party, did not abrogate donor T-cell GvT [101–103].

The protective role of human iNKT cells against GvHD has also been highlighted by several clinical studies. Nonmyeloablative conditioning with TLI/ATG prior to allo-HCT was associated with a higher iNKT/T cell ratio, increased IL-4 production, decreased incidence of GvHD, and retained GvT effect [104,105]. Patients with acute GvHD were found to have reduced numbers of total iNKT cells [65], whereas enhanced iNKT cell reconstitution following allo-HCT positively correlated with reduction in GvHD without loss of GvT effect [67]. Separately, low CD4– iNKT cell numbers in donor graft were associated with clinically significant GvHD in patients receiving HLA-identical allo-HCT [106]. Thus, increasing the iNKT cell numbers of allograft that contains few iNKT cells may provide an attractive strategy for suppressing GvHD while preventing leukemia relapse. Human CD4+ and CD4– iNKT cells likely mediate distinct effects that collectively result in a beneficial effector response for transplant recipients. CD4– iNKT cells are Th1 biased—secreting more IFN-γ than IL-4 and preferentially expressing perforin—is thought to both promote GvT and suppress GvHD. Supporting this notion are in vitro studies demonstrating that CD4– iNKT cells display direct cytotoxicity against CD1d-expressing mature myeloid DCs [106]. Human iNKT cells also express KIRs, including KIRDL1, KIR3DL2, and KIR2DL1 [107]. In an allogeneic setting, donor CD4– iNKT cells may kill host APCs in a TCR-CD1d and KIR-dependent manners to downregulate GvHD. CD4+ iNKT cells, on the other hand, can ameliorate GvHD through the production of IL-4 [22], polarizing pathogenic donor T cells toward an antiinflammatory Th2 response, and promoting the expansion of regulatory T cells [108,109].

CAR-iNKT approach

iNKT cells as a platform for CAR immunotherapy represent a novel approach, as the addition of a CAR increases their tumor-targeting specificity, in vivo persistence, and potentially tumor infiltrating capability [110]. The Metelitsa group provides the first evidence for the feasibility of engineering iNKT cells with CAR and expanding them to a clinical scale. GD2-CARs and CD19-CARs were inserted into human iNKT cells and tested against neuroblastoma and CD19+ lymphoma in vitro and in preclinical murine models [111,112]. CAR-iNKT cells were capable of killing target tumor cells without causing GvHD, whereas conventional CAR-T cells cleared the tumor but recipients also succumbed to GvHD. Furthermore, the endogenous TCR of CAR-iNKT cells remained functional, such that they were also able to kill CD1d+ tumor cells [111]. A subsequent study published by the same group

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coexpressed IL-15 with GD2-CAR in iNKT cells [97]. Coexpression of IL-15 reduced expression of exhaustion markers in CAR-iNKT cells and enhanced in vivo persistence and localization to tumor sites. Owing to their results, the first-in-human CAR-iNKT cell clinical trial is underway [97]. In their interim report consisting of three patients, the group observed no dose-limiting toxicities, expansion of CAR-iNKT cells in vivo, and localized to tumors [113]. One patient also demonstrated an objective response with regression of bone metastatic lesions. These initial results suggest that CAR-iNKT cells can be expanded to clinical scale and safely applied to treat patients with cancer. Another study reported by the Karadimitritis group further demonstrated that CD19-CAR iNKT cells can be activated by CD1d and CD19-dependent stimulation, which provided a dual-targeting strategy for CD19-CAR-iNKT cells against CD1d-expressing lymphomas in vitro and in vivo [114].

### iPSC-iNKT approach

Adoptive CAR T-cell therapy is currently limited to autologous administration. Having to make individualized CAR T-cell therapy is expensive, time consuming, and labor intensive [74]. In addition, many cancer patients are nonideal candidates owing to the chemotherapy or irradiation they received beforehand that could negatively impact the quality of their T cells. The use of donor-derived allogeneic CAR therapy provides an opportunity to treat cancer patients with “off-the-shelf” cell products. However, the risk of GvHD remains a major concern for allogeneic CAR-T cell therapy due to HLA mismatches between the donor and patient [74,115]. Advances in gene editing have allowed scientists to remove the endogenous TCR from allogeneic CAR-T cells, lowering the chances of developing GvHD. However, 100% endogenous TCR removal is not guaranteed. Because having less than 1% of TCR-expressing T cells is sufficient to cause GvHD, patients receiving these products remain at risk for developing GvHD [74,115]. Although NK cells, iNKT cells, and γδ T cells may serve as better candidates for allogeneic adoptive transfer therapy, cell products derived from donors still have residual conventional αβ T cells.

To overcome these issues, allogeneic cell therapy generated from iPSCs could be an alternative approach. iPSCs can be stored as master cell banks, continuously differentiating and generating cells for treatments, which avoid using primary lymphoid cells from patients or healthy donors as the cell source [116]. This approach standardizes manufacturing procedures and provides a solution to donor-to-donor variations, cell number, and dosage limitations of the cell products. iPSCs can also be genetically modified by gene-editing technology prior to the differentiation step. Genetically engineered cell clones can be isolated and expanded as stable cell sources in which all clones contain 100% purity of desirable characteristics [116].

It has been demonstrated that T cells and NK cells can be successfully differentiated from iPSCs in vitro [116–123]. Recently, the Kaneko group reported the generation of human iNKT cells from iPSCs [124]. They reprogrammed iNKT cells to pluripotency and then re differentiated the cells into iNKT cells in vitro. The iPSC-derived iNKT cells showed TCR-dependent proliferation and IFN-γ production after α-GalCer stimulation. In vitro cytotoxicity against the tumor cell line K562 (leukemia) and U937 (lymphoma) were also observed [124]. In a separate study, the Fujii group also generated functional iPSC-iNKT cells and then

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Benjamin_Bonavida, 978-0-12-824375-6

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tested them in vitro and in vivo [98]. These cells possessed a significant antitumor activity in a K562 xenograft mouse model, and activated human NK cells by adjuvant effect [98]. The data from this preclinical study also led to the first clinical trial using iPSC-derived iNKT cells to treat patients with head-and-neck cancer. This phase I trial is expected to last for 2 years and involves 4–18 patients.

**Challenges and perspectives**

Recent progress in our understanding of iNKT cells has paved the way for iNKT cell-based cancer therapies. However, promising findings in preclinical studies have not yet convincingly translated to similar outcomes in human clinical trials. In fact, many challenges limit the overall performance of iNKT cell-based immunotherapies, including insufficient antitumor activity, limited in vivo persistence, and immunosuppressive TME. Various approaches are currently being explored to address these concerns.

The addition of CARs to iNKT cells to more potently direct iNKT cell-mediated cytotoxicity against refractory tumors holds promise to revolutionize cancer treatment. This approach has been demonstrated to enhance antitumor efficacy against neuroblastoma in preclinical studies and is under further investigation in clinical trials. Compared to conventional CAR-T therapy, CAR-iNKT cells are less likely to cause GvHD. However, iNKT are also highly inflammatory and may induce the cytokine release/storm syndrome (CRS) as a side effect to rapid tumor lysis. Indeed, CRS is a common concern, especially for all CAR-T therapies. So far, the fast-evolving CAR-T cell therapy has accumulated valuable clinical experiences on managing CRS (e.g., anti-IL-6 antibody treatment) that can be adapted for guiding the iNKT cell-based therapies.

Expansion and persistence of iNKT cells following infusion appear to be another determinant of clinical response. Importantly, both mouse and human studies have highlighted the central role of IL-15 in iNKT cell homeostasis [125,126]. So far, transgenic expression of IL-15 in adoptively transferred iNKT cells have improved iNKT in vivo persistence without causing significant toxicity, and is therefore a potential approach for general application to maximize NKT persistence and efficacy [97].

Although iNKT cells are known to modulate immunosuppressive cells in the TME, they are frequently suppressed in cancer patients due to nutrient deprivation, hypoxia, acidity, and accumulation of toxic by-products of catabolism [55]. One strategy that can partially reverse the dysfunction of iNKT cells is through blocking inhibitory pathways such as PD-1 and CTLA-4. While these “immune checkpoints” are predominantly defined in the context of CD8+ T cells, iNKT cells also showed upregulated PD-1 expression following stimulation with α-GalCer [127–129]. Indeed, blockade of PD-1 at the time of α-GalCer injection prevents the anergy of iNKT cells [127–129]. Considering the broad use of checkpoint inhibitors in the clinic, future combination treatments may synergize the antitumor efficacies of iNKT cells. Another strategy involves the blockade of inhibitory cytokines present in the TME, such as TGF-β. The use of dominant negative receptors on T cells is also being actively explored and has shown some promises [130], suggesting a potential role in enhancing iNKT cell-mediated antitumor immunity.
Furthermore, the possibility of third-party “off-the-shelf” iNKT products derived from standardized, allogeneic sources can improve the practicality of iNKT cell therapy. With increasing focus on improving the persistence and functions of iNKT cells, it is likely that iNKT cells will move to the forefront of cancer therapy over the next few years.

Acknowledgments

L.Y. receives research funding support from the University of California, Los Angeles (BSCRC Innovation Awards, BSCRC/JCCC Ablon Scholars Award), National Institutes of Health (DP2 CA196335, P50 CA092131, P01 CA132681), California Institute for Regenerative Medicine (RB5-07089, TRAN1-08533, DISC2-11157), Stop Cancer Foundation (Research Career Development Award), and Prostate Cancer Foundation (GTSN Challenge Award for Lethal Prostate Cancer). Z. L. is a postdoctoral fellow supported by the UCLA Tumor Immunology Training Grant (USHHS Ruth L. Kirschstein Institutional National Research Service Award #T32 CA 09120). D. L. is a postdoctoral fellow supported by the UCLA Microbial Pathogenesis Training Grant (Ruth L. Kirschstein National Research Service Award AI007323). S.Z is a postdoctoral fellow supported by the UCLA Medical Scientist Training Program Grant (T32-GM008042).

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PART II

Activation of NK cells
The role of peptidases and their endogenous inhibitors in the regulation of NK cell cytotoxicity

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Abstract

Cysteine cathepsins are implicated in various physiological and pathological processes. They play important roles in immune responses involving antigen processing and presentation, cytotoxicity of natural killer (NK) cells, cytotoxic T lymphocytes (CTLs), migration and adhesion of immune cells, cytokines, growth factor regulation, and toll-like receptor signaling. In NK cells, they activate granzymes and perforin from their precursor forms, molecules that are essential for the activation of the NK cell cytotoxicity. The process is regulated by cystatin F, an endogenous cysteine peptidase inhibitor, which colocalizes with cathepsins in endosomal/lysosomal vesicles and cytotoxic granules, and is capable of direct regulation of cathepsin activity. In the tumor microenvironment, several myeloid cells and cancer stem cells secrete cystatin F, which upon internalization may induce split anergy of NK cells decreasing their antitumor cytotoxicity and allowing secretion of cytokines, and/or completely impair the NK function.

Abbreviations

\begin{itemize}
  \item \textbf{ABC}  \hspace{1cm} ATP-binding cassette
  \item \textbf{ADAM}  \hspace{1cm} disintegrin and metalloproteinase
  \item \textbf{ALDH}  \hspace{1cm} aldehyde dehydrogenase
\end{itemize}
Conflict of interest

No potential conflicts of interest were disclosed.

Introduction

Proteases participate in various processes associated with tumor development and progression. Because of their integral role in degrading the extracellular matrix and the basal lamina, they are indispensable in processes of tumor invasion, angiogenesis, and metastasis [1,2]. Furthermore, they also play a role in the release of growth factors and in cytokine activation [3]. They can promote tumor progression by regulating the apoptosis of cancer cells [4]. Proteases are also involved in the antitumor immune responses and in the enhancement of immune tolerance [5]. In addition to the extracellular proteases, intracellular proteases that are involved in cell signaling can promote adhesion, migration, and invasion of cancer cells, and their dedifferentiation in tumor stem cell remodeling and in epithelial-mesenchymal transition [2]. The involvement of proteases in tumor-promoting processes makes them potential targets for cancer treatment. Proteasome inhibitors are the most notable, as they have already been used for treating multiple myeloma and mantle cell lymphoma. Several other proteases are also likely candidates for anticancer therapy, including matrix metalloproteases (MMPs), the urokinase-type plasminogen activator (uPA) system, and several cysteine peptidases, such as cysteine cathepsins B, X, L, S, and K.

In recent years, the role of peptidases in antitumor immune responses has been uncovered. They are involved in antigen processing and presentation, cytotoxicity of natural killer (NK)
cells, cytotoxic T lymphocytes (CTLs), migration and adhesion of immune cells, cytokines, growth factor regulation, and toll-like receptor (TLR) signaling [6]. Cysteine cathepsins, in particular, are involved in various aspects of innate and adaptive immune responses. Besides the regulation of TLR signaling and cytokine activation, they are well known for their role in antigen processing and presentation [7] and the activation and migration of T lymphocytes [8]. Last, but not least, cathepsins are also important for the activation of granule-localized serine peptidases granzymes A and B and perforin in NK cells [9] and their role in regulation of NK cell function is described in more detail below.

Other peptidases can also influence the function of immune cells, including NK cells. In particular, metalloproteases from tumor cells are able to release the ligands for NK cell-activating receptors from their surface. For example, ligand B7-H6 that binds to the NK cell-activating receptor NKP30 is cleaved by “a disintegrin and metalloproteinases” ADAM-10 and ADAM-17 [10]. Similarly, ADAM-10 and ADAM-17 shed tumor-associated major histocompatibility complex (MHC) class I polypeptide-related sequence (MIC)—a ligand from tumor cell surface, which prevents the binding to NKG2D-activating receptor [11]. In addition, ADAM-10 and ADAM-17 shed ULBP-2 from glioblastoma-initiating cells [12]. Several studies have also shown that ADAM-17 is likely responsible for the cleavage of CD16 receptor from the NK cells, thereby preventing the recognition of antibody-coated target cells [13].

Matrix metalloprotease 9 (MMP9) was also found to decrease the expression of multiple NKG2D ligands—MICA, MICB, ULBP-2, and ULBP-3—on the surface of gastric cancer cell lines and clinical samples [14]. In osteosarcoma cells, MMP9 shed MICA from their surface [15]. Furthermore, MMP9 cleaves intercellular adhesion molecule 1 (ICAM-1) from the surface of tumor cells, thus preventing the adhesion of cytotoxic cells to target cells [16].

In this review, we focus on the role of cysteine cathepsins and their endogenous inhibitors, cystatins, in the regulation of NK cells and NK cell-dependent antitumor responses. We emphasize the role of cysteine cathepsins and their inhibitors derived from cancer stem cells (CSCs) and myeloid cells as likely players in tumor microenvironment that regulates NK cell effector function.

Cysteine cathepsins and NK cell function

The granule-dependent cytotoxic pathway relies on the release of cytotoxic effector molecules such as perforin and granzymes to achieve target cell killing via the activation of caspases [17]. Perforin is a calcium-dependent pore-forming protein that enables the entry of granzymes into target cells. It is synthesized as a proenzyme. It was shown that incubation with E64, a broad cysteine cathepsin inhibitor, leupeptin, or ammonium chloride, prevents the activation of perforin. The cleavage of 12–20 C-terminal residues is necessary for perforin activation. This enables the binding of the C2 domain of perforin to the membrane and the formation of pores [18]. Granzymes are members of a neutral serine peptidases family, which need the removal of N-terminal dipeptide to be converted to active form [19]. Five granzymes have been identified in humans (granzymes A, B, H, M, and K). From those, the most studied and the most abundant are granzymes A and B [20]. Lysosomotropic agents were found to inhibit NK cell cytotoxic activity by decreasing the activity of lysosomal cysteine cathepsins [21], therefore, designating this group of peptidases as potential activators of granzymes.

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This has later led to the conclusion that cytotoxic activity of NK cells was reduced by selective inhibition of cathepsin C or dipeptidyl peptidase I [22]. Based on comparable cellular distribution of cathepsin C and serine peptidases, cathepsin C was proposed as the major enzyme responsible for posttranslational processing of the granule serine peptidases [23]. Cathepsin C−/− mice exhibited normal expression of granzymes A and B, but their effector cells had deficits in cytotoxic activity. Therefore, cathepsin C was found necessary for the processing of granzymes and generating cytotoxic activity of immune effector cells [24]. However, the analysis of cytotoxic cells derived from patients with Papillon-Lefèvre syndrome revealed that cathepsin C is not the only peptidase able to activate granzymes. This rare autosomal recessive disease is strongly correlated with the loss of function mutation of cathepsin C gene [25,26]. Patients’ cytotoxic cells were still able to produce active granzymes [27–29]. Therefore, another enzyme is compensating for the lack of cathepsin C activity. One of the additional convertases was proven to be cathepsin H. Nevertheless, a mouse model lacking both cathepsin C and cathepsin H still retained residual granzyme B activity, which points to redundancy in the activation of granzyme B [30].

Studies have shown that cathepsin L is able to process perforin in vitro and that the inhibition of cathepsin L significantly decreased target cell killing. However, like in pro-granzyme activation, cathepsin L is not the only enzyme able to activate perforin, as NK cells and CTLs from cathepsin L deficient mice had an intact cytotoxic ability [31].

Another cathepsin with favored localization in cytotoxic immune cells is cathepsin W or lymphopain. When discovered, cathepsin W was detected only in the tissues related to the immune system such as spleen, lymph nodes, peripheral blood, bone marrow, and thymus. On the cellular level, it was found predominantly in cytotoxic immune cells [32,33]. Its expression is enriched in NK cells, especially after stimulation with interleukin (IL), IL-2. In contrast to other cathepsins, cathepsin W is located in the endoplasmic reticulum (ER) and not in lysosomes [34] (Fig. 1). Nevertheless, the exact function of cathepsin W in NK cells is still unknown. It was shown that it might be implicated in the cytotoxic processes, as its levels decreased during the cytotoxic attack of NK-92 cell line against target K-562 cells. In addition to that, the presence of antisense oligonucleotides against cathepsin W cDNA decreased NK-92 cell cytotoxicity [35]. Decreased expression of cathepsin W after cytotoxic activity of NK-92 cells was also confirmed in the proteomic studies [36]. Despite all this data, the cytotoxicity of effector cells in mice model deficient in cathepsin W was not altered [37]. Even though cathepsin W was confirmed to be secreted during target cell killing, the knockdown of cathepsin W by siRNA did not influence neither target cell killing nor interferon γ (IFN-γ) production. It was also shown that cathepsin W is not exclusively located in the ER but can be transported to Golgi apparatus and further transported into secretory vesicles [38]. Therefore, the role of cathepsin W in cytotoxic cells remains to be elucidated.

Cathepsin B was proposed to have a protective role in preventing cytotoxic lymphocyte self-destruction after the secretion of perforin. After T cell receptor triggering, cathepsin B levels rapidly increased on the surface of T cells. Using cathepsin B inhibitors, CTLs were sensitized to activation-induced suicide. It was speculated that cathepsin B cleaves the membrane bound perforin intermediate, which is highly susceptible to proteolysis. Same protective mechanisms were thought to be used also by NK cells [39]. This was later shown not to be true, as cytotoxic cells of cathepsin B null mice induced normal cell death of target cells and survived the cytotoxic activity both in vivo and in vitro. In addition to that, it was
shown that perforin is not efficiently activated by cathepsin B [40]. Although not directly affecting the function of NK cells, cathepsin B is implicated in the induction of apoptosis. In tumor necrosis factor α (TNFα)-treated hepatocytes, cathepsin B was released from lysosomes, which enhanced the release of cytochrome C from mitochondria and activation of effector caspases [41]. Similarly, cathepsin B is released from lysosomes after the target cells are exposed to granulysin—a killer effector molecule of NK cells and CTLs. Released cathepsin B was shown to process Bid to active tBid and consequently cause the release of cytochrome c from mitochondria (Fig. 1) [42].

**Impact of cysteine peptidase inhibitors on NK cell cytotoxicity**

The activity of cysteine peptidases is ultimately regulated by endogenous inhibitors, among them the most important are cystatins. There are three different families of cystatins: type I, type II, and type III. Type I cystatins—stefins A and B—are predominantly cytosolic proteins, although they can also appear in body fluids. They do not possess disulfide bonds and are not glycosylated [43]. In contrast, type II cystatins have two disulfide bonds. They are mainly found extracellularly and some are glycosylated. Type II cystatins are represented by cystatins C, D, E/M, F, G, S, SN, SA, CRES, testatin, and cystatins 11, 12, 13, 14 [44]. Kininogens, the last group of cystatins, contain three type II cystatin-like domains [45]. They are found in blood plasma [46]. Common function of all cystatins is to prevent excessive peptidase activity of cysteine peptidases. In cancer, the balance between cathepsins and cystatins is frequently disrupted [47,48].
Among all cystatins, the most important cystatin that regulates the function of NK cells is cystatin F. Unlike other cystatins, this type II family cystatin can be secreted in its dimeric form but can also be localized intracellularly in endosomes and lysosomes [49]. It is N-glycosylated, which enables the intracellular localization. In addition to that, N-glycosylation enables the in trans action of cystatin F. Cystatin F could be internalized by surrounding cells via the mannose-6-phosphate pathway and affects the function of cathepsins in the recipient cell [50]. Full-length cystatin F can target the function of cathepsins L, H, S, V, legumain, and also cathepsin C, but only in its activated truncated monomeric form [51,52]. The conversion of cystatin F from its inactive dimeric form is presumably catalyzed by cathepsin V [53], which cleaves 15 N-terminal amino acids from cystatin F and prevents the formation of disulfide bond between cystatin F molecules. While cystatin F is normally expressed in the immune cells, especially in NK cells and CTLs [54], it was also found to be expressed in various cancer tissues [55–57]. By expressing cystatin F, cancer cells can evade the cytotoxic effects of NK cells and CTLs. Cystatin F can be internalized by effector cells, and due to inhibition of cathepsins C, H, and L, the formation of cytotoxic granzymes and perforin could likely be disrupted. In addition to cancer cells, other immune cells that infiltrate the tumor microenvironment (TME) can contribute to increased cystatin F levels in the TME. Increased levels of cystatin F have been, therefore, suggested to induce anergy of cytotoxic immune cells [58–60].

Cancer stem cell-NK cell interaction

Human tumors, particularly those arising from solid tissues, are known to have mutational loads and cell-to-cell differences in genetic programs leading to distinct intratumoral heterogeneity [61,62]. This tumor heterogeneity represents a serious problem for the conventional tumor therapy such as radiotherapy (RT), chemotherapy, and drugs targeting particular oncogenic pathways. Population of "stem-like" CSCs represents a small proportion of cancer cells within a tumor. Their presence has been confirmed nearly in all human malignancies including leukemia [63,64] and a variety of solid tumors, such as glioblastoma [65], melanoma [66], sarcoma [67–69], osteosarcoma [70], chondrosarcoma [71], prostate [72], ovarian [73], gastric [74], lung [75], pancreatic [76,77], and breast cancer [78–81]. Their reduced proliferation rate makes them resistant to conventional cytoreductive cancer treatments in comparison to differentiated cancer cells [82,83]. In a variety of tumor types, CSCs have been shown to exploit ATP-binding cassette (ABC) transporters to actively transport chemotherapeutic agents out of the cell, thus resisting the treatment with cytotoxic chemotherapeutics [84]. RT is a usual treatment modality for many cancers and its main mode of action is the induction of single- or double-strand DNA breaks and the formation of damaging reactive oxygen species within cancer cells [85]. The resistance of CSCs to treatment-induced DNA damage is related to upregulated levels of the DNA checkpoint kinases Chk1 and Chk2 [86]. CSCs remain in quiescent state within the tumor niche [87,88]. Moreover, they have the ability to undergo asymmetric cell divisions producing an identical daughter cell and a differentiated cell which, during its subsequent division, generate the vast majority of tumor bulk thus repopulating the tumor mass after cytoreductive treatments [89].
Multiple studies have confirmed that CSCs display a generalized surface expression profile consisting of CD54, aldehyde dehydrogenase (ALDH), programmed death-ligand 1 (PD-L1), and high expression of CD44 (reviewed in Refs. [90, 91]). Some reports indicate that MHC class I is expressed by CSCs [92], while others indicate that CSCs may downregulate the expression of MHC molecules, making them an attractive target for NK cell attack [93,94]. The susceptibility of CSCs to NK cell-mediated killing has been reported in different tumor models [95,96]. For example, when patient samples comprising unsorted cells from multiple subtypes of sarcoma and multiple pancreatic adenocarcinoma were exposed to autologous NK cells the greatest decreases in the overall frequency of cells before and after NK cell killing occurred within the CSCs population [97]. Similar, efficacy in the ability of activated NK cells to kill CSCs was observed when both autologous and allogeneic NK cells were used [97]. Furthermore, an in vivo study revealed that activated NK cells transferred into NSG mice harboring orthotropic pancreatic cancer xenografts were able to preferentially kill CSCs, leading to a significant decrease in both intratumoral CSCs and tumor load [97]. Mouse models of pancreatic cancer, glioblastoma, and others, it has been discovered that, compared with more differentiated tumor cells, CSCs express elevated levels of several proteins including MICA, PVR-1, and DR5 that bind to the activating proteins, NKG2D and DNAM-1, and the apoptosis-inducing protein, TRAIL, respectively, expressed on the surface of NK cells [97,98]. In addition, it was shown that in colorectal cancer, CSCs upregulated the NK-activating receptors NKp30 and NKp44 and were susceptible to NK cell-mediated killing [95]. Melanoma cell lines with CSCs features exposed to IL-2-activated allogeneic NK cells showed an increased susceptibility to NK cell-mediated killing through upregulation of the DNAM-1 ligands, such as PVR and Nectin-2 [99]. CSCs derived from glioblastoma showed an increased susceptibility to NK cell killing by both allogeneic and autologous IL-2- and IL-15-activated NK cells [98]. Some studies showed that breast cancer-derived CSCs are resistant to NK cell killing [100], whereas others showed their sensitivity to IL-2- and IL-15-treated NK cells and augmented expression of NKG2D ligands on their surface, such as ULBP1, ULBP2, and MICA [101]. Finally, whereas CSCs are less susceptible to radiation-induced death, it was shown that after the RT treatment these cells display higher levels of stress markers and NK cell recognition ligands, making them more susceptible to NK cell cytolysis in vitro and in vivo. For example, the analysis of a tissue microarray from 12 patients with matched pre- and post-RT samples revealed a statistically significant increase in immunohistochemical expression of the ALDH1 stem-like and MICA/B markers following RT in these patients [88]. Similarly, CSCs derived from multiple solid tumor types including sarcomas, pancreatic cancers, and breast cancers were found to be more susceptible to the cytolytic effector functions of NK cells after RT [88]. In addition, in preclinical models of nonmuscle invasive bladder cancer, NK cells have also been found to synergize with chemotherapy drugs to target CSCs [102].

Apart from direct cytolysis, it has been shown that CSC-NK cell interactions led to differentiation of CSCs [90,103–106]. In vitro studies have shown that the blocking of the TNF-α and IFN-γ with specific antibodies upon treatment with split-energized NK cell supernatants engaged the oral tumor-derived oral squamous carcinoma stem cells (OS CSCs), MP2 pancreatic [103] and A549 lung cancer cells [106] in a nondifferentiated stage as measured by their decreased B7-H1 and MHC class I expression and sensibility to NK cell cytolysis. This emphasized the role of NK cell-secreted IFN-γ and TNF-α in the differentiation of CSCs. It was
further shown that the effect of both TNF-α and IFN-γ, induced by the NK cells, on tumors is important for regulating the cytotoxicity of NK cells [105]. Addition of IFN-γ augmented differentiation in A375 melanoma and MBA-MB-231 breast cancer cells and upregulated CD54, B7-H1, MHC class I, and MICA surface expression similar to the effect mediated by split-anergized NK cell supernatants [106]. Addition of anti-IFN-γ along with supernatants from split-anergized NK cells to OSCSCs increased cytokine secretion while significantly inhibiting the increase in MHC class I or CD54 surface receptor expression, while both IFN-γ and TNF-α were required to restore NK cell cytotoxicity [106]. In in vivo studies, NK cell-induced differentiation of CSCs and poorly differentiated tumors was also shown to result in the reduction of the rate of tumor growth and induction of resistance of tumor cells to NK cell-mediated cytotoxicity [103–105,107,108].

However, not all CSCs derived from tumors behave equally. Further experiments showed that brain tumor stem cell line X02GB and oral tumors OSCSCs respond differently to NK cell-mediated differentiation [109]. Upon differentiation with split-anergized NK cell supernatants X02GB cells increase, rather than decrease, inflammatory cytokine and chemokine IL-6 and IL-8, whereas NK cell cytotoxicity and IFN-γ secretion decrease [106]. Proinflammatory cytokine IL-6 is known to favor the survival of CSCs [109]. Sustained release of proinflammatory cytokines IL-6 and IL-8 by glioblastoma multiforme (GBM) in the presence of decreased IFN-γ secretion from interacting NK cells and inactivation of NK cell cytotoxic function may lead to GBM CSCs survival and suboptimal tumor differentiation [106]. This could likely be one of the underlying mechanisms for GBM’s aggressive behavior and poor prognosis in patients.

The stage of differentiation of many tumors including GBM is predictive of their sensitivity to NK cell lysis [106]. Differentiation of the CSCs by split-anergized NK cells increases their key differentiation receptors and induces tumor cell resistance to NK cell-mediated cytotoxicity [91]. On the other hand, differentiated cancer cells are more sensitive to conventional anticancer therapies or to CTL lysis. As shown by our recent results ([91], Senjor et al., in preparation), besides some myeloid cells, CSCs can also be a source of cystatin F, an inducer of NK cell split anergy.

The role of myeloid cell differentiation in NK cell function

Myeloid cell differentiation from common myeloid progenitors into distinct subsets is guided by a series of specific transcription factors including PU.1, CCAAT/enhancer-binding proteins (C/EBP) α and β, and interferon-regulatory factors (IRF) [110] (Fig. 2). PU.1 cooperates with IRF-8 to drive macrophage commitment from granulocyte/macrophage progenitors and also stimulates the development of dendritic cells (DCs), whereas C/EBPα instructs granulocytic differentiation—reviewed in Refs. [111, 112]. Myeloid cells are sensitive to the microenvironmental cues and can be further reprogrammed to distinct functional states, either supporting inflammation or immunosuppression, mainly through nuclear factor kappa B (NF-κB) and signal transducer and activator of transcription (STAT) family of transcription factors [113–116].

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Among differentially regulated genes during lineage commitment and myeloid cell differentiation are also those encoding for the peptidases and their inhibitors. IRF-8 was shown to cooperate with PU.1 to induce expression of genes encoding multiple lysosomal enzymes, including cathepsins C, L, and S as well as their inhibitor cystatin C, in DCs and macrophages [117,118]. Conversely, levels of cystatin F are downregulated by C/EBPα during monocytic and even more so during granulocytic differentiation of promonocytic cell line U937 [119]. Accordingly, activities of cathepsins C, L, and S, no longer inhibited by cystatin F, increase after differentiation of U937 cells to macrophages. Interestingly, cathepsin H activity was not affected by cystatin F in U937 cells, even though cystatin F inhibits cathepsin H in NK cells, implying that sensitivity of individual cathepsin to the inhibition varies between different cell types [59,120]. Cystatin F is unique among related cystatins, since it can be transported to the endolysosomal vesicles after synthesis [120]. Therefore, it is speculated to be the main regulator of vesicular activity of several cathepsins in immune cells. However, cystatin F is also secreted from immune cells, including CTLs, promonocytic cells, and DCs [49,50]. Indeed, cystatin F-rich myeloid cells may represent a reservoir for extracellular cystatin F, which could, after internalization into bystander cells, limit the cytotoxicity of NK cells [50,58,60] (Fig. 2).

After lineage commitment, cysteine cathepsin expression can be further modulated. Synergistic activation of STAT-3 and -6 by cytokines IL-4 and IL-6 or IL-10 drives the production and secretion of cathepsins B, C, H, L, S, and X from murine macrophages [121]. Furthermore, cathepsin expression can be upregulated in hypoxic TME, as is evident for cathepsin B, which contains hypoxia response element in its promoter region [122]. While there is no doubt that expression and activity of peptidases in immune cells are profoundly affected...

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by signaling pathways initiated via differentiating, activating or inhibitory stimuli, cathepsins and their inhibitors in turn can contribute to signal transduction. Extracellular thyropin from p41 invariant chain, which is one of the isoforms of MHC class II chaperone molecule that is involved in antigenic peptide presentation, inhibits cathepsins F, H, L, K, S, and V [123]. However, it was also shown to interfere with nuclear translocation of NF-κB p65 subunit in lipopolysaccharide-stimulated DC, thus resulting in reduced secretion of IL-12 [124]. Well described is also cathepsin X-mediated cellular signalization, through proteolytic processing of C-terminus of β2 integrin chain [125,126]. Upon activation of DC, cathepsin X translocates to the plasma membrane and enables transition of integrin receptor Mac-1 to the high-affinity form for adhesion-dependent maturation of DC [127]. Activating stimuli-induced cathepsin X periplasmic association with β2 integrin receptors is also important for adhesion and phagocytosis of U937 macrophages [128].

Cathepsins and cystatins possess also functions that are independent of their catalytic or inhibitory activity. In this regard, cystatin C has been shown to interact with transforming growth factor β (TGF-β) and interfere with binding to its receptor [129]. Notably, TGF-β signaling has been associated with impaired NK cell function in cancer [130–132]. Several other reports noted that cathepsins and their inhibitors could impact the composition of the TME, by influencing the production of immunomodulatory cytokines from myeloid cells. For example, cathepsin B is necessary for trafficking of TNF-α-containing vesicles to the plasma membrane and subsequent secretion of TNF-α from macrophages [133]. Importantly, cathepsin B deficiency inhibited the development of myeloid-derived suppressor cells (MDSCs) in vivo by reducing the levels of TNF-α [134]. Cathepsin activity is also needed for generating mature form of cyclooxygenase 2, which catalyzes the production of prostaglandin E2 [135]. TNF-α [136] and prostaglandin E2 [137,138] are both known as negative regulators of NK cell function.

Proteolytic networks play an essential role in regulating the key functions of myeloid cells during differentiation, activation, and maturation, ranging from phagocytosis, antigen presentation, cytokine production, and secretion to signaling. Moreover, peptidases have been associated with functional polarization of myeloid cells into tumor-associated macrophages (TAM) and MDSCs (reviewed in Ref. [139]). Myeloid cells exhibit remarkable plasticity that is reflected in their phenotypical heterogeneity and differential functionalities. Shaped by the TME, myeloid cells become potent suppressor of antitumor immune responses and impair the function of CTLs and NK cells [140–142]. Transition to immunosuppressive TAM [143,144] (also referred to as alternatively activated or M2 macrophages in in vitro studies) and MDSC [145,146] is marked by overexpression of cysteine peptidases. Moreover, cysteine peptidases seem to be involved in the maintenance of M2 macrophage polarization and MDSC accumulation. Inhibition of cathepsins B, L, and S by broad-spectrum inhibitor GB111-NH2 yielded apoptosis of TAM in vivo due to increased oxidative stress, which resulted in tumor regression [147]. Genetic deficiency of cathepsin B diminished MDSC numbers and reduced premalignant lesions in mouse model of hereditary adenomatous polyposis [134]. In addition, in vitro studies implicated cathepsins L [148] and K [149] in supporting M2 phenotype of macrophages.

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Conclusions

NK cells represent crucial effectors of antitumor immune responses as they are capable of targeting CSCs/undifferentiated tumors, which are resistant to most conventional antitumor therapies. In the tumor microenvironment, NK cells are exposed to several factors, which cause the loss of cytotoxic function. Importantly, cysteine cathepsins regulate the activity of granule-dependent cell cytotoxicity, which is a major mechanism NK cells utilize to kill tumors. To restore the function of NK cells, the reactivation of converting activity of cysteine cathepsins, in particular cathepsins C, H, and L could be a promising approach. To achieve this, their increased expression and activation is an option, however, in this manner, it would be difficult to target solely NK cells and not affecting cathepsins’ function in other cells.

The regulation of cystatin F, the inhibitor of cysteine cathepsins, localized in edosomal/lysosomal pathway, represents another possibility to restore NK cell cytotoxicity. We clearly showed that increased levels of active cystatin F correlate well with split anergy status of NK cells and that the uptake of extracellular cystatin F rather than its increased expression represents a source for cathepsins’ inhibition. The function of cystatin F could be regulated in several ways. Modification of its glycosylation profile could alter its cell trafficking and/or prevent its internalization. Additionally, the regulation of transcription factors could decrease its expression. Finally, its activation and monomerization from inactive dimeric form could be prevented by targeting the activating peptidase.

Nevertheless, cysteine cathepsins are involved in several other mechanisms which may also affect NK cell function. For example, they regulate CSCs and their interplay with NK cells, differentiation of myeloid cells as well as they can modulate immunosuppressive properties of MDSCs. Precise targeting of particular cathepsins involved in immunosuppressive processes in these cells may additionally improve NK cell function.

Taken together, precise regulation of cysteine cathepsins in NK cells, their target cells, and bystander cells in tumor microenvironment offers a promising opportunity for improving cancer immunotherapy and the outcome in cancer patients.

Acknowledgment

This research work was supported by the Slovenian Research Agency, grants P4-0127, J4-1776, to JK, J3-2516 to MPN.

References

5. The role of peptidases and their inhibitors in NK cells


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Novel strategies to expand supercharged NK cells with augmented capacity to withstand inactivation by tumors

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Abstract

Natural killer (NK) cells are the main immune effectors with the ability to mediate selection and differentiation of a number of different cancer stem cells/undifferentiated or poorly differentiated tumors via lysis, and secreted or membrane bound IFN-\textgreek{g} and TNF-\textgreek{a}. Tumor differentiation by NK cells leads to growth inhibition and curtailment of tumor metastasis. In this chapter, we present an overview of our recent and past findings on the biology and significance of NK cells in the selection/differentiation of stem-like tumors using \textit{in vitro} and in vivo studies conducted in the NSG and humanized-BLT mice and those of cancer patients. In addition, we discuss the significance of heightened NK cell function in a great variety of gene knockout mice, providing a potential rationale for the mechanisms of the activation of NK cells in these animals. Moreover, we report on the recent advances in NK cell expansion and therapeutic delivery, and discuss the superiority of allogeneic
supercharged NK cells over their autologous counterparts in the treatment of cancer patients. Furthermore, we discuss the potential loss of NK cell numbers and function at the neoplastic and preneoplastic stages of tumorigenesis as potential mechanisms for the induction and progression of pancreatic cancer. We also report on the novel ways of assessments of NK function within the 3D model of tumorospheres, to better represent the tumor microenvironment. Therefore, because of their indispensable role in targeting cancer stem-like/undifferentiated tumors, and a variety of other key functions of NK cells reviewed in this report, including their role in the selection and expansion of CD8+ T cells, and their potential functional inactivation by the tumors within the tumor microenvironment, which are mitigated by the mode of activation and expansion of NK cells, these cells should be placed high in the armamentarium of tumor immunotherapy. The combination of allogeneic supercharged NK cells with other therapeutic strategies such as the use of oncolytic viruses, ADCC-inducing antibodies, checkpoint inhibitors, CAR-T, CAR-NK, chemotherapeutic, and radiotherapeutic strategies can be used for the optimal treatment strategies.

Abbreviations

- ADCC: antibody-dependent cellular cytotoxicity (ADCC)
- CSCs: cancer stem cells
- Hu-BLT: humanized-bone marrow/liver/thymus
- IFN-γ: interferon-gamma
- iPSCs: induced pluripotent stem cells
- MDSs: myeloid-derived suppressor cells
- MHC-Class I: major histocompatibility complex molecule class I
- MP2: MiaPaCa-2 pancreatic cancer stem cells
- MSCs: mesenchymal stem cells
- NK cells: natural killer cells
- NSG mouse: NOD SCID gamma mouse
- OSCSCs: oral squamous cancer stem cells
- PBMCs: peripheral blood mononuclear cells
- PD-1: programmed death ligand 1
- rhIL-2: recombinant human IL-2
- TAMs: tumor-associated macrophages
- TNF-α: tumor necrosis factor-α

Conflict of interest

Authors declare no conflict of interest.

Natural killer cells: Overview and background

Natural killer (NK) cells mediate direct cytotoxicity and regulate both the innate and adaptive immune functions through the release of many pro and anti-inflammatory growth factors, cytokines and chemokines [1, 2]. They constitute 5%–15% of the peripheral blood mononuclear cells (PBMCs), and are cytotoxic effectors in the blood of healthy individuals with the ability to recognize and lyse a number of different cancer stem cells (CSCs) and undifferentiated or poorly differentiated tumors, expressing lower levels of surface MHC-class I, CD54 and PD-L1 (B7H1) receptors and higher expression of CD44 [3–6]. Although different modulations were found for the levels of expression of CD54, PD-L1, and CD44 surface receptors depending on tumor type or maturational stage of the tumors, in general we did
observe more consistent correlations of the expression of MHC-class I with the stage of the differentiation of the tumors and their susceptibility to NK cell-mediated cytotoxicity.

NK cells have two main effector functions: cytotoxicity and cytokine release. NK function is regulated by the sum of interactions between activating and inhibitory receptors on their surface and the ligands on the target cells [7]. Ligands for activating receptors are expressed on fast proliferating cells that are virally infected or malignantly transformed. Synergistic activation of multiple activating receptors by different ligands is thought to be necessary to surpass the threshold for activation. That threshold is lowered, if NK cells are previously activated in a suitable cytokine microenvironment, consisting of IL-2, IL-15, and IL-12. It is speculated that the role of inhibitory receptors on the NK cells is to prevent the killing of healthy self-cells, by binding to MHC-class I [8]. The cytotoxic activity of NK cells is executed by two distinct mechanisms. One mechanism is regulated by the cytotoxic granules containing perforin and granzymes. After the formation of the immune synapse between NK and target cells, cytotoxic granules are released, and thereby perforin alters the permeability of the target cell membrane, allowing the entry of granzymes. A cascade of reactions causes activation of caspases and eventual induction of apoptosis in the target cells [9]. Important regulators of this cytotoxic pathway are cathepsins C and L, which activate granzymes and perforin from their precursor forms, respectively. The activity of cathepsins is regulated by cystatins [10]. The second mechanism, which also causes caspase activation in the targets, is interaction of ligands on NK cells with their respective cell death receptors on target cells. Target cell death can also be induced through antibody-dependent cellular cytotoxicity (ADCC), by the interaction of the Fc region of an antibody with the CD16 receptors.

The second important effector function of NK cells is the release of cytokines and chemokines. Two major cytokines released by NK cells are IFN-γ and TNF-α [9]. Released cytokines not only affect the function of innate and adaptive immune cells, but they can also impact the differentiation of both healthy and cancer cells.

Based on their surface phenotype, NK cells are characterized as CD3− and CD56+/CD16+ cells. NK cell populations can be divided into two major groups: CD56dim and CD56bright. The majority (90%) of the NK cell population consists of CD56dim cells in peripheral blood. This population expresses the CD16 receptor and is speculated to be the mature form of NK cells that is capable of executing all effector functions. In all, 10% of the NK cell populations in the blood is characterized as CD56bright which have no or low expression of CD16 receptors in the peripheral blood. This subset is thought to be a less mature form of NK cells, although, it might also arise from a different cell lineage, or develop from the activated CD56dimCD16bright subset of NK cells which, upon interaction with the target cells, loses the expression of CD16 and increases the levels of CD56 expression. Interestingly, similar to the splint anergized NK cells (see below) in which the surface expression of CD16 is substantially diminished or completely lost, and their cytotoxicity decreased while the ability to secrete cytokines is increased, the CD56bright subset of NK cells also lacks cytotoxicity but is able to secrete cytokines [11, 12]. Tissue-resident NK cells differ from peripheral blood NK cells, as they are predominantly the CD56bright population of NK cells [12]. Furthermore, based on NK cell function and surface receptor expression, we have characterized four distinct stages of NK cell maturation. A total of 90% of the peripheral blood NK cells from healthy individuals are identified as the CD16brightCD56dimCD69− subset, which are capable of mediating cytotoxic functions,
and are able to kill CSCs/undifferentiated tumors after recognizing and binding to the target cells at their initial contact, constituting the stage one of the NK cell maturation. NK cells that acquire the CD16lowCD56brightCD69bright phenotype after binding and recognizing target cells are found to have low or no cytotoxic ability, but increased capacity to secrete cytokines. These cells are considered to be split anergized, and, therefore, constitute the second stage of the maturation of NK cells. These cells are able to regulate the function of other cells and are capable of differentiating tumor cells. If NK cells lose both the cytotoxic function and IFN-γ secreting capabilities that are seen in some cancer patients, and those suffering from chronic diseases or infections, the NK cells may enter the third stage of NK cell maturation by exhibiting disturbed cytokine and chemokine expression profiles, in addition to decreased cytotoxicity. This stage of the NK cell maturation is very similar to those seen in NK92 tumors or other transformed NK tumors as they do not mediate cytotoxicity or secrete IFN-γ but are able to produce a skewed proinflammatory cytokine pattern such as increased IL-6 and IL-10 secretions [13]. Finally, the fourth stage of NK cell maturation represents the apoptotic NK cells since the fate of functionally unresponsive NK cells may ultimately be cell death [6, 14, 15].

The different stages of NK cell maturation, therefore, greatly affect their function and impact on their target cells, which is especially important in the context of the tumor microenvironment. We have found that stage 1 NK cells are able to kill cancer stem cells and secrete cytokines that promote the differentiation of cancer cells. However, after the interaction with the target cells the cytotoxic ability of NK cells is diminished and they will enter in the stage 2 of maturation and become split anergized, as they maintain the ability to secrete cytokines [16, 17]. Split anergy in NK cells in stage 2 is different from anergy in T cells, since T cells lose all effector functions when anergic [18]. Even though split-energized NK cells are not able to kill tumor cells, they constitute an important stage in the tumor microenvironment as they are the main source of IFN-γ and TNF-α secretions that stimulate the differentiation of cancer stem cells into a more differentiated phenotype that eventually can be recognized by the cytotoxic T cells. The split anergy of NK cells can also be mimicked in in vitro conditions by activating with recombinant human IL-2 (rh-IL-2) and anti-CD16 monoclonal antibodies (mAb) [19].

In some cancer patients, the numbers and frequencies of stage 3 and 4 NK cells increase substantially, leading to ineffective NK cell-mediated functions [20, 21]. Decreased NK cell cytotoxicity in the tumor microenvironment and the peripheral blood of cancer patients as well as downmodulation of CD16 receptors on the surface of NK cells have been reported previously [22–25]. Decreased function of NK cells is associated with increased cancer risk, whereas higher activity and increased NK cell infiltration of tumor cells is associated with better prognosis [26, 27]. Therefore, if split-energized NK cells are rescued by signals from activating competent myeloid cells such as monocytes, dendritic cells, or osteoclasts, NK cells may become expanded and give rise to supercharged NK cells (see below). The ultimate fate of supercharged NK cells may be activation-induced cell death and/or may potentially give rise to a small proportion of NK cells, which are capable of maintaining the pool of competent NK cells in stages 1 and 2 of NK cell maturation. Alternatively, the small surviving progenies of supercharged NK cells could give rise to the so-called memory-like or phenotypically altered subpopulation of enhanced NK cells, which could maintain the pool of NK cells with heightened proliferative and functional potentials. These different scenarios are currently under investigation in our laboratory. Below we will define stage 2 of the NK cell maturation.

II. Activation of NK Cells
Split anergy in NK cells and the role in cellular differentiation

NK cells sorted from the healthy individuals are cytotoxic whereas those found in the mucosa are regulatory, with the ability to produce large amounts of cytokines in the absence of appreciable cytotoxicity [13]. Unlike those of the cancer patients or patients suffering from chronic diseases or infections, the peripheral blood of healthy individuals contains a fairly sterile microenvironment with potentially lower burden of antigens lacking appreciable drivers of inflammation [28, 29]. In contrast, the mucosa comes in contact with a multitude of antigens that have the ability to keep the immune cells, in particular NK cells, in an activated state, in which case the cytotoxicity of NK cells is likely decreased or is inhibited in the presence of increased cytokine secretion. Such a state of NK cells may not only drive the regulation of the immune effector function but also imparts the ability to NK cells to aid their interacting cells to function at a heightened state in order to safeguard against immune attack and lysis of surrounding healthy cells, due to the increase in differentiation of the cells by the secreted cytokines. To fully understand how NK cells function within the tumor microenvironment or during viral and bacterial infections, we need to understand how these intricate interactions take place and how tumor infiltrating NK cells are modified depending on the cues they receive from the microenvironment to either kill or differentiate tumors, or do both, or mediate neither of such functions. Therefore, the stage of split anergy in NK cells [16, 17, 30], we believe, is important for the differentiation of neighboring undifferentiated cells. In contrast, the interaction of differentiated tumor cells with the NK cells does not induce either activation or split anergy in NK cells, indicating that differentiated tumors are not targeted by the NK cells [31, 32]. We have previously demonstrated that split anergy in NK cells can also be induced by the treatment of NK cells with rh-IL-2 with anti-CD16 mAb or in the presence of rh-IL-2 and triggering of the toll-like receptors with bacteria, modeling the activation process which NK cells undergo upon interaction with tumor cells, or bacterial and potentially viral infections [14, 16, 28, 30].

Thus, split anergy in NK cells is defined as a selective loss/decrease in cytotoxicity in the presence of increased cytokine and chemokine secretions [17, 18, 33]. As indicated above, this functional stage of NK cells is important for mediating the differentiation of tumors. This potentially results in the termination of NK cell activation, as well-differentiated tumor cells are not/or targeted less by the primary activated NK cells [17, 31, 34].

Upon interaction with NK cells, why do the majority of gene-knockout mice or cells demonstrate increased NK function? Potential relationship with dedifferentiation of the cells

The list of cellular genes that augment NK cell function when deleted or decreased in tumors is increasing as more knockouts are tested for their ability to activate NK function [35]. In our previous studies, we have provided the list of genes which upon deletion in cells augment NK cell function in mice and in in vitro culture models [36, 37]. Specifically, the deletion
of NF-κB in tumors was found to increase NK cell-mediated cytotoxicity and secretion of IFN-γ significantly [38], and induce autoimmunity and inflammation in vivo [35]. Moreover, conditional knockout of STAT3 in hematopoietic cells was found to result in the induction of colitis in mice due to chronic gut inflammation [39]. Knock-down of CD44 in breast and melanoma tumors was also able to increase the expansion and functional activation of NK cells significantly [4, 40, 41]. In addition, targeted knockdown of COX2 in nontransformed healthy myeloid cells and mouse embryonic fibroblasts was found to increase expansion, and functional activation of NK cells significantly [35]. Indeed, the list of gene knockout studies in mice and in vitro tumor models, which were found to increase the activation of NK cells by us and those of the other laboratories, has been steadily increasing as more gene knockouts are being examined [35].

One common underlying mechanism for the activation of NK cells in our gene knockout studies was found to be related to the downmodulation of MHC-class I expression on both transformed and nontransformed healthy cells [4, 35, 40, 41]. Surprisingly, hyperresponsiveness of the NK cells were also seen in mice with knockouts of genes that mediate inflammation, in particular even those that are involved in NK cell signaling and activation, such as DAP10/DAP12, indicating that NK cell activation is much more complex than we have previously envisioned, involving many genes/pathways, and is likely dependent on the modulation of the stage of the differentiation of the cells by these genes/pathways [42]. Therefore, such increases in responsiveness of NK cells when key cellular genes were knockout or knockdown in interacting cells/tumors may point to the fundamental function of NK cells in targeting cells that lose ability to differentiate optimally, and that the degree of differentiation of the cells is likely the key in regulating NK cell expansion and function.

Multiple defects of NK function in cancer patients

It is well known that both cytotoxic function and IFN-γ secretion capabilities of NK cells are compromised in cancer patients [24]. Many mechanisms have been attributed to the loss of NK cell function, including downmodulation and decrease in important receptors, such as CD16 [43], NKG2D [44], zeta chain [45], and decreased survival and expansion of NK cells [46], and decreased secretion of important cytokines such as IFN-γ [46]. The loss of NK cells not only is the key factor in decreased lysis of CSCs/undifferentiated tumors, but it is also important in the decreased secretion of IFN-γ, which ultimately results in decreased differentiation of the tumors [47]. In addition, when using the same amounts of IFN-γ secreted from healthy and cancer patients’ NK cells, those from cancer patients had much lower ability to differentiate tumors when compared to IFN-γ secreted from healthy individuals [46]. Not only is the restoration of IFN-γ secretion by patient NK cells important for the effective control of tumors in cancer patients, but also the restoration of the functional capability of secreted IFN-γ is important for optimal differentiation of the tumors.
Defects in NK cells are observed at the preneoplastic stages of tumorigenesis

Pancreatic ductal adenocarcinoma (PDAC) induced by KRAS mutation, is the most severe form of pancreatic malignancy [48]. We have recently demonstrated that feeding high-fat calorie diet (HFCD) to mice with pancreatic KRAS mutation severely inhibited NK cell function at the preneoplastic stage of pancreatic cancer [28, 49]. NK cytotoxicity was decreased in the peripheral blood, spleen, pancreas, and peripancreatic tissue in mice with KRAS mutation, which were fed HFCD when compared to wild type (WT) mice fed with lean control diet (CD) [28]. NK cells cultured with autologous monocytes from mice with KRAS mutation fed HFCD exhibited decreased expansion, cytotoxicity and IFN-γ secretion.

Osteoclasts from mice with KRAS mutation fed HFCD expressed much lower levels of MHC-class I inhibitory ligands and RAE1-delta activating ligands, suggesting that both inhibitory and activating ligands for signaling of NK cells were decreased [28]. The decreased levels of MHC-class I and RAE1-delta detected on osteoclasts correlated with the generation of preneoplastic lesions (PanINs) in mice with KRAS mutation, indicating that the loss of surface receptors on osteoclasts in combination with decreased expansion and function of NK cells may be a better indicator of PanIN induction [28, 49]. Thus, our results suggested that NK cell defect induced by both genetic and environmental factors at the premalignant stage of pancreatic cancer may drive the establishment and progression of pancreatic cancers [28, 49].

Synergistic increase in IL-6 secretion in the presence of decreased IFN-γ secretion during interaction of peripancreatic adipose-derived cells with NK cells could be one mechanism by which the adipose tissue can contribute to the increased proliferation of pancreatic tumors [28]. Indeed, IL-6 is one of the major drivers of PDAC proliferation [50–54], and suppression of NK function [55]. Moreover, addition of IL-6 to tumor/NK cultures inhibited NK cell-mediated IFN-γ secretion in our previous studies [4, 13, 15, 40, 56]. Therefore, as tumors have a predilection to grow in the adipose tissue, these tissues are likely to convert tumor suppressive NK cells, to tumor-promoting cells. Blocking IL-6 may not only inhibit tumor growth, but also it may rescue NK cells from suppression induced by the peripancreatic adipose tissue or those infiltrating the tumor tissues, and offer an attractive and effective therapeutic strategy to target pancreatic tumors.

Novel strategies to expand high numbers of potent NK cells for immunotherapy: Existing successes and challenges

In order to generate large numbers of NK cells with a potent function, we opted to use osteoclasts as feeder cells. We have previously shown that osteoclasts are potent activators of NK cells, and their effect in the induction of cytotoxicity and secretion of cytokines and chemokines by NK cells is much stronger than monocytes or dendritic cells [57]. Human osteoclasts produce IL-15, IL-12, IL-18, and IFN-α, but not IFN-γ, and express lower levels of MHC-class I and II, CD14, CD11b, and CD54, and minimally upregulate MHC-class I surface expression when treated with either the combination of TNF-α and IFN-γ or when treated

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with activated NK cell supernatants known to increase MHC-class I expression [57]. Low expression of MHC-class I together with increased release of IL-15, IL-12, IL-18, and IFN-α may represent some of the underlying mechanisms by which osteoclasts are able to expand functionally potent NK cells. More importantly, osteoclasts also exhibit higher expression of NKG2D ligands [57].

Several in vitro NK expansion techniques have been developed to allow for a higher therapeutic cell dose [58, 59]. Using our strategy, we expanded highly functional NK cells at the levels that were significantly more superior to those established by other methodologies [46]. Not only superior expansion of NK cells under different experimental conditions is important for the eventual efficacy of NK cells in cancer therapy, but also their functional competency is of significance. Our ongoing studies indicated that cord blood and iPSC-derived NK cells, although able to expand large numbers of cells with the NK phenotype, they are not capable of targeting and lysing CSCs/poorly differentiated tumors, or producing sufficient amounts of IFN-γ when compared to either primary NK cells derived from peripheral blood or supercharged NK cells [37].

One of the challenges the field of NK cell immune therapeutics is presently facing is the lack of standardization among all different NK cell platforms for immune therapeutics and their functional comparisons. Such standardization should provide the basis for the selection of the best products or the combination of products for use in immunotherapy. In addition, it may also provide the rationale for why the use of such products was not successful in controlling the disease in past clinical trials. The failure of different NK cell platforms in halting the progression of cancer and reducing tumor burden in different cancer models or in different human clinical trials previously should not be inferred as the lack of significance of these cells in cancer therapeutics, but the failure of the use of the most appropriate and effective strategy in successfully targeting both the suppressive elements within the tumor microenvironment (TME) as well as controlling the growth and expansion of CSCs/poorly differentiated tumors.

Which platform is the best for cancer immunotherapy: Allogeneic or autologous NK cells?

The best NK expansion was seen when NK cells from healthy donors were used in cultures with their autologous osteoclasts. In contrast, patient NK cells with autologous osteoclasts had the most severe defect in NK expansion and function [60]. Results similar to those of cancer patients were also seen in tumor-bearing hu-BLT mice [60]. Thus, when designing immunotherapeutic strategies using autologous or allogeneic NK cells, such differences in the levels of NK expansion and function should be considered and may be crucial for the success of the therapy. In addition, to increase the effect of NK therapy, the treatment may be combined with other therapeutic strategies detailed in the following sections.

Supercharged NK cells retain their cytotoxic potential and are inactivated much less after the interaction with tumor cells

We have previously shown that cytotoxicity of NK cells become largely inactivated after their interaction with tumor cells [17]. Indeed, when assessing the NK function after different
treatment strategies, the highest inactivation of NK function after their interaction with the tumor mass was seen in the untreated primary NK cells, followed by IL-2-treated primary NK cells, as indicated below. When supercharged NK cells were compared to either untreated or rh-IL-2-treated primary NK cells for the level of inactivation of cytotoxicity after interaction with the tumor mass or tumor cells, they only lost approximately 10%-30% of their cytotoxicity when compared to rh-IL-2-treated primary NK cells, which lost approximately 60%-80% and untreated NK cells about 90%-100%. Therefore, supercharged NK cells retain their cytotoxic function and are able to mediate cytotoxicity for the second round of killing. Thus, it is possible that these cells are capable of serial killing of tumor cells, and are inactivated much less by the tumor mass. Indeed, multiple supercharged NK cells were found to bind and target glioblastoma stem cells (GSCs) in a spheroid tumor model as assessed by confocal microscopy (manuscript submitted).

Supercharged NK cells are major effectors to target GSCs in a 3D culture model

Immunotherapy, including immune checkpoint inhibitors and adoptive cell transfer using CAR-T and CAR-NK cells, has transformed the treatment of solid tumors. However, there are still no efficient immunotherapeutic approaches for the most aggressive brain tumor, glioblastoma, due to its multilayer immunosuppressive microenvironment, heterogeneity, and a lack of specific tumor antigens [61]. The NK cell-based approach is a promising approach, targeting tumor-initiating and therapeutically resistant population of GSCs [37, 62]. However, challenges exist with GBM tumors that were not seen by either oral or pancreatic tumors. We have previously shown that OSCSCs or MP2 poorly differentiated pancreatic tumors treated with supernatants from split-anergized NK cells inhibited NK cell cytotoxicity, increased CD54 and MHC-I, and blocked the secretion of cytokines and chemokines significantly, including IFN-\(\gamma\), IL-6, and IL-8 due to increased differentiation of the tumors [63]. In contrast, although split-anergized NK cell supernatant-differentiated GBM tumors inhibited IFN-\(\gamma\) released from the NK cells, it had the opposite effect on IL-6 and IL-8 secretion, in which they increased rather than decreased the secretion of IL-6 and IL-8. Thus, GBM tumors are different in that they remain persistently inflammatory even upon differentiation with immune effectors, and they increase, rather than decrease, inflammatory cytokines and chemokines IL-6 and IL-8 while decreasing IFN-\(\gamma\). This is similar to what we see in the cerebrospinal fluid (CSF) of GBM patients, since they exhibit increased IL-6 and IL-8 in the presence of decreased IFN-\(\gamma\) in CSF (manuscript submitted). Although IFN-\(\gamma\) is increased in the CSF of the GBM patients after treatment with IL-2 or IL-2 + anti-CD3/CD28, this results in the decreased ratio of IL-6 to IFN-\(\gamma\) or IL-8 to IFN-\(\gamma\); and the IFN-\(\gamma\) secreted by the immune effectors in CSF are likely to increase the differentiation of GBM tumors; however, such differentiation will result in only a further decrease in IFN-\(\gamma\) secreted from the interacting immune effectors while the levels of IL-6 or IL-8 will continuously rise, a scenario which we see when the NK cells interact with GBM tumors. Thus, even during the immunotherapeutic treatment of GBM patients with the NK cells, the levels of IL-6 or IL-8 should be targeted to minimize their effect on the growth or expansion of more tumors [63].
Trends in cancer research and immunotherapy are going toward more in vivo-like tumor models, such as multicellular tumorospheres and organoids that mimic 3D tumor structure, to better represent the cellular interactions, and oxygen and nutrient deprivation within the tumor microenvironment. Little is known how 3D architecture influences the cytotoxic potential of NK cells as most studies have used traditional 2D culture systems for cytotoxicity measurements [64, 65]. We established a 3D tumorosphere model with GSCs, in which we assessed NK cell penetration, cytotoxicity, and also NK cell-mediated effects on GSC differentiation as established by the increase in the differentiation antigens on the surface of GSCs. Moreover, the secretion of proinflammatory cytokines IFN-γ, IL-6, IL-8, and others from GSC tumorospheres was assessed after NK cell interactions.

NK cell treatment decreased the number of GSCs and increased the number of dead cells in the 3D tumorosphere model (Fig. 1). The higher the numbers of effector cells, the lower were the numbers of GSCs within the tumorospheres (Fig. 2). Supercharged NK cells were found to increase the number of dead cells substantially when compared to primary activated NK cells (manuscript in prep.) (Fig. 3). Supercharged NK cells penetrated the tumorospheres and were found to bind to the GSCs. One to three supercharged NK cells were found to bind to one GSC tumor. In addition, the GSC tumors in the tumorosphere after interaction with the NK cells exhibited higher expression of MHC class I and CD54, indicating their increased differentiation status (manuscript in prep.). Thus, these tumor models facilitated the assessment of NK function in more relevant human tumor models. However, tumors that are being targeted by the NK cells being stem-like have very large capacity to proliferate; therefore, it is possible that even though these tumors are targetable by the NK cells, by their mere potential to proliferate substantially, they may cause exhaustion of the function of NK cells as seen in cancer patients. Indeed, NK cells from cancer patients are more inactivated than their T cells.

**FIG. 1** NK cell treatment decreased the number of GSCs and increased the number of dead cells in 3D culture model. Freshly purified NK cells were treated with rh-IL-2 (1000 U/mL) before they were added to GSC tumorospheres in NK: GSC ratio 10:1. Images of GSC tumorospheres were taken 4 h after addition of NK cells using inverted fluorescence microscope. Propidium iodide (PI) staining was used to detect dead cells (red). Scale bar = 100 μm.
Therefore, restoration of NK function, and delivery of large numbers of potent NK cells will be crucial in elimination of the fast proliferating cells at the initial stages of cancer therapy, until the load of poorly differentiated tumors are under control, which will then allow other strategies such as chemotherapeutics and the CD8+ T cells to better target the remaining differentiated tumors.

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Supercharged NK cells preferentially expand CD8+ T cells

Osteoclast-expanded supercharged NK cells from healthy donors expand for a longer period of time (days 30–60) before the small fraction of contaminating T cells start expanding resulting in the decline in the percentages of NK cells and expansion of CD8+ T cells. There is a faster kinetics of expansion of CD8+ T cells (days 12–24) with osteoclast-expanded supercharged NK cells from cancer patients when compared to healthy controls. Therefore, this could be one reason why the numbers of NK cells may decline in the tumor microenvironment as these cells will result in the expansion of CD8+ T cells. Supercharged NK cells showed the ability to lyse receptor-activated CD4+ T cells but not CD8+ T cells in a target cell visualization assay (TVA). When comparing CD8+ T cells cultured with osteoclasts, CD8+ T cells expanded with supercharged NK cells exhibited the highest activation and secretion of IFN-γ. Accordingly, oral tumor-bearing hu-BLT mice injected with supercharged NK cells demonstrated an increase in numbers and function of CD8+ T cells [29]. Thus, supercharged NK cells are important in the selection and expansion of CD8+ T cells by increased targeting of CD4+ T cells [66]. Supercharged NK cells expand effector and central memory CD8+ T cells [66].

NK cell immunotherapy may enhance the effect of chemotherapy and radiation

CSCs/undifferentiated tumors were shown to be chemoresistant due to their increased expression of multidrug resistance and DNA mismatch repair genes [67]. Increased survival and selection of CSCs/undifferentiated tumors after chemotherapy results in relapse, metastasis, and invasion of tumors [68, 69]. Thus, our findings indicated that CSCs/undifferentiated tumors, even though are highly susceptible to NK cell-mediated cytotoxicity, they are quite resistant to either cis-diamminedichloroplatinum (CDDP), a chemotherapeutic drug also known as cisplatin, or radiation-induced cell death, whereas their differentiated counterparts, even though are resistant to NK cell-mediated cytotoxicity, are susceptible to both CDDP and radiation-induced cell death [4].

CDDP induced significant cell death in oral squamous carcinoma cells (OSCCs) when compared to oral squamous cancer stem cells (OSCSCs). Similarly, OSCCs were highly susceptible to radiation treatment, whereas OSCSCs did not undergo cell death after radiation [4]. Differentiation of OSCSCs or MP2 pancreatic poorly differentiated/CSC tumors with supernatants from NK cells resulted in a significant increase in their susceptibility to CDDP, whereas undifferentiated OSCSCs or MP2 cells remained relatively resistant to CDDP. Melanoma tumors stably expressing shRNA against firefly luciferase (A375shLUC) demonstrated higher differentiation antigens, and were significantly more susceptible to CDDP-mediated cell death even though they were resistant to NK cell-mediated cytotoxicity whereas those which had knock-down of CD44 were susceptible to NK cell-mediated cytotoxicity while they were more resistant to CDDP. Differentiation with NK cell supernatants increased CDDP-mediated cell death of CD44 knock-down cells substantially, whereas it made them more resistant to NK cell-mediated cytotoxicity [4]. These data suggest that
knockdowns of cellular genes, and their reversion to a less differentiated phenotype may activate NK cell-mediated cytotoxicity but it may also lead to the resistance of these cells to chemotherapeutic agents. Thus, the stage of differentiation may be a determinant of tumor susceptibility to NK cell-mediated cytotoxicity as well as their response to chemotherapeutic drugs. Therefore, a tailored therapeutic scheme using a combination of NK immunotherapy and chemotherapy is important for the efficient elimination of both CSCs/undifferentiated and differentiated tumors present in TME.

NK cells can target CSCs and their differentiated counterparts through direct lysis and/or ADCC, respectively

NK cells can eliminate both undifferentiated and differentiated tumors through direct killing and through antibody-dependent cellular cytotoxicity (ADCC) respectively [70–72]. Indeed, if antibodies against specific receptors expressed on differentiated tumors are available, even though NK cells will not be able to lyse these cells directly, they can eliminate such tumors through ADCC. Such observations were made with regard to the increased expression of MICA/MICB and PD-L1 on differentiated tumors, and much less on CSCs/undifferentiated tumors, and the differentiated tumors were found to be targeted greatly through NK cell-mediated ADCC while minimally through direct lysis [4, 73]. Similarly, antibodies targeting specific receptors on CSCs/undifferentiated tumors should be able to lyse these tumors through NK cell-mediated ADCC as well as direct lysis. Interestingly, when NK cell-mediated ADCC was assessed against poorly differentiated OSCSCs no significant augmentation in cytotoxicity could be seen even though there was an increased surface receptor expression for that specific receptor [73]. We are currently delineating the differences in the killing of well-differentiated and poorly differentiated tumors through direct killing and ADCC, and the mechanisms governing each killing method. Moreover, sensitizing antibodies such as IL-4 mAbs have also been used in combination with standard chemotherapy to successfully target CD133bright CSCs of colon cancer [74]. A large number of antibodies targeting tumors such as anti-EGFR, anti-HER2, and anti-PD-L1 antibodies, to name a few, have been generated and are currently being used to treat patients. These antibodies could potentially not only kill tumors directly but also indirectly through NK cell-mediated ADCC [75]; therefore, delivery of competent NK cells to the patient should be able to kill CSCs/poorly differentiated tumors directly, as well as well-differentiated tumors through antibody-mediated cytotoxicity. Therefore, NK cells are indispensable effectors of the immune system as they could target tumor cells with diverse mechanisms.

Genetically modified oncolytic viruses have gained popularity as exciting therapeutic modality for cancer treatment with approved agents for cancer therapy [76]. Genetically engineered viruses kill cancer both directly by infection and lysis of tumors, and indirectly by inducing host cytotoxic immuno-responses to cancer by modulating the
imunosuppressive nature of tumor microenvironment. Immune cell infiltration in tumors is predictive of response to immunotherapies, with the type I and type II interferon (IFN) pathways being the key players in the activation of adaptive immunity [77].

Oncolytic viruses (OVs) enhance immune activation against tumors through multiple mechanisms. OVs enhance infiltration of tumors by immune cells, upregulate costimulatory molecules on cancer cells, enhance immunogenic cell death and antigen release [78], and activate the type I IFN pathway. Preclinical [79] and clinical evidence [80] clearly indicate that OV’s turn immunologically “cold” tumors into “hot” tumors. Treatment of refractory disseminated melanoma with agents targeting immune checkpoint inhibition is greatly enhanced by oncolytic herpes viral therapy [81].

OV therapies not only activate T cells, but also NK cells. In a recent preclinical study of Newcastle Disease oncolytic viruses (NDV), intratumoral therapy of B16 melanoma with oncolytic NDV was shown to induce inflammatory responses leading to lymphocytic infiltrates and antitumor effect locally and in distant tumors without virus spread. Immune cell depletion studies showed that both NK and CD8+ T cells were essential for the therapeutic effect of OVs [79]. NDV was also shown to activate NK cells [81]. It appears that NK cells are key immune cells for early inflammatory responses and IFN-γ production after OV therapy, while CD8+ T cells are responsible for long-term antigen-specific tumor control [79].

Although NK cells may kill infected cancer cells and limit the amplification of OVs, studies have found that NK cells often have positive effects on therapeutic outcomes of OVs [82–85]. Different types of oncolytic viruses have been engineered to target and lyse pancreatic tumors. They have been engineered to not only be effective in lysing the tumors but also to allow effective activation of immune effectors, in particular NK cells [86, 87]. Virus-infected cancer cells tend to downregulate their MHC-class I, making them good targets for NK cells [82, 83].

This ability of the virus to downmodulate MHC-class I on tumors occurs both in poorly differentiated as well as in well-differentiated tumors. We have found that different preparations of oncolytic viruses have different potencies in lysing the tumor and activating the function of NK cells [88].

Combination therapy with NK cells and immune checkpoint inhibitors

Treatment with check-point inhibitors has shown success in certain cancers and for select group of patients. Many recent reports have indicated the ability of NK cells to become activated through checkpoint inhibitions [89–91]. In particular, the role of anti-PD-1/PD-L1 axis in NK cell inhibition and the ability of antibody to anti-PD-1 to activate NK cells have been studied in many laboratories including ours [89, 91]. We have shown previously that when tumors are implanted in the oral cavity [29] or in the pancreas of hu-BLT mice [60], and injected either with supercharged NK cells or with anti-PD-1 antibody, both were able to activate immune cells to secrete higher amounts of IFN-γ when compared with tumor implanted mice in the absence of these agents [37]. Remarkably, infusion of NK cells and treatment with anti-PD-1 antibody had a synergistic effect, and increased IFN-γ secretion.
substantially when compared to the infusion of each agent alone [37]. The increased secretion of IFN-γ can be both from the NK cells and T cells as activated NK cells can also activate T cells, thereby providing increased expression of PD-1 on the T and NK cells, making them more susceptible to the activation through anti-PD-1 antibody. Anti-PD-1 antibody treatment had differential effect on cytotoxicity vs IFN-γ secretion in hu-BLT mice implanted with oral tumors and infused with supercharged NK cells and fed with AJ2 probiotic bacteria. Whereas NK cell-mediated cytotoxicity was increased by the treatment of anti-PD-1 antibody, secretion of IFN-γ was variable due likely to the plateauing effect of IFN-γ secretion by the treatment of supercharged NK cells in the presence of AJ2 feeding [29]. Nonetheless, there is a clear effect of anti-PD-1 antibody treatment when combined with supercharged NK cells in the presence of probiotic bacteria AJ2 [29].

More recent work has implicated other checkpoint inhibitors such as TIGIT in NK cell function, and their blockade was shown to activate NK cells [92, 93]. Future immunotherapeutic strategies may make use of the blockade of several different checkpoint inhibitors for effective targeting of tumor cells by the NK cells.

Conclusion

NK cells have proven to be indispensable for cancer therapy; however, only recently we have started to appreciate the scope of the significance of these cells in cancer therapy. Identification of cancer stem cells or poorly differentiated tumors as prime targets of NK cells has finally shown the indispensable role that these effectors play in the successful treatment of cancer patients, as NK cells are potentially one of the main if not the main effectors that target these cells. In addition, studies from our laboratory and those of the others have shown significant suppression in the functions of NK cells and T cells in cancer patients, indicating that successful cancer therapy will require restoration of both NK and T cell functions in cancer patients as each are likely designed to target specific subsets of tumor cells perhaps with opposing degrees of cellular differentiation. NK cells are likely targeting CSCs/undifferentiated tumors with no or lower MHC-class I expression that are not targets of T cells since these cells are known to target tumors with higher expressions of MHC-class I. NK cells mediate successful control of the tumor cells, by their direct cytolytic effect and/or through ADCC or indirectly through differentiation of tumor cells by IFN-γ, which increases the efficacy of chemotherapeutic and radiotherapeutic targeting strategies. Because of significant changes in the cancer patients’ immune environment, selection of allogeneic or autologous NK cell immunotherapy should be considered carefully as many cancer patients have defective NK cell function. In addition, strategies should be designed to allow maintenance of good NK expansion and function in cancer patients since NK cells are likely to limit the expansion of TAMs, Tregs and MDSCs, MSCs and fibroblasts; all of which are immunosuppressive cells and constitute the hallmarks of aggressive tumors. Large numbers of allogeneic supercharged NK cells can be combined with other immunotherapeutic strategies such as oncolytic viruses, ADCC-inducing antibodies, check point inhibitors, CAR-T, CAR-NK, and chemotherapeutic and radiotherapeutic strategies for the ultimate goal of tumor eradication.

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Acknowledgments

The authors acknowledge the help of numerous undergraduate, graduate, postdoctoral fellows, and visiting scholars and faculty members for their excellent contribution to the work presented in this chapter. In addition, we are grateful to our funding agencies and donors for supporting the work presented in this chapter.

Statement of originality

The authors declare that the work published in this chapter is original.

References


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References

II. Activation of NK Cells


NK cell-mediated immunotherapy: The exquisite role of PGC-1α in metabolic reprogramming

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Abstract

Natural killer (NK) cells use germline-encoded nonclonotypic receptors to recognize transformed malignant cells. Due to this innate ability, NK cells possess exceptional potentials to be used against a spectrum of cancer cells in the clinic. Among tumor-infiltrating lymphocytes (TILs), NK cells constitute one of the major subsets. Nevertheless, malignancy represents a failed immune response. Immunosuppressive tumor microenvironment (TME) augments a metabolically unfavorable condition repressing immune cell activation. Therefore, a metabolic reprogramming is obligatory for mounting a successful antitumor response. Within the TME, NK cells must adapt to a changed nutrient environment and metabolic demands to mount an immune response. As immune cells transition from a quiescent state to an active state, they must increase energy production and synthesize metabolites necessary for cell growth and proliferation and processes associated with effector functions. This includes cytokine synthesis and cytolytic vesicle formation and mobilization. The high energetic and synthetic demand of these processes means NK cells need to be reprogrammed to enable energy production and biosynthesis. Filling the knowledge gap in immune metabolism is central to the successful utilization of NK cells in immunotherapeutic approaches. Here, we summarize the recent developments on one of the key elements, PGC-1α, that plays a requisite role in the metabolic reprogramming of NK cells.
Metabolic adaptation of NK cell function

Metabolic reprogramming is an obligatory step in activating immune cell proliferation, differentiation, and initiation of effector functions [1]. In multiple immune cell types, including NK cells, activation induces changes in the metabolite pool and flux through specific pathways as a means of meeting increased energetic and biosynthetic demand and initiating metabolite-based signaling [2–7]. Metabolic reprogramming was described initially in transformed cells as an increase in glycolysis [8]. However, changes in the regulation of individual metabolic enzymes and metabolite levels can have unique regulatory effects on specific cellular functions such as cytokine gene translation and cell differentiation [9–12]. Augmenting cellular metabolism also increases the efficacy of cell-based immunotherapy and that augmenting glycolysis or oxidative phosphorylation can have unique effects on donor cells, such as regulating the balance between short-term effector and memory cell formation [13–15]. Therefore, it is critical to identify portions of cellular metabolism utilized by NK cells to meet the bioenergetic and biosynthetic demands of an immune response.

Following activation, NK cells increase glycolysis and oxidative phosphorylation (OX-PHOS), which are critical for NK cell effector functions (Fig. 1). Glycolytic inhibition, through treatment with the glycolysis inhibitor 2-deoxyglucose (2-DG), or mechanistic target of rapamycin (mTOR) deletion, results in reduced cytokine and granzyme B (Gzm-B) production and disrupts the ability of NK cells to control MCMV infection [6, 15, 16]. Treatment of NK cells with the OX-PHOS inhibitor oligomycin results in reduced IFN-γ production. However, oligomycin treatment also increases the glycolytic activity by 2.5-fold, suggesting that the mitochondrial activity is also a critical part of metabolic reprogramming in NK cells [16]. Corroborating these data, when NK cells are cultured in the absence of glutamine and the presence of the fatty acid oxidation (FAO) inhibitor etomoxir, thereby eliminating critical mitochondrial fuel sources, stimulation via the NKR killer cell lectin-like receptor subfamily B, member 1 (NK1.1) results in significantly reduced IFN-γ production [2]. Interestingly, identical treatments administered prior to cytokine-mediated activation with IL-12 and IL-18 do
Changing the nutrient environment alters NK cell function

Core metabolic pathways are required for NK cell functions. (Left) Ligation of NKR results in the production of inflammatory cytokines and release of cytotoxic vesicles critical for control of both transformed and infected cells. (Right) NKR ligation in the presence of various metabolic inhibitors reduces the production of effector molecules in a manner that inhibits the ability of NK cells to control immune challenge. While the production of IFN-γ and GzmB are reduced following metabolic inhibition, glycolytic disruption does not block the production of other proinflammatory cytokines, suggesting a level of specificity in metabolic regulation of NK cell effector functions.

In addition to a dependence on activation mechanism, NK cell metabolism also changes with different nutrient environments (Fig. 2). NK cells analyzed from the peripheral blood of obese patients exhibit reduced glycolysis and OX-PHOS compared to those isolated from lean patients, and this diminished metabolic capacity is associated with significantly decreased IFN-γ and Gzm-B production [17]. A similar study found an inverse correlation between body mass index (BMI) score and the ability of peripheral blood NK cells to mediate degranulation and produce cytokines during coculture experiments [18].

While these studies demonstrate that obesity is associated with reduced function in peripheral blood NK cells, analysis of adipose tissue-resident NK cells during obesity has produced conflicting results. O’Rourke et al. found that adipose tissue-resident NK cells increased the expression of the activation receptor NKG2D and a more inflammatory phenotype in obese patients than lean controls [19]. Recent data from Shoae-Hassani et al., however, found that

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compared to lean controls, adipose tissue-resident NK cells from obese patients exhibited a decrease in the expression of the activation receptors NCR2 and NCR3, and that cells were less responsive to in vitro stimulation in coculture experiments [20]. In addition, several studies using mouse models show obesity induces a hyperinflammatory phenotype in adipose tissue-resident NK cells compared to controls [21–23]. The fact that obesity and the varying nutrient environments presented in different tissues can alter NK cell activity in the absence of any additional pathogenic challenge demonstrates how changes in the nutrient environment can impact cell function (Fig. 2).

It is critical to understand this connection between metabolism and function from a clinical perspective because altered metabolism can contribute to immune suppression. In addition to obesity, NK cell dysfunction has also been linked to type 2 diabetes (T2D). NK cells isolated from patients with T2D exhibit reduced cytotoxic potential compared to those isolated from metabolically healthy controls, and NK cells from patients with T2D and colon cancer exhibit a nearly complete loss of cytotoxic function [24, 25]. Similarly, tumor progression, and the

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associated changes in nutrient availability in the tumor microenvironment (TME), can also impact cell metabolism in a way that leads to reduced antitumor functions (Fig. 2).

Previous work demonstrates that tumor cells take up and sequester glucose away from immune cells and that blocking tumor cell glycolysis rescues immune cell function [26]. In NK cells, specifically, TME-induced activation of fructose-1,6-bisphosphatase 1 (FBP1) leads to reduced glycolysis and the ability to mediate effector functions during tumor progression [27]. Notably, inhibition of FBP1 augmented the ability of NK cells to produce cytokines and mediate cytotoxicity against target cells, demonstrating a clear link between metabolism and activity [27]. Together, these studies exhibit the dependence of NK cell function on the metabolic environment and how changing nutrient availability can affect cell metabolism in a way that impacts cellular function (Fig. 1). Therefore, it is critical to identify metabolic regulators and portions of cell metabolism required for NK cell effector functions to fully understand how NK cells regulate effector functions during an immune response.

Several regulatory mechanisms have been identified that initiate and sustain metabolic changes in NK cells following activation. For example, in cytokine-stimulated NK cells, mTOR mediates increases in glycolysis and nutrient uptake essential for cytokine production and control of viral infection [6, 15, 16]. Similar results were seen in human NK cells following cytokine-mediated activation [4]. Also, the mTOR complex 1 and mTOR complex 2 are also required for maturation and maintaining NK cell homeostasis in peripheral tissues [28].

Transcriptionally, increase in hypoxia-inducible factor 1-alpha (HIF-1α) activation has been shown to drive glycolytic gene transcription and augment cytokine production, while activation of sterol regulatory element-binding protein 1c (SREBP1c) increases expression of citrate-malate shuttle genes required to maintain elevated levels of glycolysis and OX-PHOS [12, 29]. Recent work also shows activation of c-Myc leads to increased amino acid uptake required to sustain increased metabolic activity in cytokine-stimulated NK cells [30]. Studies using human NK cells have also demonstrated that increased pyruvate kinase muscle isozyme M2 (PKM2) expression is associated with increased glycolysis and greater metabolic flexibility during cell-mediated cytotoxicity [31]. Together, these studies have been integral in defining how NK cells regulate glycolysis and overall metabolic activity to sustain effector functions during an immune response.

Mitochondrial regulation of NK cells through PGC-1α

Recent studies have focused to identify how mitochondrial metabolism and activity are regulated in NK cells. Since loss of mitochondrial function inhibits NK cell-mediated cytokine production and NK cells must mediate effector functions in a TME where glucose availability is limited, a mitochondrial regulation is predicted to be a critical aspect of NK cell metabolism. Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) is a transcriptional coactivator encoded by the Ppargc1a gene that drives transcription of nuclear-encoded genes critical for mitochondrial function [16, 32–34] and is one of three members of the PGC-1 family (PGC-1α/β and PGC-1α-related coactivator). PGC-1α’s genetic activity is primarily performed in the nucleus, where PGC-1α does not bind DNA directly but contains docking sites for various transcription factors that directly interact with target gene promoters (Fig. 3).

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PGC-1α’s binding partners include several nuclear receptor transcription factors such as nuclear response factor 1 and 2 (NRF-1/2), estrogen receptor α and β (ERα/β), and peroxisome proliferator-activated receptor α, β, and γ (PPARα/β/γ). These interactions are mediated through an LXXLL motif (Fig. 3) and several variants spread throughout the N-terminal and central region of the protein [32, 33, 35]. In addition to these nuclear receptor family proteins, PGC-1α also interacts with several nonnuclear receptor transcription factors, including forkhead box protein O1 (FoxO1), SREBP, and myocyte enhancer factor 2 (MEF2). Following binding to these various transcription factors and docking at target-gene promoters in the nucleus, PGC-1α augments gene transcription through an N-terminal activation domain that binds proteins with histone acetyltransferase activity capable of increasing transcription following histone modification [36]. PGC-1α also contains a C-terminal RNA-binding domain associated with different SR proteins capable of pre-mRNA processing [37]. PGC-1α augments transcription of nuclear-encoded mitochondrial genes through an activation domain that controls the opening of target-gene promoters and through regulation of pre-mRNA processing (Fig. 3).

Through interactions with different transcription factors, PGC-1α mediates a variety of tissue-specific functions associated with increased oxidative phosphorylation, including uncoupled thermogenesis in brown adipose tissue (BAT), mitochondrial biogenesis in skeletal muscle, and FAO in the liver [32, 38–40]. These changes in mitochondrial function allow the different cell types to maintain their function despite changing nutrient environments. These functions enable PGC-1α to control global mitochondrial biogenesis and activity through the expression of multiple proteins, including mitochondrial transcription factor A (TFAM) and several electron transport chain (ETC) and ATP synthase subunits (Fig. 3) [41, 42]. PGC-1α also mediates activation of several specific mitochondrial pathways, including FAO and glutaminolysis, through increased expression of several genes, including carnitine palmitoyltransferases (CPTs) and glutaminases (GLSs), respectively [34, 43, 44]. PGC-1α
is also capable of regulating mitochondrial nutrient utilization and pyruvate metabolism through increased expression of pyruvate dehydrogenase kinases (PDKs) (Fig. 3) [45–47]. PGC-1α acts as a master regulator of mitochondrial activity and biogenesis through its ability to regulate myriad mitochondrial functions.

**Regulation of Ppargc1a gene expression**

PGC-1α’s activity is controlled by a combination of transcriptional regulation and post-translational modifications that alter its ability to augment target-gene transcription. Transcriptional regulation of PGC-1α is controlled by binding sites in the promoter region for three different transcription factors shown to be critical for PGC-1α expression: cAMP response element-binding protein (CREB), activating transcription factor 2 (ATF2), and MEF2 [48–51]. The promoter region also contains a FoxO1-binding site, but the exact role in mediating PGC-1α expression is controversial [52]. These transcription factors increase PGC-1α expression in response to energetic stress, with the role of each transcription factor varying from tissue to tissue (Fig. 4).

For example, in adipose tissue, exposure to cold temperatures activates B3-adrenergic receptor signaling, which subsequently stimulates the cAMP-protein kinase A (PKA)-CREB axis and...
enables CREB to mediate PGC-1α expression [53, 54]. This axis also stimulates p38 mitogen-activated protein kinase (MAPK), which can phosphorylate and activate ATF2, further increasing PGC-1α expression [55]. In muscle cells, calcium signaling stimulated at a neuromuscular junction can lead to activation of both calcium/calmodulin-dependent protein kinase type IV (CaMKIV) and calcineurin, which in turn stimulate CREB and MEF2, respectively [50, 56, 57]. During exercise, muscle cells activate p38 MAPK, which increases PGC-1α expression in an ATF2-dependent manner [49]. In hepatocytes, fasting and subsequent increases in systemic glucagon activate cAMP and p38 MAPK signaling, stimulating CREB-mediated increases in PGC-1α expression [48, 58]. These different transcriptional pathways enable PGC-1α expression to be controlled by energetic stress in a tissue-specific manner (Fig. 4).

**Posttranslational regulation of PGC-1α function**

Similar to the transcriptional regulation of PGC-1α, posttranslational modifications that control PGC-1α activity also depend on the energy state and energy demand of the cell (Fig. 5). PGC-1α contains numerous phosphorylation sites that are targeted by kinases responsive to changing cellular metabolism. AMP-activated protein kinase (AMPK), for example, is activated by increases in the AMP/ATP ratio, and AMPK in turn directly phosphorylates and augments the transcriptional activity of PGC-1α [59]. Metabolic changes can also induce inhibitory phosphorylation of PGC-1α, as insulin signaling in hepatocytes activates Akt, which subsequently phosphorylates PGC-1α in a manner that prevents localization to its target-gene promoters (Fig. 5) [60]. While these phosphorylation events affect PGC-1α activity by regulating the ability to form complexes at target-gene promoters, several kinases regulate PGC-1α activity by controlling its stability.

With a half-life of approximately 2 h, PGC-1α is an intrinsically disordered protein subject to rapid proteasome-mediated degradation even without prior ubiquitin ligation [61–63].

**FIG. 5** PGC-1α function is controlled by posttranslational modification. Following protein synthesis, the function of PGC-1α is regulated by myriad posttranslational modifications that control both protein stability and transcriptional activity. These modifications are provided by different signaling proteins responsible for activating (green) or inhibiting (red) PGC-1α. GSK3β and p38 phosphorylate PGC-1α at different threonine residues that either promote or inhibit ubiquitination and subsequent proteasome-mediated degradation, respectively. AMPK and Akt also work in opposition. Phosphorylation of serine 538 by AMPK increases transcription of PGC-1α-target genes, while phosphorylation of serine 570 by Akt blocks PGC-1α-mediated gene expression. The transcriptional activity of PGC-1α is also controlled by acetylated lysine residues that span the length of the protein. In the case of PGC-1α, acetylation by the acetyltransferase GCN5 blocks its transcriptional activity, and removal of these marks by SirT1 restores its ability to mediate target-gene expression.

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PGC-1α stability can also be reduced by phosphorylation by glycogen synthase kinase 3 beta (GSK3β), which enhances its proteasomal degradation following ubiquitination [64, 65]. Therefore, extending the half-life of PGC-1α represents a critical mechanism by which its activity can be augmented during times of increased energy demand. In muscle cells and hepatocytes, stimulation through cytokines or free fatty acids respectively leads to activation of p38 MAPK [61, 66]. In addition to increased transcription of PGC-1α, p38 can also directly phosphorylate PGC-1α, which increases the stability of the protein by almost threefold [61, 66]. In muscle cells, this leads to increased mitochondrial gene transcription required for cellular respiration and energy expenditure and is also required for gluconeogenesis in hepatocytes [61, 66]. Thus, p38-mediated phosphorylation of PGC-1α increases protein stability to enable cells to adjust to increased energy demand (Fig. 5). Together, these phosphorylation events enable PGC-1α to respond to changes in the energy state of the cell.

In addition to phosphorylation, the cellular energy state also alters the activity of specific acetyltransferases and deacetylases that control PGC-1α function (Fig. 5). PGC-1α contains various acetylation sites along the length of the protein that is targeted by the acetyltransferase GCN5 [67, 68]. Acetylation of these residues by GCN5 inhibits the transcriptional activity of PGC-1α by disrupting its binding to target gene promoters [67]. Expression of GCN5 is increased during caloric excess and decreased during caloric restriction, thus providing another mechanism by which energy status is linked to PGC-1α function [69].

Similar to the mechanism by which GCN5 disruption reduces PGC-1α acetylation and increases its transcriptional activity, sirtuin-1 (SirT-1) also augments PGC-1α function through deacetylation (Fig. 5). SirT-1 levels directly affect PGC-1α acetylation and the resulting transcriptional activity, and knockdown or overexpression of SirT-1 directly results in decreased or increased PGC-1α activity, respectively [68, 70]. The deacetylase activity of SirT-1 depends on NAD as a cofactor, and its activity is increased by increasing amounts of NAD or increased NAD/NADH ratios [71]. Thus, during an energy deficiency, such as during fasting or exercise, when there is a greater need for mitochondrial function and NAD/NADH ratios are high, increased SirT-1 activity results in decreased PGC-1α acetylation and augments transcriptional activity [72, 73]. This dynamic has been demonstrated in both muscle cells and hepatocytes, where increased SirT-1 activity results in PGC-1α-dependent increases in mitochondrial respiration and gluconeogenesis, respectively [70, 72, 73]. Together, GCN5 and SirT-1 act as sensors that directly communicate the energy state of the cell to PGC-1α through posttranslational modification (Fig. 5).

### The functions and tissue-specificity of PGC-1α

Following activation, PGC-1α increases transcription of target genes capable of regulating myriad metabolic functions, including mitochondrial biogenesis, gluconeogenesis, and fatty acid oxidation. However, not all target genes are activated simultaneously, and the specific gene expression profile can vary both among and between cell types, with the outcome being controlled both by the activation condition and the specific transcription factor to which PGC-1α binds. In adipocytes, for example, exposure to cold temperatures results in a PGC-α-dependent increase in uncoupling protein-1 (UCP-1) that increases heat generation and drives the transition from white adipose tissue to brown adipose tissue (Fig. 6) [32, 43].

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On the other hand, in hepatocytes, PGC-1α activation through fasting increases transcription of \( \text{Pck1} \) and \( \text{G6pc} \), which are required for gluconeogenesis [38, 74]. This gluconeogenesis profile is induced through the interaction between PGC-1α and HNF4. Liver-specific functions can also be augmented by PGC-1α, as the interaction between PGC-1α and HNF4 induces expression of the gluconeogenesis genes \( \text{Pck1} \) and \( \text{G6pc} \). The PGC-1α-induced gene expression profile can also vary within a given tissue type through binding of different transcription factors. In skeletal muscle, for example, the PGC-1α-Pparα interaction induces FAO genes, while the PGC-1α-NRF-1 interaction induces expression of genes that drive mitochondrial biogenesis.

The specific functions of PGC-1α in regulating mitochondrial activity have been well studied in tissues that are essential for maintaining energy homeostasis. The liver, adipose tissue, and skeletal muscle are examples of tissues with well-defined roles in adapting to changing nutrient environments. While lymphocytes are not classically involved in maintaining energy homeostasis, the effect of changing nutrient environments on immune cell function is a well-established relationship. As part of the effort to understand the precise mechanisms by which nutrient conditions and the resultant metabolic changes impact immune cell activity, recent evidence has demonstrated the ability of PGC-1α to modify mitochondrial activity in

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lymphocytes. In a genetic deletion model, loss of PGC-1α in Foxp3+ cells abrogated their suppressive function [40, 76].

PGC-1α has also been shown to play a role in maintaining mitochondrial function in proinflammatory and cytotoxic T cells during both chronic infection and within a TME [77, 78]. Analyses of mitochondrial function in T cells isolated from the tumor microenvironment or mice infected with chronic lymphocytic choriomeningitis virus demonstrate suppressed mitochondrial function associated with reduced PGC-1α activity. When cells from nondraining lymph nodes or uninfected mice were analyzed, mitochondrial activity was maintained, demonstrating that the reliance upon PGC-1α for lymphocyte function depends on the nutrient environment. During both infection and tumor challenge, overexpression of PGC-1α was sufficient to rescue mitochondrial activity and lymphocyte function, establishing a clear link between PGC-1α-dependent mitochondrial function and lymphocyte activity. In terms of NK cells, knockdown of PGC-1α in human NK cells following culture in high doses of IL-2 results in reduced cytotoxic potential along with cellular respiration [79, 80]. Similarly, NK cells isolated from elderly patients and cultured in high doses of IL-2 exhibit reduced NK cell function and mitochondrial activity associated with decreased PGC-1α levels compared to cells isolated from younger control patients. These earlier studies determined the function of PGC-1α in maintaining the cytotoxicity potentials of cultured NK cells.

Functions of PGC-1α in NK cells during bacterial infection and tumor clearance

Mitochondrial reprogramming to adjust to changing nutrient environments energetic requirements during the NK cell-medicated immune responses is not well understood. NK cells augment transcription of genes critical for mitochondrial function and specific PGC-1α-target genes during Listeria monocytogenes infection [81] and PGC-1α is required for optimal NK cell-mediated cytokine production and cytotoxicity. Analysis of mitochondrial nutrient regulatory pathways revealed that glutaminolysis and FAO were required for NK cell-mediated cytokine production. Conversely, pyruvate dehydrogenase (PDH) activity was dispensable for IFN-γ production, while the function of the kinase inhibits PDH, pyruvate dehydrogenase kinase (PDK), was required. PGC-1α inhibits PDH by augmenting the expression of PDKs [45]. Lack of PGC-1α augmented PDH activity, while it was unchanged in wild-type NK cells following stimulation via NKG2D. Since suboptimal NK cell effector functions are correlated with increased PDH activity in PGC-1α-deficient NK cells, PGC-1α may regulate mitochondrial nutrient utilization by mediating blockade of pyruvate into the tricarboxylic acid (TCA) cycle, while glutaminolysis and FAO serve as primary sources of mitochondrial fuel [81].

An essential role for mitochondrial function in maintaining NK cell efficacy in a tumor microenvironment. The ability of tumor cells to sequester glucose from immune cells is well established [26, 82–84], and the previous reports demonstrate the importance of PGC-1α in maintaining mitochondrial health in a tumor microenvironment [78]. B16F10 lung melanoma model suggests that PGC-1α is a critical part of metabolic reprogramming during the NK cell response to tumor growth [81]. Ppurgc1a cKO NK cells were unable to control the development of lung metastases to the level of WT NK cells, and they also exhibited disrupted

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mitochondrial function when analyzed ex vivo. The RT-qPCR analysis revealed reduced expression of PGC-1α-target genes, suggesting that PGC-1α actively regulates mitochondrial function in a TME to enable NK cells to respond optimally to tumor growth. Previous work has demonstrated the ability of metabolic regulation to augment cellular function in an immunotherapy setting. For example, overexpression of Pck1, the enzyme responsible for the synthesis of phosphoenolpyruvate, prolongs calcium signaling in activated T cells and enhances the antitumor efficacy of these cells following adoptive transfer [11]. Similarly, chimeric antigen receptors engineered to promote mitochondrial metabolism provide T cells with increased persistence and a more memory-like phenotype following transduction [14]. These studies exemplify the translational potential of immune cell metabolism and demonstrate the importance of elucidating mechanisms of metabolic regulation.

In NK cells, transcriptional changes alter the expression of metabolic enzymes to meet their energetic and biosynthetic demands. When NK cells are activated in vitro via the cytokines IL-15, IL-12/18, or IL-2, measurements of oxygen consumption rate (OCR) and lactic acid production [extracellular acidification rate (ECAR)] show an increase in both mitochondrial and glycolytic activity [2, 6, 16]. Similarly, both infection and tumor models show that glycolytic activity is required for optimal NK cell immune responses in vivo [6, 15, 27]. Previous work has also demonstrated a clear link between NK cell dysfunction and altered cellular metabolism in cells isolated from T2D and cancer patients [24]. Identifying regulatory mechanisms that control metabolic function during the immune challenge is, therefore, critical for understanding NK cell biology and the requirements optimal effector functions.

Multiple studies have demonstrated increases in glucose uptake, and glycolytic activity is transcriptionally regulated, where NK cell activation increases transcription of the glycolytic enzymes Glut1, Hex2, and Ldha [16, 29]. In addition to glycolytic enzymes, previous work has demonstrated other key metabolic regulators, including the citrate-malate shuttle genes Acly and Slc25a1 and the amino acid transporter Slc7a5, are also transcriptionally upregulated in activated NK cells [12, 30]. These studies point to a specific, transcription-based metabolic program that alters cellular metabolism during the initiation of NK cell effector functions. Gene set enrichment analyses (GSEA) of NK cells following infection with L. monocytogenes demonstrated robust activation of inflammatory gene signatures as well as increased expression of genes responsible for driving glycolysis [81]. This is consistent with previous work in MCMV infection models, where administration of 2-DG or mTOR deletion inhibits the function of NK cells [6, 15]. Collectively, these studies confirm that the glycolysis is a necessary component of NK cell effector functions.

### Respiratory requirements of human NK cells

Previous reports have established the importance of PGC-1α in cultured human NK cells for cytotoxicity [79, 80]. These studies show that the following culture in high doses of IL-2, siRNA-mediated knockdown of PGC-1α is associated with suboptimal NK cell-mediated cytotoxicity in coculture experiments with K562 target cells [79, 80]. Recent studies corroborate previous findings in terms of cytotoxicity and show PGC-1α is also required for optimal cytokine and chemokine production in human NK cells. In addition, the importance of PGC-1α...
extends beyond cultured human NK cells such that inhibition of PGC-1α results in reduced mitochondrial activity and effector functions in naïve human NK cells. During tumor growth, transformed cells are capable of sequestering glucose away from lymphocytes, thus creating a metabolically challenging environment that can limit the effectiveness of infiltrating immune cells [26, 82–84]. Thus, impaired mitochondrial function contributes to immune cell dysfunction and that PGC-1α represents a potential pathway through which mitochondrial metabolism could be augmented during an immune response. Given that adoptive transfers of NK cells into patients have demonstrated efficacy against multiple cancer types [85–87], these findings have the potential to optimize cell-based immunotherapy through metabolic regulation that enables NK cells to better meet their metabolic demand within a tumor microenvironment.

Mitochondrial nutrient utilization is essential for NK cells to mediate effector functions

Mitochondrial functions are tightly associated with PGC-1α-mediated gene transcription [34, 81, 88–90]. Blockade using pharmacological inhibitors showed that the mitochondrial nutrient acquisition pathways controlled by PGC-1α-target genes were required for NK cell-mediated IFN-γ production. Studies show that glutaminolysis and FAO are required for cytokine production, while pyruvate entry into the TCA cycle is dispensable, and in fact, PDK-mediated suppression of PDH is required for NK cell effector functions. These data suggest that glutaminolysis and FAO serve as the primary sources of mitochondrial metabolites in activated NK cells, while pyruvate is primarily utilized outside of the TCA cycle.

PGC-1α-deficient NK cells augment PDH activity following activation with NKG2D [81]. This increase does not occur in WT NK cells, suggesting PGC-1α limits pyruvate utilization by the TCA cycle through PDK expression to enable optimal NK cell-mediated cytokine production. While the precise mechanism by which PDH inhibition contributes to IFN-γ production in NK cells is unknown, one potential mechanism by which this may occur is increasing pyruvate flux through the lactate dehydrogenase enzyme (LDH). Previous work in T cells shows that T cell receptor (TCR) stimulation results in PDK-mediated inhibition of PDH and subsequently increases the LDH-mediated pyruvate to lactate conversion [91]. LDH contains a binding site for AU-rich elements present in the 3' UTR of some transcripts and binding of LDH has been shown to initiate posttranscriptional regulation of these mRNAs [91, 92]. Following TCR stimulation, increased flux of pyruvate through LDH occupies the enzyme and prevents it from binding to the 3' UTR of Ifng mRNA, thus enabling increased IFN-γ production from activated T cells. This is one of several mechanisms established that link glycolytic enzymes to posttranscriptional regulation of cytokine production [91, 93]. These findings point to a model in which regulation of mitochondrial nutrient acquisition is a critical part of NK cell metabolic reprogramming, and PGC-1α may enable greater utilization of alternative fuel sources during an NK cell immune response. Several recent studies emphasize the importance of maintaining bioenergetics during cell-based immunotherapy [11, 13, 14, 26, 94]. Thus, a transcriptional regulation of numerous genes involved in these pathways by PGC-1α could be augmented to provide greater metabolic flexibility and enable optimal NK cell function during

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infection or in a TME. Future studies should explore strategies designed to augment the mitochondrial function of NK cells through genetic or culture-based mechanisms for the treatment of multiple tumor types.

The specificity of metabolic reprogramming in NK cells

PGC-1α is required for optimal NK cell effector functions in specific inflammatory and nutrient environments; thus, these studies provide another example of the context-dependent nature of metabolism regulation in immune cells that have broadened the definition of metabolic reprogramming beyond the Warburg effect. Early work to define metabolic reprogramming in lymphocytes was primarily performed in T cells, where several studies found that cellular activation induced the same augmented glycolytic increases that define the Warburg effect [8]. Multiple studies comparing metabolism in resting cells and activated cells revealed anywhere between a 20- and 50-fold increase in glycolysis that constituted a switch from mitochondrial metabolism utilized by resting cells [95–97]. While this classic example of the Warburg effect occurs in some T cells, the metabolic profile of activated T cells has since been expanded to include metabolic changes specific to different subsets. Tregs and memory T cells, for example, preferentially utilize OX-PHOS rather than glycolysis, and glycolytic inhibition can promote the formation of these subsets [98–101].

Similar variation has been observed in NK cells, where activation mechanism and metabolic context induce varying metabolic regulation. For example, activation with IL-2 or IL-12 induces very little change in metabolism, while activation with IL-15 alone or IL-2 and IL-12 together cause significant increases in glycolysis and mitochondrial metabolism [2, 6, 16]. NKR-mediated activation also induced very little change in glycolysis or OX-PHOS during that stimulation through NKR’s potently induces cytotoxicity cytokine production. In addition to cell metabolism changes, treatment with various metabolic inhibitors and analysis of cytokine production following IL-12/18 or NKR-mediated activation demonstrates the metabolic requirements for effector functions also vary with activation conditions.

NK cells do not undergo clonal expansion as part of the immune response and thus have diminished proliferative burden compared to expanding T cells. Metabolic characterization reveals that while effector T cells upregulate glycolysis more than 20-fold [102], not all activation conditions induce glycolytic increases in NK cells and those that do only increase glycolytic rates several fold [2, 6, 16]. It is also likely that immune cells with different functions require different metabolic programs, and consequently that different activation mechanisms would induce different metabolic changes. This dynamic is particularly interesting in NK cells, where different activation mechanisms can produce unique functional outcomes when used individually instead of always requiring prior sensitization through one receptor, as is the case with the TCR. NK cells could thus be used to define metabolic changes associated with specific activation mechanisms and unique functional outcomes, such as cytokine mRNA production, cytotoxic granule mobilization, or proliferation, and to identify the mechanistic link that makes a given metabolic program necessary. This also strongly suggests that an effective metabolic reprogramming of NK cells is required to formulate disease-specific therapeutic approaches.
Metabolism-based regulation of lymphocyte function for immunotherapy

Metabolism-based regulation of immune cell effector functions was first observed when studies in T cells showed increasing glutamine levels in vitro or in vivo augmented cytokine production and altered increased CD4:CD8 ratios [103–105]. These studies also provide initial evidence metabolic reprogramming can impact lymphocyte functions outside of its original purpose of driving proliferation. Subsequent work has demonstrated that the unique role metabolic pathways play in providing direct regulation of lymphocyte effector functions. Differentially regulating the effector functions such as antitumor cytotoxicity and proinflammatory cytokine production is essential for effective immunotherapeutic approaches with minimal side effects [106]. Analysis of etomoxir-treated WT NK cells suggests that FAO contributes to cytokine secretion independent of ATP production, highlighting a critical aspect of metabolic reprogramming in providing direct regulation of lymphocyte effector functions [81].

Analysis of metabolic reprogramming in immune cells has demonstrated the ability of metabolism to regulate myriad cellular functions including differentiation, calcium signaling, and cytokine production. For example, recent studies have elucidated the role of HIF-1α-dependent glycolysis in controlling differentiation between proinflammatory T h17 and antiinflammatory T reg cells [98–100]. Deletion of HIF-1α or inhibition of glycolysis with 2-DG during in vitro T cell activation results in decreased T h17 differentiation, while paradoxically promoting T reg differentiation [99]. Similar regulation has also been demonstrated in T h1 cells, as activation of naïve CD4 T cells in the absence of glutamine under conditions that generally induce T h1 skewing not only disrupts T h1 skewing but also promotes T reg formation [10]. In addition to differentiation, metabolic reprogramming can also directly regulate calcium signaling. This occurs when increases in the rate of glycolysis augment the production of phosphoenolpyruvate (PEP), which in turn binds to and inhibits the function of the calcium reuptake receptor Sarco/ER Ca(2+)-ATPase (SERCA) [11]. Increased PEP production consequently results in prolonged calcium signaling, and PEP-overproducing T cells were shown to restrict tumor growth in vivo [11]. Several studies have also shown that metabolic enzymes or metabolic intermediates can directly regulate cytokine production by controlling mRNA translation or promoter acetylation to augment transcription [9, 91, 93].

While direct metabolic regulation has yet to be demonstrated in NK cells, several studies have elucidated the importance of specific metabolic enzymes for NK cell effector functions. For example, inhibition of the citrate-malate shuttle enzyme ATP-citrate lyase (ACLY) results in reduced production of IFN-γ and GzmB following cytokine-mediated activation [12]. FAO enzyme CPT1 and the glutaminolysis enzyme GLS1 are required for NK cell-mediated cytokine production, while PDH is dispensable despite its ability to fuel the TCA cycle. These studies demonstrate that individual metabolic enzymes are required for NK cell effector functions and point to metabolism-based regulatory mechanisms of cytotoxicity and cytokine production. Similar to what has been demonstrated in T cells, identifying these mechanisms is necessary to define the extent to which metabolic regulation controls lymphocyte effector functions and achieve a comprehensive understanding of the relationship between metabolism and immune cells during an immune response.

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It is also critical to understand these mechanisms from a clinical perspective, as many of the parameters that factor into the efficacy of cell-based immunotherapy, in which immune cells are transferred into patients, can be controlled by metabolism. The persistence of transferred cells, for example, has been shown to be associated with improved antitumor immunity and patient survival in both preclinical models and actual clinical trials [107–110]. Subsequent studies have demonstrated a link between persistence and cell metabolism, and sorting cells based on mitochondrial parameters has been shown to be effective in separating short- and long-lived cells [111–113]. In addition, T cell differentiation and memory formation are important factors in determining graft persistence, and direct alteration of mitochondrial function in vitro can be used to promote memory formation that is retained in vivo following engraftment [5, 101, 114, 115]. Along with longevity and persistence, metabolism can also be used to promote antitumor functions. Increasing glycolysis by augmenting glucose transporter 1 (GLUT1) levels or AKT/mTOR activity can rescue T cell dysfunction associated with an animal model of B cell acute lymphoblastic leukemia [116]; and multiple studies demonstrate that augmenting the mitochondrial function of T cells prior to engraftment synergizes with antiprogrammed cell death protein 1 (PD-1) therapy to improve tumor control [13, 117]. These studies highlight the translational potential of utilizing metabolism to optimize lymphocyte function during immunotherapy—particularly in cell-based immunotherapy, where genetic modification prior to cell engraftment presents an ideal setting for metabolic perturbation.

Summary and future outlook

As the major lymphocyte subset of the innate immunity, NK cells target and clear transformed or virally infected cells through cell-mediated cytotoxicity and cytokine production. In immune cells, this activation induces changes in the cellular metabolite pool and flux through specific metabolic pathways as a means of meeting increased energetic and biosynthetic demand. The role of this metabolic reprogramming also includes direct propagation of unique signaling and control of immune effector functions. Modulation of metabolic pathways in immune cells has proven to be effective in augmenting cellular functions. Recent studies have suggested that a metabolic reprogramming is required for the NK cells to maintain normal rates of growth and proliferation and regulating multiple cellular functions including cytokine translation, calcium signaling, and cell fate determination. Therefore, utilization of NK cells in immunotherapy and their unique role in innate immunity necessitates identification of specific metabolic changes and metabolite-based signaling critical for their effector functions. Human NK cells fall into specialized individual subsets [118, 119]. These subsets were classified based on their single-cell transcriptomics, which define them either developmentally or functionally divergent clusters. A systematic understanding of these subsets in terms of their effector functions is essential in the formulations of NK cell-based immunotherapies. Most importantly, the independent metabolic requirements and pathways used by these NK cell subsets are critical in formulating better clinical outcomes. Thus, the future work should focus to determine the metabolic reprogramming of select subsets of NK cells to improve the clinical efficacy.
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References

This work was supported in part by NIH R01 AI102893 and NCI R01 CA179363 (S.M.); HRHM Program of MACC Fund (S.M.); Nicholas Family Foundation (S.M.); Gardetto Family (S.M.); MCW-Cancer Center-Large Seed Grant (S.M.); MACC Fund (S.M.); Ann’s Hope Melanoma Foundation (S.M.); and Advancing Healthier Wisconsin (S.M.). Z.G. conceived and wrote the chapter and generated the figures. S.M. edited the text.

Acknowledgments

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7. NK cell-mediated immunotherapy


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References


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[77] Bengsch B, Johnson AL, Kurachi M, Oodorizzi PM, Pawelke KE, Attanasio J, Stelekati E, McLane LM, Paley MA, Delgoffe GM, Wherry EJ. Bioenergetic insufficiencies due to metabolic alterations regulated by the inhibitory receptor PD-1 are an early driver of CD8+ T cell exhaustion. Immunity 2016;45:358–73.


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References


7. NK cell-mediated immunotherapy


IL-15 and IL-15Rα: Something old, something new, and something blue

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Abstract

NK cell-based immunotherapies have gained traction in the clinic for the treatment of cancer due to the ability of NK cells to directly lyse tumor cells. NK cells are being exploited in clinical trials using autologous and allogeneic NK cell infusion strategies alone or with hematopoietic stem cell transplantation. These strategies have rapidly evolved to exploit the therapeutic effects of NK cells transduced with chimeric antigen receptors and the utilization of IL-15 to induce NK cell persistence and expansion that seems to dominate current clinical trials. IL-15 is a member of the common receptor gamma chain (γc) family, which also includes IL-2, IL-4, IL-7, IL-9, and IL-21. This group of cytokines has a broad pleiotropic activity on both the innate and adaptive immune systems, with important therapeutic ramifications. The protein form of IL-15 contains two N-linked glycosylation sites at the C-terminus of the IL-15 protein, at N79 and N112, and disulfide bonds at positions C42-C88 and C35-C85, the former being homologous to the C—C within IL-2. The IL-15 heterotrimeric receptor complex consists of a private IL-15 specific alpha subunit IL-15Rα (CD215), the IL-2/IL-15 receptor β (CD122), and the common γc receptor subunit (CD132) which are shared by the IL-2 receptor. The ability of IL-15 to stimulate NK cell responses, and its well-documented antitumor activity in preclinical models, support the development of clinical studies of IL-15 in cancer. IL-15 showed acceptable toxicity profiles in mouse and primate models and, thus, is increasingly recognized as a promising cytokine for the treatment of hematological and solid malignancies. IL-15 is currently being tested clinically in a number of formulations to enhance immunotherapy for several indications. Clinical studies of IL-15 or IL-15 superagonists, consisting of IL-15 linked to IL-15Rα portions, have been initiated; studies combining IL-15 with adoptive transfer of NK cells are ongoing in cancer patients. This review traces how our understanding of the complex regulation of IL-15 has evolved in time and how accumulated knowledge from the bench was translated in the clinic.

Abbreviations

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<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>DC</td>
<td>dendritic cells</td>
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<td>ER</td>
<td>endoplasmic reticulum</td>
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IL-15 is a 14–15-kDa glycoprotein encoded by a 34-kb region mapping to the human chromosome 4q31 and the central region of the mouse chromosome 8 [1, 2]. The genomic structure of human IL-15 contains nine exons with a similar intron/exon structure and an estimated size between the murine and human IL-15 genes [1, 2]. Earlier studies have identified two isoforms with two different signal peptides encoding for the same mature IL-15 protein [3, 4]. One isoform consists of a 316-bp 5' untranslated region (UTR), a 486-bp coding sequence, and a 400-bp 3'UTR that is translated into an IL-15 precursor protein with a long signal peptide (LSP) of 48 amino acids, which is encoded within the exons 3–5. The other isoform of IL-15 mRNA has a short signal peptide (SSP) of 21 amino acids encoded by the exons 4A and 5. In humans and mice, both SSP and LSP isoforms produce the same mature protein; however, the two differ in tissue distribution (Table 1) and intracellular trafficking [15]. LSP-IL-15 is targeted to the Golgi apparatus, early endosomes, and the endoplasmic reticulum (ER) secretory pathway, whereas SSP-IL-15 is not secreted and remains exclusively in intracellular compartments [5, 6, 16]. Using different combinations of signal peptides, Tagaya et al. showed that the SSP form regulates the fate of the mature protein by controlling the intracellular trafficking to cellular compartments.

### TABLE 1  
**Tissue-specific distribution of IL-15 isoform.**

- LSP form is largely expressed in the skeletal muscle, placenta, heart, lung, liver, thymus, and kidney [3, 5–8]
- High expression of SSP form is found in the heart, thymus, and appendix. Weak expression of SSP form was also observed in gall bladder, pancreas, and testis [5, 9]
- Both SSP and LSP forms are expressed in activated monocytes/macrophages and several cell lines [5, 10, 11]
- Lymphoma-derived L591 and HuT102 cells express mostly LSP whereas thymocyte-derived Cole cell line express mostly the SSP form [5, 10–12]
- Two isoforms of IL-15 one lacking exon 6 and the other lacking a portion of exon 7 were identified in mouse intestinal epithelium. Both encode in-frame proteins using the LSP and appear to inhibit full-length IL-15-mediated proliferation [13]
- The isoform lacking exon 6 was further identified in activated immune cells such as macrophages and B cells. This form was described as a natural antagonist for IL-15 [14]
nonendoplasmic reticulum sites, whereas the LSP form regulates both the rate of protein translation and the function [5]. As a result, proteins associated with the SSP form do not enter the ER and are not secreted but rather stored intracellularly in the nuclear and cytoplasmic components [5, 10, 12, 17]. In contrast, proteins associated with the LSP form enter the ER where the LSP is fully processed, and the protein is N-glycosylated and secreted by the classical Golgi route [5–7, 12]. The demonstration that there are both secreted and intracellular fates raised the possibility that IL-15 may act both as an intercellular signaling factor (LPS-IL-15) and in intracellular signaling (SSP-IL-15) [7]. It should be noted that the biological significance of IL-15 as a cytokine with an intracellular fate remains to be determined.

IL-15 is inefficiently secreted unless IL-15Rα comes to the rescue

Signal peptides determine the intracellular localization and potential secretion of the associated mature protein. For IL-15, the SSP form is not secreted and remains exclusively in the intracellular compartments, whereas the LSP form is directed to the secretory pathway [5, 6, 16]. However, despite evidence of its localization in the ER, LSP-IL-15 is produced inefficiently as a secretory protein by mammalian cells, almost 2–3 logs lower than that of a more typical cytokine such as IL-2 or IL-4 [5, 12, 17]. Given the extraordinarily high affinity with which IL-15 and IL-15Rα interact (~5 × 10−11 M) (Table 2), earlier efforts focused on elucidating the relationship between IL-15 and its specific receptor, IL-15Rα. Accumulating evidence from genetic and cell transfer experiments converged to show that IL15Rα is required as an intracellular chaperone for IL-15 secretion as well as an extracellular scaffold that presents IL-15 to responsive cells [18, 33–43]. The observation that IL-15Rα−/− cells fail to secrete IL-15 clearly demonstrated the requirement of IL-15Rα for IL-15 secretion [44]. It was also reported that the detection of IL-15 protein was progressively diminished by the presence of increasing amounts of IL-15Rα protein, indicating that epitopes of IL-15 detected by anti-IL-15 antibodies are blocked by high affinity interactions between IL-15 and IL-15Rα [44].

Along these lines, transfection of cells with IL-15 lacking the IL-15 binding (sushi) domain failed to yield the secreted IL-15, further demonstrating that IL-15 secretion requires binding to IL-15Rα [45]. It should be noted that IL-15 is not required for the production of IL-15Rα since the expression of IL-15Rα is efficiently induced in stimulated IL-15−/− cells [44]. IL-15Rα-dependent secretion of IL-15 is currently the accepted model for IL-15 secretion, and further evidence showing that IL-15 mediates physiological activity, not as a monomer but predominantly in complex with IL15Rα, is summarized in Table 3.

That efficient secretion of LSP-IL-15 requires the simultaneous expression of IL-15Rα is nowadays undisputable, but what about the SSP-IL-15 form? Can interaction with IL-15Rα “rescue” this isoform resulting in its secretion? Earlier work from Bergamaschi et al. provided compelling evidence that the SSP-IL-15 isoform, which was previously thought to be exclusively intracellular, can be secreted if IL-15Rα is simultaneously expressed [50]. In particular, it was possible to visualize SSP-IL-15 at the cell surface when IL-15Rα is coexpressed both by confocal microscopy and flow cytometry. Further experiments based on coinjection of SSP-IL-15 and IL-15Rα-expressing plasmids into mice resulted in increased plasma levels of bioactive SSP-IL-15, capable of inducing in vivo the expansion of IL-15-responding cells [50]. However, it is important to report that even in the presence of IL-15Rα, the stability of SSP-IL-15 is
TABLE 2  IL-15Rα versus IL2Rα.

- The heterotrimeric IL-15R is composed of a beta subunit (IL-2R/15Rβ) that is shared with the IL-2R, a common gamma subunit (γc) shared with IL-2, IL-4, IL-7, IL-9 and IL-21, and a unique alpha subunit (IL-15Rα) that confers receptor specificity to IL-15 [18–20]

- The IL-2R/15Rβ subunit is a 525 aa receptor consisting of a 214 aa extracellular segment, a 25 aa transmembrane region, and a 286 aa cytoplasmic domain [21]. The human γc consists of a 233 aa extracellular domain, a 28 aa transmembrane domain, and an 86 aa cytoplasmic region [22]. IL-15Rα has a 173 aa extracellular domain, a single 21 aa membrane-spanning region, and a 37 aa cytoplasmic domain [23, 24]

- Structure of IL-2Rα and the IL-15Rα revealed the presence of a conserved protein-binding motif and similar intron/exon structure [18, 22]

- Alternative splicing generating different forms of IL-15Rα has been reported [1, 18, 24–27]

- The full-length IL-15Rα transcript was detected in numerous tissues and cell lines, demonstrating a much wider distribution than the IL-2Rα [23]

- Three different IL-2R complexes exist: IL-2Rα binds IL-2 with low affinity (Ka ~10⁸ M⁻¹) without transducing a signal; the heterodimeric IL-2Rβγ binds IL-2 with intermediate affinity (Ka ~10⁷ M⁻¹) and transduces intracellular signals; and the heterotrimeric IL-2Rβγ binds IL-2 with high affinity (Ka ~10¹⁵ M⁻¹) and also signals [28–30]

- IL-15Rα by itself has an extremely high affinity for IL-15, almost 100-fold higher than the affinity of the IL-2Rα for IL-2 [18]

- The full-length IL-15Rα alone was sufficient for high-affinity (Ka >10¹¹ M⁻¹) binding of IL-15, but, similar to IL-2Rα, it played no role in signal transduction [15]. The high affinity of IL-15Rα for IL-15 is in stark contrast to the IL-2Rα, which has low affinity for IL-2 in the absence of the IL-2Rβγ. Thus, IL-15Rα binds IL-15 with high affinity but transduces signals only in the presence of the IL-2/15Rβγ [15]

- The crystal structures of the heterodimer IL-15/IL-15Rα and the quaternary IL-15/IL-15Rα/IL-2Rβ/γc complex have been reported [31, 32]

- IL-15 has two distinct binding sites, site I for the binding to IL-2Rβ and site II for the binding to IL-2Rγc. In contrast, IL-15Rα does not contain IL-2Rβ, with a distance of >15 Å separating the subunits at their closest point [31, 32]

substantially lower than the stability of LSP-IL-15, thereby resulting in lower secretion and steady-state levels of SSP-IL-15/IL-15Rα complexes. Although a secreted version of SSP-IL-15 does not exist, we cannot rule out, based on the above observations, the possibility that the bioactivity of this isoform is mediated by an as yet uncharacterized IL-15Rα isoform.

Biological significance of SSP-IL-15 remains puzzling

The production of a cytokine with an intracellular fate is not typical of interleukin systems. Since its discovery, the identification of an intracellular form of IL-15 raised interest and
further evidence of its distribution in the nucleus was puzzling. The questions of how SSP-IL-15 is retained in the nucleus and whether it has a biological significance remain unsolved. It was speculated that this intracellular form may serve as a reservoir of IL-15 that can be released upon damage and destruction of producer cells during inflammation [5]. This is an attractive hypothesis given of the augmented levels of IL-15 in inflammatory settings [6, 7, 17, 51–55]; however, it remains unverified. Another hypothesis emerged from evidence that rapid and low levels of SSP-IL-15 can be secreted upon coexpression of IL-15Rα [50]. Accordingly, it was proposed that SSP-IL-15 may be important for expressing a form of IL-15 with a lower magnitude or duration of biological effects. Such a mechanism could be used to produce lower levels of IL-15 in specific tissues such as the thymus. Perhaps thymic regulation for particular T cell populations requires either low levels or short-acting IL-15, in which case SSP-IL-15/IL-15Rα complexes can achieve this objective by providing lower production of this cytokine [50]. Another proposed role for the SSP form of IL-15 is the regulation of endogenous production of IL-15 through negative feedback [8, 9, 16, 56]. For instance, the coexpression of LSP-IL-15 and SSP-IL-15 in the presence of IL-15Rα resulted in lower levels of bioactive IL-15 [50]. Likewise, the increasing ratio of delivered SSP/LSP IL-15 DNA resulted in inferior bioactivity of IL-15 [50]. However, the most compelling evidence supporting a regulatory role of the SSP form came from transgenic mice expressing SSP-IL15 [8]. Nishimura et al. generated two lines of transgenic mice expressing either SSP or LSP for IL-15 under the control of an MHC class I promoter. Analysis of these mice showed that LSP-IL-15 transgenic mice expressed significant levels of secreted IL-15 and exhibited resistance to Salmonella infection. In contrast, a large amount of intracellular IL-15 protein was detected but hardly secreted extracellularly in SSP-IL-15 transgenic mice. An interesting

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observation from this study is the impaired production of endogenous IL-15 in SSP-IL-15 transgenic mice, resulting in increased susceptibility to Salmonella [8]. The finding that overexpression of SSP limits the production of endogenous IL-15 supports a role of this form in the negative feedback of endogenous production of IL-15.

Alternative splicing is a common regulatory mechanism used to generate variants of many biologically and immunologically important molecules such as IL-2, IL-4, IL-6, and IL-10 [57]. For these cytokines, splice variants are believed to be natural inhibitors of cytokine signaling, essentially acting as dominant negative forms of the cytokine that compete with the full-length cytokine for receptor binding [58–60]. In the case of IL-15, the SSP form could have binding affinity for yet an uncharacterized IL-15Rα isoform that may result in the formation of cytokine/receptor complexes with different biochemical features, capable of equipping the cell with the ability to produce different levels and functions of bioactive IL-15. In view of the deleterious role of IL-15 in driving or exacerbating inflammation [61–63], it is plausible to suggest that negative control mechanisms mediated by antagonistic IL-15 isoforms such as the SSP form may be needed to help dampen IL-15-mediated inflammatory events. Much further work is needed to understand the biological significance of having a form of IL-15 with an intracellular fate.

Where does the IL-15/IL-15Rα complex form?

The observation that coexpression of IL-15Rα had no effects on the mRNA levels of IL-15 suggested that protein trafficking and stability rather than mRNA stability are responsible for the outcome of IL-15 secretion in the presence versus absence of IL-15Rα [50]. Various secretory pathways that enable cells to control the release of cytokines into their tissue environment have been characterized [64–66]. These pathways operate on the basis of recognized general principles for regulating protein secretion: secreted proteins commonly are translated as precursor proteins, which possess signal peptides at their N termini that target them to the secretory pathway [67, 68]. Upon translocation across the endoplasmic reticulum (ER), this signal peptide is removed by signal peptidases and initial glycosylation can occur [69, 70]. Subsequently, proteins are transported within vesicles to the Golgi compartments, in which additional glycosylation trimming occurs [71–73]. Finally, secretory vesicles deliver mature proteins at the cell surface for secretion, either constitutively or upon specific stimuli [74–76]. By biochemical methods in combination with mutational analyses, by employing specific inhibitors and an analysis of N-linked glycosylation in membrane fractions and supernatants, earlier work examined the secretory route of IL-15 and elucidated how IL-15 is chaperoned through the secretory pathway by complexing with its own high-affinity receptor [45]. Like many transmembrane and secreted proteins, IL-15 undergoes N-linked glycosylation in the ER, a form that is sensitive to EndoH digestion [7]. N-linked sugars are then removed from IL-15 in the Golgi during further processing to generate mature forms of IL-15 that are resistant to EndoH [77]. To determine where IL-15/IL-15Rα complexes are assembled, cells were stimulated and lysed, immunoprecipitated with anti-IL-15Rα antibodies, and treated with EndoH. The result that IL-15/IL-15Rα complexes contain EndoH-sensitive IL-15 provided evidence that IL-15/IL-15Rα complexes form in the ER [44]. Duitman et al. further
elucidated that IL-15 translocation into the ER occurs independently of the presence of IL-15Rα. Subsequently, however, IL-15 is transported through the Golgi apparatus only in association with IL-15Rα and then secreted via the classical secretory pathway as a complex [45]. Incubation of IL-15 produced in the supernatants with Endo H showed that the N glycosylation of the secreted IL-15 is Endo H resistant, indicating that IL-15 secreted in the supernatant exists predominantly in a complex form with IL-15Rα [45]. Thus, interaction between IL-15 and IL-15Rα in the ER is required for IL-15 stabilization resulting in the formation of IL-15/IL-15Rα heterodimeric complexes that are either cell-associated and retained in the plasma membrane or released into the medium after proteolytic cleavage [43]. It should be noted that mechanisms by which a cognate receptor chain contributes to the mobilization and secretion of its ligand have been previously reported. For instance, in the case of IL-4, both receptor and ligand are independently transported and stored in granules [66]. In contrast, IL-15 requires complex formation with IL-15Rα in the ER and its secretion is independent of secretory granules [45]. Given its key role in IL-15 secretion, IL-15Rα is increasingly viewed as a component of the heterodimeric cytokine rather than just a member of the cytokine receptor.

View of IL-15 trans-presentation from different angles

In a simplistic way, IL-15 trans-presentation can be defined as follows: IL-15 bound to the IL-15Rα chain on one producing cell is offered to an IL-15-responsive cell which must minimally express β and γc subunits of IL-2R (Table 3). However, as our understanding of the mode of action of IL-15 evolved during the last decade, different modes of trans-presentation

FIG. 1 Three modes of IL-15 trans-presentation. **Left panel:** A dendritic cell (DC) trans-presents IL15Rα–IL-15 to Natural Killer cells (NK) cells that express IL-2Rβγc chains. This interaction occurs at the DC–NK cell synapse at the plasma membrane. **Middle panel:** IL-15Rα–IL-15 is cleaved from the plasma membrane by a metalloprotease and the soluble form binds to βγc chains of NK cells. This complex could be endocytosed. **Right panel:** The full, membrane-associated IL-15Rα–IL-15–IL2Rγc trans-cellular complex is trans-endocytosed, bringing along a vesicle snatched from the plasma membrane of the DC. In this configuration, trans-presentation persists inside NK cells. This is a different type of endosome into which the quaternary IL-15 complex is trans-endocytosed along with a fragment of the DC plasma membrane, which results in a very different topology. DC membrane proteins that came along have the potential to influence the outcome. Figure courtesy of Dr. Eric Long, Laboratory of Immunogenetics, National Institute of Allergy and Infectious Diseases, National Institutes of Health.
with distinct functional outcomes have emerged (Fig. 1). First, in the well-documented trans-presentation mode, the heterodimer IL-15/IL-15Rα complex on one cell binds to the β-γc subunits of IL-2R expressed by another cell, and signal occurs at the immunologic synapse between presenting and responding cells [34]. A beneficial aspect of this interaction is the potential for regulation by other receptor–ligand interactions occurring at the synapse between presenting and responding cells. By using this mode of trans-presentation, the interaction of DCs as presenting cells with NK cells as responding cells results in the formation of an immunologic synapse with characteristics of activating and inhibitory synapses, called a regulatory synapse [78–81]. In the second model, the IL-15/IL-15Rα complex is transferred from presenting cells to responding cells through the activity of cell surface metalloproteases [82]. This process is dependent on the proteolytic cleavage of IL-15Rα (also termed IL-15Rα shedding), which allows IL-15/IL-15Rα complexes to separate from the presenting cells and enter by endocytosis into the responding cells via β-γc subunits of IL-2R. Once separated from presenting cells, responding cells are able to store and use (recycle) the IL-15/IL-15Rα complex for their own proliferation and survival [83].

Beside its role in the separation of the complex from presenting cells, the proteolytic cleavage of IL-15Rα allows a conformational change in the positioning of the cleaved IL-15Rα within the high-affinity quaternary complex resembling the cis-presentation mode, and this allosteric effect was shown to be required for efficient IL-15 trans-presentation [83, 84]. A recent study by Antona et al. described a third mode of IL-15 trans-presentation that involves the transfer of the IL-15/IL-15Rα complex from presenting cells to responding cells independently of IL-15Rα shedding [85]. In this case, the entire membrane-associated IL-15/IL-15Rα complex is snatched from the plasma membrane of presenting cells and trans-endocytosed into responding cells (β-γc-expressing cells) where trans-presentation persists [85]. The persistence of IL-15 trans-presentation in endosomes as an interaction between two receptors, each anchored on a different membrane, may provide an advantage. For instance, once trans-endocytosed, IL-15/IL-15Rα bound to IL-2Rβ-γc may be sheltered from control by other receptor-ligand interactions occurring at the DC-NK immunologic synapse [81]. Also, trans-endocytosis of IL-15/IL-15Rα, as opposed to the endocytosed shed complex, appears to have distinct functional consequences [85]. In particular, shed IL-15/IL-15Rα from presenting cells contributes to efficient signaling for NK cell survival [83], whereas uptake of the entire membrane-associated IL-15/IL-15Rα complex via trans-endocytosis from presenting cells contributes to NK cell proliferation [85]. Although the precise basis for the various modes of IL-15 trans-presentation is not entirely understood, these recent observations extend our current understanding of IL-15 trans-presentation, in that the cellular compartment in which receptor-ligand interaction occurs can have an effect on functional outcome.

Beside cell-cell interaction, trans-presentation may also occur between soluble IL-15/IL-15Rα complexes found in biological fluids [84, 86] and responding cells expressing β-γc subunits of IL2R. Although the precise basis for the various modes of IL-15/IL-15Rα complex secretion is currently incompletely understood, the decision to produce soluble versus membrane-bound IL-15 complexes seems to be strongly dependent on the splice version of the IL-15Rα [25]. Müller et al. described multiple IL-15Rα isoforms in humans that appear to determine the fate of IL-15 complex secretion [25]. In particular, the isoform containing the Ex2A domain was shown to prevent the release of IL-15/IL-15Rα heterodimers from the cell membranes, thereby facilitating its trans-presentation function, whereas the IC3 isoform that
Pleading on behalf of the soluble IL-15 monomer

does not contain the Ex2A domain instead functions as a soluble secreted cytokine [25]. Although it has been suggested that membrane-bound complexes possess a competitive advantage over soluble complexes due to increased local concentration or valency of membrane-bound complexes during cell-cell contact [44], compelling evidence shows that soluble IL-15/IL-15Rα complexes can survive for a long time with a strong biological activity in the bloodstream [43].

Although at first glance the process of IL-15 secretion and presentation seems cumbersome compared to that of other cytokines, it provides a sophisticated mechanism to fine-tune the release of IL-15 and regulates signaling via different modes of trans-presentation with different functional outcomes.

**What is the fate of uncomplexed IL-15?**

The coexpression of IL-15 and IL-15Rα in the same mammalian cell leads to rapid intracellular association in the endoplasmic reticulum, stabilization of both molecules, and efficient transport of the heterodimeric complex to the cell surface, where it is bioactive [43, 50]. How about uncomplexed IL-15? What is its fate? It has been shown that uncomplexed IL-15 fails to continue its way through the secretory pathway resulting in aborted production. The observation that a large portion of IL-15 is rapidly degraded immediately after synthesis in the absence of IL-15Rα demonstrated a key role of IL-15Rα in the stability of IL-15 [43]. Stabilization of IL-15 in the presence of IL-15Rα was demonstrated directly by measuring the decay of IL-15 after blocking protein synthesis by cycloheximide [43]. Results showed a half-life of ~1 h when IL-15 is expressed alone, whereas the presence of IL-15Rα significantly improved IL-15 stability up to ~20 h [43, 50].

Along these lines, blocking of proteasomes in conditions where IL-15 is expressed alone resulted in increased intracellular accumulation of IL-15, indicating that IL-15 is rapidly degraded after synthesis through the proteasome, unless complexed with IL-15Rα [7, 43–45]. IL-15Rα binding to IL-15 early after synthesis is therefore required to prevent the rapid movement of unbound, unstable IL-15 to the proteasome degradation pathway, as well as to promote trafficking of the complex to the cell surface [50]. Such complexes prevent free IL-15 from undergoing ER-associated protein degradation, a well-established process by which “misfolded” proteins in the ER are proteolyzed in ubiquitin- and proteasome-dependent processes [87]. Although it is recognized that the formation of IL-15/IL-15Rα complexes is the primary mechanism underlying IL-15 secretion, evidence of free IL-15 in certain pathological conditions [61–63] and after physical exercise [88–91] pleads for an alternative mode of IL-15 secretion, yet to be characterized.

Pleading on behalf of the soluble IL-15 monomer

It has been well established that development and homeostasis of NK cells requires that both IL-15 and IL-15Rα are expressed as a heterodimeric complex by producing cells [35–37, 92], arguing that the monomeric form of IL-15 is irrelevant for physiological responses to this cytokine. While this is true for NK cells, data from other physiological and pathological conditions argue for a role of the soluble monomer IL-15. For instance, blocking IL-15 with an
antagonist mutant IL-15/Fc protein or a soluble IL-15Rα proved to be efficient in preventing the induction of allergic inflammation [62] and progression of collagen-induced arthritis [61]. Studies from skeletal muscle reported that amounts of serum IL-15 increase acutely following physical exercise in both humans and mice [88–91]. Transgenic mice with a global or muscle-specific deletion of IL-15Rα revealed robust amounts of IL-15 in serum despite a lack of IL-15Rα [90, 93, 94]. To determine whether a free form of IL-15 readily exists in the serum, Anderson et al. developed a new and more sensitive technique for measurement of IL-15. Contrary to previously published work, they reported that most IL-15 in mouse serum exists in the free state and proposed that IL-15 may be secreted in the free form by an unknown pathway independently of IL-15Rα [95]. An alternative mode, which is not mutually exclusive with the currently accepted model of IL-15 secretion for providing free IL-15 in the serum, is the possible dissociation of the IL-15 monomer after the heterodimer complex is released from the producing cell. However, the demonstration that IL-15/IL-15Rα heterodimers are highly stable complexes that do not release appreciable amounts of free IL-15 to neighboring IL-15Rα-bearing cells [44] rather refutes this possibility.

Regardless of the mode of its physiological secretion, IL-15 in complex with IL-15Rα, as opposed to free IL-15, has the advantage of enhanced bioavailability and functional activity [40, 41, 95]. Mice treated with IL-15 alone or a complex of IL-15 and IL-15Rα reported a half-life of 30–60 min for IL-15 alone, whereas data from complexed IL-15 revealed an extended half-life of 20 h [40, 41, 95]. Likewise, the biological activity of soluble IL-15 is significantly improved when it is provided as a complex with IL-15Rα. Indeed, results from treatments with the free form of IL-15 showed a modest effect, whereas administration of IL-15/IL-15Rα complexes resulted in strong expansion of IL-15-responding cells [40, 41, 95]. Specifically, a dose of 0.1 μg of IL-15 combined with 0.6 μg of IL-15Rα induced a level of proliferation of responding cells similar to that of 5 μg of free IL-15, indicating a 50-fold increase of activity when IL-15 is coadministered with IL-15Rα [40, 41, 95]. In the context of clinical use, recombinant human IL-15 has been produced as a monomer and tested in preclinical studies to evaluate safety, toxicity, pharmacokinetic, and pharmacodynamic properties [96]. Although the treatment of rhesus macaques with monomeric IL-15 yielded increased numbers of NK cells [97–100], this form posed multiple challenges for clinical use due to its instability and rapid plasma clearance [99, 100]. In contrast, administration of the IL-15/IL-15Rα heterodimer reconstituted from purified subunits resulted in sustained plasma IL-15 levels and in robust expansion of NK cells in mice, demonstrating pharmacokinetics and in vivo bioactivity superior to single chain IL-15 [101]. It should be underscored that the superior bioactivity of IL-15 described in this heterodimeric formulation is mainly the result of the presence of IL-15Rα contributing to increased stability of the cytokine in vivo [101]. Using a series of IL-15 transgenic expressing different forms and levels of this cytokine, Polansky et al. examined the activity of IL-15 when available as soluble monomers [102]. Transgenic mice expressing high levels of free IL-15 were crossed into a Il15Rα−/−/Il15−/− background to ensure that the pathway of IL-15Rα-dependent secretion of IL-15 is abrogated. In these mice, in which the only source of IL-15 available is the free monomer form, the development of NK cells failed in most sites (bone marrow, spleen, and blood), except in the liver where normal reconstitution of NK cells was observed [102]. Although it is tempting to propose that the soluble IL-15 monomer can support liver NK cell homeostasis if provided at high enough levels, evidence of NK cell exhaustion in response to a high dose of IL-15 or prolonged exposure to IL-15 treatment challenges this possibility (see chapter “Less is more” is the rule of thumb for IL-15). Also, it should be noted that
free IL-15 in the serum could not be detected in these transgenic mice despite evidence of free IL-15 in cell lysates and supernatants [102]. Thus, observations from these mice should be considered only in the context where free IL-15 is present in the vicinity of producing cells rather than disseminating a systemic effect.

In summary, although the detection of free IL-15 monomers in the serum remains puzzling, identified properties—stability and bioactivity—of both forms of IL-15 provide a strong rationale for further evaluation of the complexed form rather than the monomeric form for clinical use.

**“Less is more” is the rule of thumb for IL-15**

Complex and tight control of IL-15 secretion and presentation is unusual for most cytokines characterized thus far, arguing that IL-15, if overused, is somehow deleterious to the host. In the context of prolonged proinflammatory exposure, Fehniger et al. showed robust expansion of NK and T cells, resulting in fatal lymphocytic leukemia cells in transgenic mice developed to overexpress IL-15 by eliminating posttranscriptional checkpoints [103]. Further studies from the same group reported that large granular lymphocytic leukemia can be induced in vitro from prolonged culture (>6 months exposure) of human NK cells with IL-15 [104]. While neither of these studies investigated NK exhaustion or dysfunction in these contexts, they do provide evidence for the detrimental effects of prolonged IL-15 exposure and the resulting dysfunction that can be attributed to exhaustion. In a recent study, Álvarez et al. evaluated the occurrence of NK cell exhaustion after chronic stimulation with IL-15 (for >5 days) and proposed a paradigm of NK cell exhaustion similar to T cell exhaustion, such that prolonged exposure sustains NK cell proliferation before it leads to decreased effector function [105]. Still, the most compelling evidence demonstrating that prolonged treatment of IL-15 results in NK cell exhaustion came from a comparative study in which mice received either transient or prolonged treatment with IL-15/IL-15Rα complex [106]. Whereas transitory stimulation increased the number of activated NK cells and significantly enhanced their effector function, prolonged stimulation by IL-15/IL-15Rα complexes led to a marked accumulation of mature NK cells with considerably impaired activation, cytotoxicity, proliferative activity, and altered balance of activating and inhibitory receptors [106]. It should be noted that depletion of CD8 or CD4 T cells and absence of T and B cells did not change NK cell hyperresponsiveness in this model, indicating that extrinsic factors are unlikely to be culprits. Frusto et al. further demonstrated that two repetitive injections of IL-15 exhausted NK cells, whereas a single injection was efficient in inducing NK cell proliferation [107]. However, unlike previously described [106], observations from this study clearly implicated a role of T cells in NK cell hyporesponsiveness induced by repetitive exposures of IL-15 [107].

IL-15-induced NK dysfunction was also investigated in human NK cell responses when exposed to either continuous or intermittent treatments with soluble monomeric IL-15 [108]. Results informed that continuous exposure to IL-15 decreased NK cell survival secondary to alterations in NK cell metabolism. When compared with NK cells treated intermittently with IL-15, NK cells continuously treated with IL-15 displayed a reduced mitochondrial respiration profile that is dependent on fatty acid oxidation. This phenotype was described to be associated with criteria of NK cell exhaustion including diminished signaling, decreased

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function, and reduced tumor control [108]. It should be highlighted that treating NK cells with a single dose of IL-15 monomer for a week did not result in NK cell hyporesponsiveness, indicating that continuous exposure to IL-15, without the benefit of a break, is responsible for the induction of NK cell exhaustion [108]. In a preclinical study, multiple cycles of injection with an IL-15 agonist were revealed to be detrimental for NK cell expansion in SIV+ macaque blood [109]. Likewise, results from a human phase I study conducted in refractory solid tumor cancer patients reported that one IL-15 infusion was efficient in inducing NK cell expansion in the blood of patients, whereas a second infusion resulted in a decreased response, consistent with NK cell exhaustion [110].

Collectively, results from these studies indicate that NK cell exhaustion in response to repeated IL-15 stimulations is a common denominator, regardless of the form of IL-15. Therefore, the methodology of dosing will likely have a great effect on NK cell responses; while weekly subcutaneous dosing might elicit strong NK cell expansion and function, repetitive intravenous dosing or continuous infusion might have an opposite effect by driving NK cell exhaustion, although drug half-life and availability will have an important role in this process. In this regard, the need to reduce the number of IL-15 treatments for clinical use provides another rationale for consideration of the complexed form rather than the monomeric form of IL-15, given the superior stability of IL-15 in complex with IL-15Rα [40, 41, 95–101], which presumably requires less repetitive treatments to sustain NK cell survival and activation in the therapeutic settings.

How does this knowledge translate in the clinic?

Soluble recombinant (r) IL-15 was initially examined for therapeutic potential [111], and data from several preclinical mouse tumor models showed antitumor effects [112–115]. Indeed, administration of rIL-15 promoted antitumor efficacy associated with enhanced NK and CD8+ T cell functions in multiple murine carcinoma models [114], and prolonged the survival of mice with metastatic colon cancer [115]. Nevertheless, IL-15 preparations administered as monotherapy were ineffective due to multiple factors including the: (i) dose-limiting toxicities, (ii) actions of immunological checkpoints, and (iii) short survival in vivo. Dose-limiting toxicities were observed at the doses required to induce immune and antitumor responses, and as a result, alternative dosing strategies are being examined for feasibility, safety, and efficacy of rIL-15 treatment. Also, the administration of IL-15 alone was not optimal because it activates the immune system negative regulatory checkpoints that dampen the immune response [116, 117]. To circumvent such checkpoints, trials of rIL-15 in combination with other anticancer agents were initiated. For instance, tumor-bearing mice receiving rIL-15 with antibodies to CTLA-4 and PD-L1 exhibited marked prolongation of survival compared to mice receiving IL-15 with either agent alone [115, 117]. In murine models of lymphoma and adult T cell leukemia, rIL-15 enhanced the antitumor efficacy of both rituximab (anti-CD20) and alemtuzumab (anti-CD52), respectively. Notably, the enhanced antitumor effects observed with these combinations were associated with augmented antibody-dependent, cell-mediated cytotoxicity mediated by NK cells [118]. These results provided the scientific basis for trials with rIL-15 combined with alemtuzumab for patients...
with adult T-cell leukemia (ATL) (NCT02689453), with obinutuzumab (anti-CD20) for patients with chronic lymphocyte leukemia (CLL) (NCT03759184), with mogamulizumab (anti-CCR4) for patients with ATL and Mycosis Fungoides/Sezary Syndrome (NCT04185220), with ipilimumab (anti-CTLA4) and nivolumab (anti-PD-1) for patients with refractory cancers (NCT03388632), and with avelumab (anti-PDL-1) in patients with mature T-cell malignancies (NCT03905135) and Clear-Cell Renal Carcinoma (NCT04150562).

Despite enhanced antitumor efficacy in these combinations, the efficacy of rIL-15 is limited by its short half-life due to instability, ranging from <1 h in mice to 2.5–12 h in patients depending on the route of administration and IL-15Rα availability [110, 119]. To improve the efficacy of IL-15 therapy, various other preclinical approaches have been explored, including coadministration of anti-CD40 to induce IL-15Rα expression, and the covalent binding of IL-15 to soluble IL-15Rα to mimic trans-presentation and increase the biostability of IL-15. Both of these approaches produced greater antitumor responses than IL-15 alone [42, 114, 115, 120], allowing an array of alternative IL-15 agents associated with IL-15Rα to be introduced clinically [39, 43, 86, 101, 121–127]. These include a heterodimer consisting of IL-15 and IL-15Rα [43, 86, 101, 126], RLI, a fusion protein consisting of IL-15 linked to the cytokine binding (sushi) domain of IL-15Rα [39, 121], RLI-anti-CD20 and RLI-CD20, which are RLI linked to anti-CD20 or GD2 [123, 124], ALT-803, a mutated (N72D) IL-15 linked to the sushi domain of IL-15Rα that is fused to an IgG-Fc fragment [124, 127], and the ALT-803 scaffold fused to four single-chains of rituximab (N-803) [125, 128].

These complexes, or IL-15 superagonists, have greatly increased IL-15 potency and serum stability compared to rIL-15, with half-lives of 7–20 h in mice [101, 121]. In particular, N-803 was detectable in patient’s serum up to 4–7 days after subcutaneous injection [129]. N-803 restricted tumor growth and improved the median overall survival of mice with colon and breast tumors, among others, and this antitumor effect was further improved upon combination with anti-CTLA4 and/or anti-PD-L1 [130, 131]. The overall lymphocyte numbers in the blood and the spleen of rhesus macaques were significantly increased upon treatment with hetIL-15 [126, 132]. Likewise, expansion of NK and CD8+ T cell populations was associated with a reduction in B16 melanoma and pancreatic tumor growth by IL-15/IL-15Rα-Fc [133], or B16 melanoma and CT26 colon tumor burden by RLI [121, 134]; and the combination of RLI with anti-PD-1 therapy further improved responses against CT26 murine colon tumors [135]. Given the current clinical results, IL-15 superagonists, as opposed to rIL-15, appear to have an advantage in mediating greater expansion of NK cells without inducing significant toxicity. This is not surprising given the compelling evidence of increased stability/half-life and bioactivity provided by IL-15Rα in the heterodimeric formulations compared to IL-15 monomers.

A different approach to enhance IL-15 activity exploits the immunostimulatory properties of DC vaccines to enable trans-presentation of IL-15. IL-15 has been shown to be effective when administered as a vaccine adjuvant in preclinical models of cancer and infectious diseases [116]. Earlier work by Steel et al. reported that animals vaccinated with DC expressing IL-15 and truncated neu gene remained tumor-free much longer than those vaccinated with neu alone; and this effect was further increased by modifying the DC vaccine to express IL-15Rα along with IL-15 [136]. Subsequent work by Dr. Lion’s group corroborated that the incorporation of the IL-15 trans-presentation mechanism into currently used DC vaccines by means of mRNA electroporation increased their immunostimulatory properties as
demonstrated by the enhancement of phenotypic NK cell activation and NK cell-mediated killing of tumor cells [137] and superior expansion of tumor-specific CD8+ T-cells [138]. Since mRNA electroporation is broadly accepted to introduce tumor antigens into DC, in situ cotransfection with IL-15 and IL-15Rα mRNA has raised significant interest in clinical use of DC vaccines capable of fulfilling two goals: antigen presentation and IL-15 trans-presentation. Future studies will unravel the potential of such a strategy to achieve clinical efficacy in cancer patients.

**Conclusion**

The current clinical results demonstrate that IL-15 in complex with IL-15Rα is promising for the treatment of hematological and solid malignancies. Further studies of IL-15 therapy in combination with NK cell immune therapy, checkpoint inhibitors, or DC vaccines may provide better opportunities of cancer treatment in a broader patient population [111]. The next step in IL-15 therapies may lie in understanding the interaction of IL-15 with the tumor environment. For instance, while continuous exposure to IL-15 exhausts NK cells, combinations of IL-15 with other cytokines such as IL-12 and IL-18 generate instead a memory function [139, 140]. The action of IL-15 in promoting NK cell survival and boosting NK cell activity for therapeutical purposes will likely depend on the availability of other cytokines in the tumor environment that may cancel IL-15 effects; one cytokine with such property is TGF-β [141–143]. Therefore, a better understanding of the context-dependent effects from IL-15 exposure as well as the narrow window between activation and dysfunction of NK cells may be the next step in IL-15 therapy.

**References**


II. Activation of NK cells

II. Activation of NK cells
8. IL-15 and IL-15Rα


II. Activation of NK cells
References


8. IL-15 and IL-15Rα


[105] Alvarez M, et al. Regulation of murine NK cell exhaustion through the activation of the DNA damage repair pathway. JCI Insight 2019;5.


II. Activation of NK cells
References


II. Activation of NK cells
Induction of NK cells from stem cells
Advances in pluripotent stem cell-derived natural killer cells for cancer immunotherapy

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Abstract
Adoptive cell therapy using natural killer (NK) cells is rapidly emerging as a promising method to treat a variety of malignancies. NK cells can now be routinely derived in vitro from human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs). An iPSC-based platform to generate NK cells for adoptive therapy overcomes a number of constraints associated with the use of other available NK cell sources for generating uniform, genetically engineered NK cell therapy products with enhanced activity. iPSC-derived NK cells can now be produced at a clinical scale, and within the last few years several genetically engineered iPSC-NK cell products have entered clinical trials to treat cancer, underscoring their therapeutic potential. Here we review current progress made in the generation of NK cells from hESCs and iPSCs, including key studies identifying novel strategies to generate genetically modified iPSC-derived NK cells with augmented antitumor function and improved therapeutic efficacy, and explore iPSC-derived NK cell products that are currently being evaluated in clinical trials.

Abbreviations
Ab antibody
ADCC antibody-dependent cellular cytotoxicity
AML acute myeloid leukemia
CAR chimeric antigen receptor
CD cluster of differentiation
CIS/CISH cytokine-inducible SH2-containing protein

*These authors contributed equally.
Conflict of interest

MJC and KY declare no conflicts of interest.

DSK is a founder and consultant for Shoreline Biosciences. He also consults for Qihan Bio and VISICell Medical.

Introduction

While adoptive cell therapy using tumor-targeted lymphocytes has gained considerable interest in the last decade, this field actually goes back to the 1980s when studies using autologous tumor infiltrating lymphocytes (TILs) demonstrated the ability to mediate regressions in some metastatic malignancies refractory to standard chemotherapy [1]. Since this time, advances in genetic modification technology, most notably the use of chimeric antigen receptors (CARs) to more specifically and effectively target tumors, led to the approval of Tisagenlecleucel (Kymriah) and Axicabtagene Ciloleucel (Yescarta), for the treatment of refractory B-Acute Lymphoblastic Leukemia in 2017 by the United States Food and Drug Administration (US FDA). These cell products, which both express CARs that target CD19 expressed on B cells, were the first US FDA-approved cell therapies that rely on the adoptive transfer of tumor-targeting lymphocytes for cancer treatment. Most recently, a new CAR-T cell therapy, Brexucabtagene Autoleucel (Tecartus), was also approved in 2020 to treat refractory Mantle Cell Lymphoma [2]. CAR-T therapy has shown remarkable efficacy in treating refractory B cell malignancies—with numerous Phase I and II studies reporting over 80% response rates [3–7]. The success of CAR-T cell therapy has fueled the development of other effective adoptive cell therapy options for treatment of more challenging tumor types.

These currently approved CAR-T cell-based therapies rely on the use of autologous ex vivo modified and expanded T cells harvested through leukapheresis from patients [8]. While autologous methods minimize some risks of therapy failure as they are not subject to immune-mediated graft rejection, there remain some drawbacks associated with this approach. Patient-to-patient variability on the quality of cells harvested can affect the outcome of the treatment. In addition to inherent genetic variations across patients that can influence the T cell phenotype, the disease and/or prior chemotherapy or radiotherapy can also affect the quality of the cells harvested and used for autologous therapy. Indeed, for CAR-T cell production, high naïve T cell content in the leukapheresis product is correlated with good ex vivo expansion of CAR-T cells and eventual clinical response, but these cells are selectively depleted by chemotherapy used to treat pediatric solid and lymphoid malignancies [9, 10].

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In rare events, leukemic cell contamination within the transduced T cell population led to the generation of CAR-expressing tumor cells, which makes them resistant to CAR-T cytotoxicity via masking of the CD19 epitope [11]. Patients treated with CAR-T cell therapy also experience potentially lethal complications, most notably cytokine release syndrome (CRS) and neurotoxicity [12–15]. Additionally, the current production of Kymriah and Yescarta takes approximately 3–4 weeks before the engineered CAR-T cells can be transferred back into the patient [16–18], during which period the disease can progress before the patient can receive the cell therapy. To overcome such challenges associated with current autologous cell therapy regimens, there is increasing interest in the development of ‘off-the-shelf’ cell therapies to provide a safe, consistent clinical cell product that is always readily available for full dose administration.

In this context, natural killer (NK) cells are gaining considerable interest as a suitable source to generate off-the-shelf cell therapy products. Unlike T cells, NK cells recognize and kill virus-infected [19] and tumor cells [20, 21] without the necessity for specific antigen sensitization. While T cell therapies require donor and recipient human leukocyte antigen (HLA) matching to avoid graft-versus-host disease (GvHD), NK cells can be administered across HLA types without causing GvHD [22]. Notably, NK cells display enhanced cytotoxicity against HLA-mismatched tumor targets due to the lack of NK inhibition via the binding of inhibitory killer cell immunoglobulin-like receptors (KIR) to their HLA ligands. Tumors also downregulate HLA class I expression as a mechanism to evade immunosurveillance by CD8+ cytotoxic T cells [23]. However, the absence of HLA class I expression can make the tumors more susceptible to NK cell killing due to the loss of inhibitory signals transmitted via KIRs and other NK receptors that engage with HLA molecules. Several early trials demonstrated complete remissions in patients with poor-prognosis acute myeloid leukemia (AML) when treated with haploidentical peripheral blood (PB)-NK cells, with good in vivo expansion and persistence [24–27]. Most recently, Phase I and II studies utilizing allogeneic adoptive transfer of ex vivo expanded, HLA-mismatched, cord blood-derived (CB)-NK cells engineered to express an anti-CD19 CAR and IL-15 to treat 11 patients with CD19-positive relapsed or refractory B cell malignancies showed objective response in 73% of patients [28]. Furthermore, even following infusion of up to $10^7$ NK cells/kg, none of the patients developed CRS, neurotoxicity, or GvHD [28]. Therefore, NK cell-based therapies have so far demonstrated both efficacy and safety, and may possess several advantages over T cell-based approaches.

PB- and CB-NK cells, as well as established NK cell tumor lines (e.g., NK-92 and KHYG-1), are NK cell sources that have demonstrated tumor clearing efficacy in adoptive cell therapy settings. However, each of these NK cell sources presents its own set of shortcomings that may hinder their progress toward their use as consistent and effective off-the-shelf therapies. While CB contains a higher proportion of NK cells compared to PB, it also contains an increased percentage of less mature, less cytotoxic CD56brightCD16lo/− NK cells [29–31]. Variability in the NK cell yield can also be influenced by a number of factors, including donor variability [32] and NK cell yield postpurification, which typically involve the depletion of CD3+, CD19+, and CD14+ cells via magnetic cell separation prior to expansion for therapy [28]. The removal of CD3+ cells is particularly important as T cell contamination in the final infusion product can potentially result in GvHD [33]. PB- and CB-NK cells can be efficiently expanded ex vivo via the use of irradiated artificial antigen presenting cells (aAPCs) to reach a

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clinical scale [34, 35]. Following isolation, CB- and PB-NK cells can also be difficult to genetically manipulate using traditional transfection methods, which remains a barrier toward generating homogeneous, gene-altered NK cells with enhanced activity. While there has been some progress in improving transduction methods, such as the use of charge-altering releasable transporters to deliver mRNA [36], baboon envelope pseudotyped lentiviral vectors [37], and ribonucleoprotein electroporation [38, 39], these methods still produce a heterogeneous, nonuniform population of genetically modified NK cells. In contrast, NK cell lines such as NK-92 and KHYG-1 provide defined, homogeneous cell populations that expand indefinitely in culture and are more amenable to genetic alterations [40]. However, these malignant NK-92 and KHYG-1 cells lack a number of receptors typically expressed on NK cells [40]. In particular, NK-92 do not express CD16, an Fc receptor that plays an important role in activating antibody-dependent cellular cytotoxicity (ADCC), a key pathway that mediates NK cell cytotoxicity when combined with therapeutic antibodies. However, they can be engineered to express absent receptors such as CD16, as well as other xenogenic constructs to enhance cytotoxicity [41, 42]. What is most concerning about these cell lines is that they are aneuploid and need to be irradiated prior to administration for safety reasons, a treatment which abrogates their ability to expand and persist in vivo and therefore limits their antitumor efficacy [40].

To circumvent hurdles associated with using these available NK cell sources, the use of NK cells generated from pluripotent stem cells, specifically human induced pluripotent stem cells (iPSCs), for adoptive cell therapy is emerging as a promising strategy to generate enhanced, uniform off-the-shelf NK cell therapy products. In this chapter, we will discuss recent advances in the use of iPSCs in regenerative medicine and cell therapy, refined methods to derive NK cells from iPSCs to a clinical scale, and strategies being investigated at both preclinical and clinical stages to generate iPSC-derived NK cells with enhanced cytotoxic activity to effectively combat a variety of malignancies (Fig. 1).

Pluripotent stem cells as a source for cellular therapy

hESCs and iPSCs have the capacity to grow indefinitely in an undifferentiated state via self-renewal. Thomson et al. originally described the first derivation of hESCs that are capable of differentiating into the three primary germ layers [43]. Subsequently, Yamanaka et al. reported successful generation of iPSCs—terminally differentiated somatic cells that are reprogrammed to a pluripotent state. iPSCs were initially generated using mouse cells [44], followed later with human fibroblasts and other somatic cells [45–47]. iPSCs can be derived by the introduction of transcription factors, termed pluripotency factors (e.g., KLF4, OCT3/4, SOX2, c-MYC, LIN28), in terminally differentiated somatic cells [44, 45, 48, 49]. Initial methods used viral vectors to deliver transcription factors to the cells, resulting in genomic integration of the viral construct and constitutive expression [44–46]. Advancements in iPSC induction technology have reported using nonviral delivery of pluripotency factors for reprogramming, including the use of synthetic mRNA [50], recombinant proteins [51], and CRISPR-Cas9 activators [52]. Advancements in iPSC technology, in particular the ease with which one can generate them, have made pluripotent stem cells more widely accessible to
FIG. 1 Strategies to engineer human pluripotent stem cells to derive NK cells with improved antitumor activity. (A) hESCs and iPSCs can be modified through introduction of exogenous DNA (lower panel), then differentiated into ESC/iPSC-derived NK cells that express chimeric or other modified receptors (upper panel), including increased affinity and cleavage-resistant CD16, CARs, and IL-15/IL-15R fusion constructs. (B) Genes that code for proteins which contribute to fratricide (CD38) or inhibit cytotoxic activity (CIS) can be removed via CRISPR-Cas9-mediated genetic disruptions (lower panel) in hESCs/iPSCs, enabling the derivation of a homogeneous population of 'enhanced' ESC/iPSC-derived NK cells for immunotherapy (upper panel).
study. As such, iPSCs have been instrumental in accelerating human stem cell biology research and the potential for iPSC-derived clinical therapies is expanding.

In the decade since their development, iPSCs have moved to the forefront of regenerative medicine. The first-in-human clinical trial using iPSC-derived retinal pigment epithelium (RPE) cells began in 2014 in Japan to treat age-related macular degeneration (AMD) [53]. Consistent with a previous study using hESC-derived RPE transplantation for the treatment of AMD and Stargardt’s macular dystrophy, there was no evidence of graft rejection nor adverse proliferation of the iPSC-derived RPE in the >12 month follow-up [54, 55]. Best-corrected visual acuity did not improve or worsen, demonstrating a lack of further degeneration from AMD and safety from iPSC-derived RPE transplant [54]. The first US-based clinical trial using iPSC-derived RPE to treat AMD followed in 2019 (NCT04339764) [56]. Clinical trials utilizing iPSCs and their derivatives have now expanded to treat diseases across the world including Parkinson’s disease (UMIN000033564), heart failure (NCT03763136), spinal cord injuries (jRCTa031190228), graft-versus-host disease (NCT02923375). Thus, iPSCs hold great promise to be used as a safe and reliable source of cells and tissues that can effectively treat a variety of diseases for which curative treatments currently do not exist.

Generation of NK cells from human pluripotent stem cells

The feasibility of generating hematopoietic lineage cells from hESCs and iPSCs has also opened possibilities for the generation and use of PSC-derived hematopoietic cells for adoptive cell therapy. Methods describing in vitro generation of CD34+ hematopoietic progenitor cells [57], then subsequently essentially all specific myeloid, erythroid, and lymphoid lineage cells [58–61], were described soon after the derivation of hESCs. Initial work refined hematopoietic differentiation from hESCs, which were successfully translated to human iPSCs [62–64]. Additional studies to dissect in vitro hematopoietic differentiation from hESCs/iPSCs demonstrated the role of stage-specific Activin-Nodal and WNT-β-Catenin signaling pathways to promote either the generation of primitive (CD235a–) or definitive (CD235a+) hematopoietic progenitors [65]. Primitive hematopoietic progenitor cells differentiate into a limited number of cells in the hematopoietic lineage including erythrocytes, megakaryocytes, and macrophages, and resemble extra-embryonic hematopoietic progenitors that arise in the yolk sac during fetal development [66]. One interesting recent study demonstrated that NK cells derived from primitive, Wnt-independent derived HOXA+CD34+ progenitors produce NK cells that predominantly degranulate upon activation and are more cytotoxic than NK cells generated from HOXA–CD34+Wnt-dependent derived definitive hematopoietic progenitors that are biased for inflammatory cytokine upregulation and exhibit less cytolytic activity [67].

Improvement in the methods to generate NK cells from hESCs/iPSCs now enables the production of homogeneous, functional NK cells at a clinical scale [68]. Our initial method to derive cells of hematopoietic origin involved coculturing of hESCs with irradiated stromal cell lines—initially with M2-10B4 or S17 to generate CD34+CD45+ hematopoietic progenitors, then with AFT024 or EL08-1D2 along with supplemental IL-7, FLT-3-L, IL-15, IL-3, and SCF to produce CD45+CD56+ NK cells [68, 69]. Our group has subsequently refined this method further to eliminate the use of serum-containing media and stromal cells, making
them more compatible for clinical use. This approach uses a “spin embryoid body (EB)” protocol to generate hematopoietic progenitor-containing EBs from hESCs/iPSCs by culturing with BMP4, VEGF, and SCF, followed by IL-7, SCF, FLT-3 L, IL-15, and IL-3 to direct NK cell differentiation [68, 70]. In addition to circumventing feeder use, the improved method also demonstrated that intact EBs can be directly introduced to NK cell differentiation conditions without the need to sort for CD34+ hematopoietic progenitor cells to generate mature NK cells. With these improved methods, hESCs/iPSCs can now be differentiated to fully functional, homogeneous, mature NK cells in 3–4 weeks [71]. These NK cells can also be further expanded in the presence of IL-2 and aAPCs expressing 4-1BBL and membrane-bound IL-21 [35] and can generate over $10^9$ cells from $10^6$ starting undifferentiated hESCs/iPSCs [68, 71].

hESC and iPSC-derived NK cells recapitulate many key features of primary NK cells. They express typical NK cell markers such as CD56, CD94, NKG2D, NKp44, NKp46, CD16, and KIRs, and exhibit potent cytotoxicity toward diverse solid tumors and hematological malignancies [69, 72, 73]. Interestingly, a higher proportion of iPSC-NK cells express the inhibitory receptor NKG2A compared to PB- and CB-NK cells [68, 69]. iPSC-NK cells display variable KIR expression, though expression of these proteins does not seem to correlate with cytotoxic activity, suggesting that NK cells generated through in vitro differentiation may be less susceptible to KIR-HLA interaction-mediated inhibition [73]. hESC and iPSC-NK cells also kill primary HIV-1-infected CD4+ cells and prevent viral replication as effectively as PB and CB-derived NK cells [74]. Similar to CB- and PB-NK cells, hESC/iPSC-derived NK cells elicit cytotoxicity against target cells via lytic granule release containing perforins and granzymes, proinflammatory cytokine upregulation (e.g., IFNγ and TNFα), and direct cell contact-mediated apoptosis through Fas-FasL and TRAIL-TRAIL-R1/R2 interaction [34, 75]. Importantly, they also express CD16, which can mediate ADCC [69]. Primary NK cells are broadly categorized as CD56brightCD16− and CD56dimCD16+, with the latter possessing a more mature, cytotoxic phenotype that predominates in peripheral blood, spleen, bone marrow, and lungs [76]. In contrast, hESC-/iPSC-derived NK cells typically express homogeneous levels of CD56 that do not correlate with CD16 expression.

The iPSC-NK-based cell therapy platform addresses a number of challenges associated with the use of other NK cell sources for generating effective, uniform NK cells for off-the-shelf use. iPSCs are genetically tractable, so genetic deletions, mutations, or transgenes that enhance NK cytotoxicity can easily be introduced while iPSCs are in an undifferentiated state. The introduction of CRISPR-Cas9 and transgenes often produce off-target effects that lead to unintended genetic alterations, especially when introducing multiple alterations simultaneously, which have led to chromosomal translocations [77, 78]. The use of an iPSC-based model allows for individual clone isolation and characterization for off-target genetic alterations and abnormalities via whole-genome sequencing and karyotyping [79]. This approach also enables the sequential introduction of multiple genetic alterations to further augment NK cell cytotoxicity while minimizing off-target mutations. Once engineered and cloned, these iPSCs can be banked to serve as a master clone to consistently produce NK cells of identical phenotype over time. Using this approach, several iPSC-NK products have already entered clinical trials. The following sections discuss recent developments and strategies being investigated to augment iPSC-NK cell activity to produce next-generation, off-the-shelf NK cell therapies.
Improving iPSC-NK cell metabolic fitness and in vivo persistence via CISH deletion

NK cells require exogenous cytokines to proliferate and mediate cytotoxicity. IL-2 stimulates NK cell proliferation, differentiation, survival, and cytotoxicity, yet high IL-2, exogenous in humans with cancer, can lead to toxicity through capillary leak syndrome, as well as activate regulatory T (Treg) cells that can impede T cell and NK cell activities [80, 81]. IL-15 augments many of these same cytotoxic functions in NK cells (and cytotoxic T cells) without activating Tregs and is generally well tolerated [82]. For this reason, NK cells with increased sensitivity to IL-15 provide an attractive option as they can be dosed in combination with minimal concentrations of IL-15 for tumor immunotherapy.

The IL-15 receptor shares two of the same subunits as the IL-2R (CD122 and CD132), as well as the IL-15 receptor alpha (IL-15Rα). IL-15 binds the IL-15Rα with high affinity and is presented to adjacent IL-15R-expressing cells [83], primarily by the activated monocytes [84]. The binding of IL-15 to IL-15R triggers an intracellular signaling cascade via the JAK/STAT pathway that induces the expression of IL-15 responsive genes, including CIS (cytokine-inducible SH2 containing protein), encoded by the gene CISH [85]. CIS directly binds JAK1, inhibiting downstream STAT signaling, and simultaneously recruits an E3 ubiquitin ligase complex that directs JAK1 to proteasomal degradation, thus inhibiting further IL-15 signaling [85]. CISH−/− mice exhibited greater tumor control in a metastatic murine breast cancer model than their wild-type counterparts, indicating that CIS may also be an important checkpoint in human anticancer responses.

To determine the ability of CIS to regulate human NK cell activity, we generated CISH−/− iPSCs using CRISPR-Cas9 technology and differentiated these into CISH-deleted iPSC-NK cells that phenotypically resemble WT iPSC-NK cells [86]. As expected, these CISH−/− iPSC-NK cells exhibited greater proliferation in low IL-15 conditions, as well as increased killing of human tumor cells in comparison to their wild-type counterparts, correlating with increased degranulation and production of IFNγ. Notably, the CISH−/− iPSC-NK cells demonstrated increased metabolic fitness with higher basal glycolysis and maximal respiration potential than WT iPSC-NK and PB-NK cells after 3 or 7 days in IL-15-limited conditions. This increased cytotoxicity and responsiveness to limited exogenous cytokines translated to significantly increased survival curves in a murine xenograft model of acute myeloid leukemia, with a majority of mice achieving complete tumor clearance and survival beyond day 120, compared with none of the mice treated with WT iPSC-NK cells reaching this endpoint [86]. Together, these studies demonstrate that CISH−/− iPSC-NK cells are more cytotoxic, exhibit better persistence, and can effectively function at lower cytokine concentrations than WT iPSC-NK cells to provide an attractive therapeutic option for the treatment of refractory malignancies [86].

Stabilized CD16 expression enhances ADCC mediated by iPSC-NK cells

As previously mentioned, NK cells express the activating immunoglobulin gamma Fc receptor IIIa (FcγRIIIa), also known as CD16a. CD16a induces ADCC in NK cells following...
recognition of the Fc region of IgG antibodies bound to their target antigens. Indeed, NK cell-mediated ADCC has been documented as a key component of many monoclonal antibody therapies in humans, including the anti-CD20 rituximab and the anti-EGFR cetuximab (reviewed in Ref. [87]).

CD16a has multiple allelic variants in humans, resulting in differential binding affinities to the Fc region of IgG antibodies. Indeed, expression of a naturally occurring variation of CD16a in humans, termed ‘high affinity’ CD16 (F158V), led to improved antitumor responses in patients treated with rituximab [88] or cetuximab [89]. Furthermore, CD16a is cleaved from the surface of activated NK cells by the metalloprotease ADAM17, resulting in decreased ADCC [90, 91]. Mutagenesis of this ADAM17 cleavage site blocks CD16a shedding following NK cell activation [90]. Our lab recently evaluated a genetically engineered hybrid CD16 molecule containing both of these mutations, termed high-affinity noncleavable CD16a (hnCD16), in iPSC-derived NK cells for its ability to mediate increased antitumor function [79]. We demonstrated that hnCD16-iPSC-NK cells maintained unchanged CD16 surface expression and demonstrated increased IFNγ production and cytotoxic granule release when stimulated with Burkitt’s Lymphoma (Raji), ovarian cancer (SKOV-3), and oral squamous cell carcinoma (Cal27) cells in combination with anticancer monoclonal antibodies when compared to WT iPSC-NK cells [79]. This increased activation correlated with greater killing of target cells in vitro. In vivo efficacy was demonstrated first using an intraperitoneal B cell lymphoma model, where anti-CD20 rituximab antibodies in combination with hnCD16-iPSC-NK cells conferred significantly greater protection than PB-NK cells + rituximab, with several mice completely clearing all tumor cells. Furthermore, these findings were reproduced in a systemic B cell lymphoma xenograft model (intravenous injection of tumor cells, followed by intravenous infusion of NK cells) in combination with rituximab, and in an intraperitoneal ovarian cancer model where the hnCD16-iPSC-derived NK cells were introduced in combination with an anti-HER2 mAb [79].

Augmentation of iPSC-NK cell cytotoxicity with chimeric antigen receptors

Multiple studies now demonstrate the cytotoxicity of both T cells and NK cells can be enhanced through the introduction of CARs. As they were initially developed for T cells, most CAR constructs used in NK cells contain T cell-oriented intracellular signaling domains, often composed of the CD28 and/or 4-1BB signaling domains and the CD3ζ chain [92]. These CARs are typically introduced into cord blood-derived-NK cells or PB-NK cells using viral vectors [93, 94]. Indeed, the efficiency of the infused CAR-NK product in one major study targeting CD19 was 49.0%, with a range of 22.7%–66.5%, and typically contains a small amount of contaminating CAR-T cells [93]. In addition, a review of over 25 CAR-T studies either published or listed on ClinicalTrials.gov revealed that the median amount of CAR⁺ T cells within each trial ranged from below 20% to greater than 95%, with an overall median across all studies of approximately 55% [95]. Here the advantages of iPSC-derived NK cells become apparent, as iPSCs can be engineered to express a CAR construct, then differentiated into iPSC-NK cells from a clonal population, resulting in a homogeneous CAR-iPSC-NK cell product.

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Furthermore, the baseline population of iPSCs can be genetically edited (i.e., hnCD16 or CISH\(^{-/-}\)) in combination with CAR expression, further enhancing their antitumor efficacy. Additionally, CAR-NK cells have demonstrated better safety profiles than CAR-T cells while maintaining similar efficacy against tumors [93, 96, 97].

**Design and testing of novel NK cell-specific CARs**

Our group previously developed novel CARs designed with NK cell-specific signaling domains (NK-CARs) to evaluate their activity compared to commonly used CAR constructs designed to activate T cells [92]. Following the expression of multiple different NK-based CAR constructs in NK-92 cells, the antimesothelin (meso) CAR4 construct consisting of an antimeso scFv, the CD8a hinge region, an NKG2D transmembrane domain, a 2B4 costimulatory domain, and the CD3\(\zeta\) intracellular signaling domain exhibited the greatest activity against target cells and was selected for further assessment in iPSC-derived NK cells. Importantly, unlike for the introduction of CAR constructs into CB- and PB-NK cells using viral vectors, our group introduced the NK-CAR4 construct into iPSCs using the nonviral Piggybac transposon method, then differentiated these into a homogeneous CAR-iPSC-NK cell population [97]. Importantly, the antimeso-CAR4-iPSC-NK cells conferred significantly greater cytotoxicity against mesothelin-expressing tumor targets than untransduced PB-NK cells. Indeed, when directly compared in a mesothelin-positive ovarian cancer mouse xenograft model, antimeso-CAR4-iPSC-NK cells significantly controlled the tumor burden, displayed greater in vivo persistence, and increased the overall survival time when compared with unmodified PB-NK, WT iPSC-NK, or modified iPSC-NK cells expressing an antimeso-CAR designed for T cells, containing the CD28, 4-1BB, and CD3\(\zeta\) signaling domains. Further, the antimeso-CAR4-iPSC-NK cells were compared with antimeso-CAR-T cells in a xenograft model, and similar antitumor effects were observed. However, mice treated with the antimeso-CAR-T cells were found to have significantly decreased body weight compared to those treated with antimeso-CAR4-iPSC-NK cells, indicating that the mice were suffering adverse effects. Indeed, by day 20 postlymphocyte infusion, inflammatory cytokines (IFN\(\gamma\), TNF\(\alpha\), and IL-6) levels were dramatically elevated in mice treated with CAR-T cells compared with antimeso-CAR4-iPSC-NK cells. These CAR-T cell-treated mice likely died not from the tumor, but from a combination of CRS and GvHD that was not present in CAR-NK cell-treated mice. These data demonstrate that CAR-iPSC-NK cells provide a viable and potentially safer alternative to CAR-T cells.

**CAR-iPSC-NK cells targeting the solid tumor antigen glypican-3**

The success of CAR-iPSC-NK cells in preclinical solid tumor models suggests they will be ideal candidates for human therapies. The Kaneko laboratory developed CAR-iPSC-NK/ILC (innate lymphoid cell) cells that target the oncofetal antigen glypican-3 (GPC3) that is overexpressed in 60 tumor types [98], including hepatocellular carcinoma, squamous cell lung cancers, and ovarian clear cell carcinoma, but rarely seen in healthy adult tissue [99]. Their study sought to simultaneously determine whether CAR-iPSC-NK/ILC cells are a
viable immunotherapy option to target difficult to treat solid tumors and assess safety concerns with using stem cell-derived therapies [99]. Following validation of their construct in T cells, iPSCs were transduced with a lentivirus encoding the anti-GPC3 CAR construct containing the CD28, 4-1BB, and CD3ζ intracellular signaling domains and subsequently differentiated into CAR-iPSC-NK/ILC cells. These anti-GPC3-CAR-iPSC-NK/ILC cells effectively killed GPC3⁺ SK-Hep cells through germline-encoded natural cytotoxicity receptors (NCRs); however, the introduced CAR greatly increased this specific killing of GPC3⁺-transduced SK-Hep cells. Treatment with the anti-GPC3-CAR-iPSC-NK/ILC cells led to improved survival in a mouse xenograft model using KOC7c clear cell carcinoma cells, with increased mean survival time from approximately 44 days for mice just treated with PBS, to approximately 65 days for mice that received anti-GPC3 CAR-NK/ILC treatment. However, as the control injections were PBS and not WT (non-CAR-expressing) iPSC-NK/ILCs, it is difficult to differentiate the role of the CAR in this improved survival compared to the innate cytotoxic activity of the NK/ILC cells [99]. Importantly, this study also assessed the toxicity and tumorigenicity of the CAR-iPSC-NK/ILC cells and demonstrated there were no residual iPSCs in the final cell product that could form tumors or teratomas in the recipient mice; nor did it observe any significant toxic effects after multiple doses compared to control mice, both important concerns in iPSC-derived therapies [99].

Current iPSC-NK cell products and clinical trials

One of the key players in the translation of iPSC-derived NK cell therapies from the bench to the bedside is Fate Therapeutics (San Diego, CA) who conducted the first clinical trial of nonmodified iPSC-derived NK cells (FT500) in the US [100]. Following lymphodepletion, patients with advanced solid tumors were treated with multiple doses of FT500 in combination with checkpoint inhibitors (NCT03841110) [100]. Importantly, no patients exhibited dose-limiting toxicities, nor was GvHD, CRS, or neurotoxicity reported, suggesting that these iPSC-NK cells are safe and well tolerated [101]. Indeed, iPSC-NK cells are poised to become an off-the-shelf therapy, similar to other nonpersonalized immunotherapy tools such as therapeutic monoclonal antibodies, with one major advantage being reduced production and safety testing costs compared to current autologous CAR-T cell-based therapies that are produced on an individualized patient-specific basis [102].

The second iPSC-derived NK cell product now in clinical trials is FT516 (Fate Therapeutics), an iPSC-derived NK cell product expressing hnCD16 (described above) as a monotherapy for the treatment of relapsed/refractory AML and in combination with monoclonal antibody therapy to treat relapsed/refractory B cell lymphoma [100]. FT516 was the first ever genetically engineered iPSC-derived product approved for clinical testing (NCT04023071). As outlined above, these cells retain the ability to kill tumor cells through germline-encoded NCRs and exhibit greater antitumor function in the context of specific mAbs against target antigens. Indeed, while initial clinical trials focused on B cell malignancies [100], FT516 is now being used in combination with mAbs against solid tumors (NCT04551885) to provide greater on-tumor specificity than nonengineered iPSC-derived NK cells.
Perhaps of most interest is the ability to generate ‘stacked’ iPSC-NK cells that harbor multiple modifications, i.e., encoding antigen-specific CARs, the hnCD16 receptor, and can produce activating cytokines that function in an autocrine manner. Indeed, FT596 is a Fate Therapeutics product composed of iPSC-derived NK cells expressing an anti-CD19 CAR4 construct, the hnCD16 receptor, and an IL-15R fusion construct to enable improved NK cell persistence in the absence of exogenous cytokines [93, 103]. FT596 is now being investigated in an open-label phase 1 clinical trial as a monotherapy, and in combination with anti-CD20 antibodies for the treatment of advanced B cell lymphoma and CLL (NCT04245722). Another NK cell product to soon enter clinical trials is FT576 that contains the anti-CD19 CAR, hnCD16, the IL-15R fusion construct and has been genetically edited to remove CD38, enabling the use of anti-CD38 mAbs to target multiple myeloma without causing fratricide of CD38+ NK cells [104, 105].

Conclusions

Pluripotent stem cell-derived NK cells provide an important and exciting addition to the immunotherapy repertoire. hESC-/iPSC-derived NK cells exhibit intrinsic cytotoxic activity against many tumor types and can be used in allogeneic settings without genetic modifications, providing a substantial advantage over genetically modified T cells. Furthermore, the genetic tractability of iPSCs has enabled the development of novel engineered cell therapy products that delete specific genes and/or introduce activating constructs to enhance persistence and cytotoxicity. Together, this approach results in a standardized, homogeneous, and clonally derived population of immune cells that can be generated and readily infused in an off-the-shelf manner at a reduced cost in comparison to autologous CAR-T cell therapies. We eagerly await additional results from the current clinical trials evaluating iPSC-derived NK cells and anticipate that these effectors will become an important part of the anticancer immunotherapy arsenal.

Acknowledgments

The authors apologize to their colleagues whose works were not cited here due to space limitations. The authors also thank current and former members of the Kaufman laboratory for their helpful discussions and contributions to the work summarized in this chapter.

Research in the Kaufman laboratory is supported by the NIH/NCI, the California Institute for Regenerative Medicine (CIRM), and the Sanford Stem Cell Center at UCSD.

References


9. Advances in natural killer cells for cancer immunotherapy


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References
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[66] Fraser ST. The modern primitives: applying new technological approaches to explore the biology of the earliest red blood cells. ISRN Hematol 2013;2013:568928.


[72] Bock AM, Knorr D, Kaufman DS. Development, expansion, and in vivo monitoring of human NK cells from human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs). J Vis Exp 2013;74, e50337.


III. Induction of NK cells from stem cells


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Phenotypic plasticity: The emergence of cancer stem cells, collective cell migration, and the impact on immune surveillance

Caterina A.M. La Porta

Abstract

The presence of subclones of tumor cells with a more aggressive phenotype and their role in metastasis are discussed in this chapter. Phenotypic plasticity of tumor cells has an important role for immune surveillance and here we show how it impacts metastasis. Another important issue is the appearance of metastasis as an independent disease, where on one side, the possibility to hit the plasticity of tumor cells and on the other side, the microenvironment can lead to a successful therapeutic strategy.

Abbreviations

- CC: cancer cell
- CSC: cancer stem cells
- CTC: cancer tumor cell
- EMT: epithelial mesenchymal transition
- NK: natural killer

Conflict of interest

No potential conflicts of interest were disclosed.
Tumor heterogeneity and metastasis

Nowadays, metastasis is the main cause of cancer mortality. In fact, early cancer detection is a critical step for a successful treatment. Usually the presence of a cancer is discovered by chance or after a screening. Technological improvements in radiological imaging, laboratory tests such as cancer markers, or genetic testing improved significantly in the last 10 years, leading to the discovery of a primary tumor earlier. The latter means, as a consequence, a better outcome. On the other hand, metastasis is often fatal. The main goals, considering the failure of the current strategies, are to find new ideas to treat metastasis. To achieve these goals, it is crucial to better understand the biological characteristics of metastatic cells and then design a targeted and personalized therapeutic strategy. In this context, immunosurveillance also plays a crucial role and can be strictly related to the biological features of the metastatic cells.

According to the recent literature, primary tumors show a high heterogeneity, while metastases seem to display more aggressive subclones. In particular, next-generation sequencing data revealed that a primary tumor possesses on average over 10,000 somatic mutations: about 2–8 in “driver” genes that confer a selective growth advantage, and 30–60 protein-coding changes in “passenger” genes that may alter other cellular functions [1]. The mechanisms underlying subclonal diversification include genomic instability, homologous recombination deficiency, chromosome instability, etc. In recent years, many reports have identified a more aggressive subpopulation inside the tumors, the CSCs [2]. Moreover, the recent studies on single cells produced new details about subclonal frequencies and their evolution during solid tumor progression. Single-nucleus sequencing of breast tumors showed that copy number evolution occurred in short bursts early in tumor evolution, whereas point mutations evolved gradually over time to produce more extensive clonal diversity [3]. These data suggest that targeting multiple clones would give a better response than targeting a single clone at least for primary tumors. However, metastasis seems to be carried out by rare cells with unique cellular and molecular properties [4]. Single-cell investigations now enable the identification and characterization of such cells, including their localization in primary tumors, and the effect on the environment. In this connection, next-generation sequencing studies of bulk tumor samples seem to indicate that metastasis is initiated by a subclone of the primary tumor. For example, whole-genome sequencing identified numerous point mutations in the brain metastatic tumor than in the paired primary tumor [5]. Similar disparities in mutation frequencies have been reported in paired tumors and metastases from patients with pancreatic and renal cancers [5]. The latter evidence supports a subclonal model for metastasis initiation [5, 6]. However, another report showed that metastases often have unique mutations that were not found in paired primary tumors [5]. In this connection, recent reports showed that metastatic lesions similarly derive from early dissemination events [7, 8].

Single-cell genome analyses of patient CTCs provide insight into the genotype of potential metastasis-initiating cells. CTCs display substantial subclonal diversity, suggesting that cells of various genotypes are capable of entering the circulation [9]. Another study found that CTCs in patients with breast cancer often possess variants that are not found in the primary tumor, indicating that they either represent a rare subclone or occur after dissemination [10]. As it is clear that metastatic propensity is not encoded exclusively at the genetic level [11], it will be necessary to investigate other programs (for example, transcriptomic and epigenetic).
driving metastatic progression at the single-cell resolution. Single-cell multiplex qPCR technology has shown that metastasis is initiated by cells with stem cell and EMT-like characteristics in patient-derived xenograft models of breast cancer [12]. A recent scRNA-seq study of human head-and-neck cancers further implicated EMT transition in metastasis [13]. Altogether, single-cell approaches are emerging as valuable tools in dissecting those complexities from genomic, transcriptomic, and proteomic perspectives and in potentially determining the molecular signatures of every cell and its destiny during the course of the disease. However, to overcome the noise of data or batch effect due to the fact that the data are not homogeneous, new informatic tools are needed. In a different context, our group developed new algorithms to solve this problem [14] that could be used for this purpose.

Another important aspect my group has deeply investigated in recent years in collaborations with many international groups is the movement of collective cancer cells and their relationship with their invasive capacity [15–17]. A deep discussion of collective cell migration and its statistical features are reported in a recent book [18]. Adhesion molecules such as E-cadherin have been shown to be deregulated as part of the EMT during progressing cancers [19] helping tumor dissemination [20–22]. As well as by adherens junctions, the ability of moving cells to engage with neighboring cells and move collectively is modulated by micro-environmental conditions. These include cell density [23, 24], the topology of extracellular matrix (ECM) which guides moving cells [25–29], and the effects caused by volume exclusion between cells that align reciprocally while migrating [30].

In this connection, topographical single-cell sequencing analysis showed that multiple clones comigrated through the basement membrane of breast ducts and into adjacent tissues to establish invasive breast carcinomas [31]. This is consistent with the previous work showing collective cell migration at the invasive front of tumors, as well as observations that CTC clusters in the bloodstream are more effective than single CTCs for seeding metastasis [32, 33]. In a recent paper, we found that cadherins and extracellular matrix confinement cooperate to determine unjamming transitions and stepwise epithelial fluidization toward, ultimately, cell individualization [34].

Phenotypic plasticity is an emerging property of cancer cells and the possibility of the tumor cells to move from a CC to CSC is allowed by factors produced by the environment or niche [35]. The EMT is usually referred as cell plasticity and the recent literatures showed that this process can be a multiple-step one in which cells expresses a mixture of E and M features, giving rise to hybrid E/M states [36, 37]. In a recent comment, I discussed with a physicist colleague that cells undergoing EMT can induce a jamming versus unjamming transition (JUT) through the reduction of cell–cell adhesion, a control parameter driving the JUT [35]. This aspect appears interesting since understanding how tissue fluidizes in a stepwise manner will be useful for conceptualizing different types of invasion plasticity and transitions from collective to single-cell migration for cancer invasion and metastatic escape [34].

Furthermore, our group has extensively studied the molecular mechanisms underling phenotypic switching in human melanoma [38, 39]. In particular, recently, we identified hsa-mir-222-5p as a key factor for melanoma plasticity [38, 39]. In fact, we showed that CSCs can self-renew or give rise to CSCs and CCs and CCs cells can also revert into the CSC state [38]. The tumor niche or the microenvironment controls the switching between the two biological states [38, 39]. We also showed that hsa-mir-222-5p was the inhibiting factor released by CSCs controlling the phenotypic switching [39]. In fact, we demonstrated that this miRNA
was highly expressed before the overshoot of CSCs from the CCs [38]. More recently, we also showed that it is released into the conditioned media (CM) [39]. Furthermore, its downregulation had important consequences for tumor aggressiveness: the cells no longer express CSC markers and their migration capability was impaired [39]. Investigating the relationship between the hsa-mir-222-5p and the Wnt pathway, which plays a crucial role in melanoma plasticity, we found that, at the steady-state, hsa-mir-222 is expressed by the cells and released outside, acting on paracrine cells (Fig. 1). Under this condition, the level of expression of LEF1, a key downstream target of the Wnt signaling pathway, showed an increase upon silencing of hsa-mir-222 [39]. The canonical Wnt signaling is activated when the Wnt ligand binds the Frizzled and LRP5/6 receptors. This leads to the stabilization of β-catenin, which is transferred from the cytoplasm to nucleus where it activates LEF1 (see Fig. 1). It is known that MITF, which is directly induced by LEF1, acts as an activator of hsa-mir-222 [40]. Hence, activation of the Wnt pathway leads to increased hsa-mir-222 expression. On the other hand, it has been reported that hsa-mir-222 can inhibit the translation of Frizzled [41, 42], thus suppressing the activity of this pathway (Fig. 1). The complete picture that emerges from these studies suggests that hsa-mir-222 is the switch inhibitor regulating the feedback loop as illustrated in Fig. 1.

In conclusions, our report provides guidance for therapeutic interventions targeting the switching into CSCs. Focusing on miRNA 222-5p.

![Figure 1](image_url)

**FIG. 1** Impact of mir222-5p on the Wnt pathway. (A) The activation of Wnt pathway leads to increase the level of miRNA 222 which can be released (B) and acts on the paracrine cells controlling the phenotypic switching.

III. Induction of NK Cells from Stem Cells
Immune surveillance and tumor plasticity

Phenotypic plasticity of tumor cells has serious implications in terms of immunological recognition and killing of the tumor [41]. Tumor cell plasticity allows tumor cells to dedifferentiate or to undergo a plastic mesenchymal-like phenotype, which, during tumor progression, enables them to adapt to and resist conventional therapeutic strategies. Accumulating evidence indicates the existence of a link between EMT-like processes and altered immune functions. In vivo immunoediting of breast tumor cells by CD8 T cells creates immune-resistant tumor cells via incomplete EMT [43, 44]. Acquisition of the EMT phenotype in various MCF-7 derivatives was shown to be associated with impairment of target susceptibility to CTLs by a mechanism involving the induction of autophagy [45].

These results suggest that EMT and autophagy might represent conceptual realms for immunotherapeutic strategies to block immune escape. In contrast, induction of EMT in human keratinocytes and colorectal cancer cells was shown to strongly upregulate the NKG2D (natural killer group 2D) ligands MHC class I chain-related molecules A and B (MICA/B) and ULBP1–3 [46]. This study also showed that triggering EMT rendered cancer cells more susceptible to NKG2D-mediated killing by NK cells, suggesting that EMT is a relevant checkpoint in the control of tumor progression through NKG2D-mediated immune responses [46]. A large number of studies has also highlighted that cytoskeleton remodeling is a key determinant in the interaction between tumor cells and immune cells through immune synapse formation [47]. Immunoediting allows tumors to evade immune destruction by becoming less immunogenic or more immunosuppressive. Such adaptability, achieved through cell reprogramming, reflects an important property of tumors called immune-induced plasticity.

Recent evidence showed that CSCs have the ability to hide from the immune system ab initio, evading form the immunosurveillance phase [48]. Other work highlighted that immune escape depends on an intrinsic property of quiescent stem cells and that the latency and immune evasion of metastatic cancer cells is strictly correlated with the acquisition of a slow-cycling stem-cell-like state [49]. By gene-set-enrichment analysis, the authors revealed that these cells show stem-like phenotype, express SOX2 and SOX9 transcription factors, and self-impose a slow-cycling state by the autocrine production of a WNT inhibitor, DDK1 [49]. Upon entering quiescence LCC cells evade NK-mediated immune surveillance through a broad downregulation of ULBP NK cells activators [49]. In the proposed model, after infiltrating target organs, LCC proliferating cells are killed by NK cells, but a minority of them enter a quiescence state and remain latent for an extended time [49]. As such, they retain a metastasis-initiating power as shown by NK depletion, a condition that may mimic a transient decrease in immune surveillance [49]. Hence, the immune-privileged status is not an intrinsic property of CSCs, but is linked to the ability to enter a quiescent condition [50].

Conclusion and future perspectives

Cancer was initially thought to be a disease of cells, then of genes, and then of genomes. It may be more accurate to consider cancer as a disease whose phenotype is due by a continuous talking between the cancer cells and the microenvironment. Phenotypic switching allows
tumor cells to plastically change their phenotype, thanks to the factors released by them as well as responding to signals released from the microenvironment. The possibility to impair phenotypic switching and, therefore, tumor plasticity appears a successful strategy and the recent advances in this field are starting to identify possible targets and detangle the molecular mechanisms. Further studies should be done on this direction guiding the pharmacological approach to metastasis. Metastasis should be considered as another disease with respect to the primary tumors and an interesting approach might be to hit the mechanisms that allow the cells to plastic adapt to the microenvironment becoming invisible to the classical drugs and to the immune surveillance.

This chapter had tried to point out the main aspects to start new investigations in this direction.

References

References


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Benjamin_Bonavida, 978-0-12-824375-6

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PART IV

CAR-NK cells in immunotherapy
CHAPTER 11

Using CAR-NK cells to overcome the host resistance to antibody immunotherapy and immune checkpoint blockade therapy

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Abstract

The objective of this review is to promote the idea of using CAR-NK cells to overcome host resistance to therapeutic antibody and immune checkpoint blockade immunotherapies in the current clinical practice. Antibody (Ab) and immune checkpoint blockade therapy (ICBT) are promising cancer immunotherapeutic approaches. Immune cells from the host are required for both therapies to be effective. For instance, natural killer (NK) cells are required for therapeutic Ab to kill tumor cells by mediating antibody-dependent cell-mediated cytotoxicity (ADCC), whereas PD-1-positive immune cells are needed for immune checkpoint inhibitors (ICIs) to be effective in cancer treatment. However, clinical data suggest that host resistance can and actually often contributes to reduce the effects of antibody and ICBT in patients. Specifically, NK cell impairment is often observed in cancer patients and can contribute to the host resistance to therapeutic antibodies, while the lack of PD-1-positive immune infiltration contributes to the host resistance to ICBT. Here, we propose to use chimeric antigen receptor (CAR)-modified NK cells to overcome host resistances. Our published study demonstrated that the tissue factor (also known as CD142)-recognizing CAR-NK cells could mediate ADCC as effector cells via CD16, while they also could directly kill cancer cells via CAR. These findings suggest that CAR-NK cells may enhance the antibody efficacy by overcoming NK impairment in cancer patients. CAR-NK cells are also positive for expression of PD-1, which may increase PD-1-positive immune infiltration in the tumor microenvironment when combined with ICBT, so the latter can benefit not only responders, but also nonresponding patients due to the lack of immune infiltration. We anticipate that CAR-NK cells in combination with antibody and ICBT will achieve an optimal clinical outcome for more cancer patients.
Abbreviations

ADCC: antibody-dependent cell-mediated cytotoxicity
CAR: chimeric antigen receptor
CD: cluster of differentiation
ICBT: immune checkpoint blockade therapy
ICI: immune checkpoint inhibitors
ICON: tissue factor-targeted immunoconjugates
L-ICON: tissue factor-targeted fVII light chain ICON
MDSC: myeloid-derived suppressor cells
NK: natural killer cells
PD-L1: programmed death-ligand 1
PD-1: programmed cell death-1
TF: tissue factor

Conflict of interest

Z.H. is coinventor of ICON and fVII-targeted PDT (previously with the Yale University) and the inventor of L-ICON1, L-ICON3, and TF-CAR technologies (with the Ohio State University). Z.H. is scientific cofounder of L-ICON3 technology-based startup company, Eikonoklastes Therapeutics.

Introduction

Natural killer (NK) cells are a class of lymphocytes distinct from T and B cells and play an important role in the innate immune system for direct killing of virus-infected cells and cancer cells as well as in antibody-mediated killing in antibody immunotherapy of cancer [1–3]. NK cells are large granular lymphocytes lacking of CD3 and T-cell receptors and compose about 10%–20% of the peripheral blood lymphocytes (PBL) in healthy individuals [1, 3, 4]. They express certain cell surface markers such as CD16 and CD56 in humans and NK-1.1/NK-2.1 and CD49b in mice. CD56+ and CD3− can be used as distinct surface markers for the identification of human NK cells, whereas CD16 is the receptor for the Fc portion of the immunoglobulin gamma (IgG Fc) to mediate antibody-dependent cell-mediated cytotoxicity (ADCC).

The adoptive transfer of chimeric antigen receptor (CAR)-modified T and NK cells represents a promising cancer immunotherapy approach. The concept of the CAR is based upon the idea of expressing novel receptors on the T or NK cell surface that would enable the T and NK cell to identify corresponding antigens on the surface of a target cell. The basic CAR construct consists of an extracellular antigen-recognition domain, usually single-chain antibody variable fragments (scFv), attached to an extracellular spacer domain, a transmembrane domain of CD28, and a signaling cytoplasmic domain such as 4-1BB (CD137), OX40 (CD134), DAP10, ICOS, and CD3ζeta chain (CD3ζ) [5].

One of the most advanced CAR applications is the use of CAR-T cells targeting CD19, a surface antigen on B cell malignancies, which have demonstrated antitumor efficacy in patients with these cancers [6]. However, early-phase clinical trials of CAR-T therapy also showed that this treatment is frequently associated with side effects [6], some even causing life-threatening toxicity [7], partly due to the fact that current CAR targets are expressed by both the malignant cells and normal cells, such as CD19, which is also expressed by normal B cells throughout the B cell lineage [6]. To overcome these challenges, it would be ideal to develop CAR-T or CAR-NK cells (Fig. 1) that could target a surface molecule that is commonly

IV. CAR-NK cells in immunotherapy
yet selectively expressed by several major tumor compartments, including but not limited to, the cancer cell, cancer stem cell (to eliminate metastasis and recurrence), and tumor vascular endothelial cell (to shut down blood supply then to starve cancer cells), whereas it is not expressed on normal peripheral blood mononuclear cells (PBMC, such as B, T, NK cells, monocytes, etc.) and resting vascular endothelial cells (to avoid side effects).

**FIG. 1** The idea of using CAR-NK cells in combination with antibody immunotherapy to overcome NK cell impairment, in which CAR-NK cells can independently kill target cancer cells via CAR and also can serve as CD16-positive effector cells for mediating ADCC against cancer cells. An ideal cancer antigen, for example, tissue factor (TF, also known as CD142) as a new target in TNBC and as an example of cancer antigen for Ab therapy, should be commonly yet selectively expressed by multiple tumor compartments, including but not limited to, the cancer cells, cancer stem cells, and tumor vascular endothelial cells, whereas it is negatively, minimally, or restrictedly expressed in normal cells. ICON and L-ICON: TF-targeting antibody-like immunoconjugates.

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**Challenges in antibody immunotherapy: NK cell impairment and its negative impact on therapeutic antibody immunotherapy**

The NK cell is often impaired (in number and/or function) in animals and cancer patients [8–10], contributing to host resistance to antibody therapy [9, 11, 12] and associated with a higher risk of recurrence [13, 14]. In 1979, Herberman et al. summarized that the NK impairment was noted in tumor-bearing mice and in patients that had large tumor loads [15]. Later, more studies reported that the NK ADCC activity was impaired in patients with cancer, including melanoma [16], breast [9, 17, 18], lung [19], gastric [12], esophageal [20, 21], and...
hepatocellular carcinoma [22–24], pancreatic [25], neuroendocrine tumor of gastrointestinal tract [26], ovarian [27, 28], and urologic cancers [29] as well as leukemia [30–33], lymphoma [34, 35], and myeloma [36–38]. In our published preclinical animal study of a human head and neck cancer (tongue cancer) [11], we found that ICON (Fig. 2), the first generation of tissue factor-targeted antibody-like immunoconjugate [39–41], could completely eradicate established human tongue tumor xenografts in SCID/CB-17 mice that have functional NK cells [42] whereas it was less effective in the SCID/Beige mice that do not have functional NK cells [43]. Furthermore, using flow cytometry assaying peripheral blood NK percentage in the ICON-treated mice, we further observed that not only the function but also the level/percentage of NK cells was crucial for ICON to completely eradicate established tumor xenografts in host mice [11].

In breast cancer, for example, the dysfunction of NK cells accompanies human breast tumor progression [9] and is correlated with decreased NK cell function, most notably cytotoxicity [9]. Moreover, tumor-infiltrating NK cells had more pronounced impairment of their cytotoxic potential than peripheral NK cells [9]. Moreover, NK activity can decline with age [44], which may contribute to higher incidence of cancer developed in elderly populations. As a result, NK dysfunction contributed to the impaired Ab ADCC, for example, Herceptin (an anti-HER2 Ab) therapy in gastric cancer patients [12]. Taken together, these preclinical and clinical observations suggest that overcoming NK cell impairment may help the current and future antibody therapies, e.g., tissue factor-targeted immunoconjugates (called ICON and L-ICON) (Fig. 2), including the first (ICON) [11, 39–41], second (L-ICON1) [45], and third-generation ICON (L-ICON3, patent pending), achieve an optimal clinical outcome and reduce the risk of recurrence in cancer patients. Here, we propose to overcome the impairment of host NK cells by using chimeric antigen receptor-modified NK (CAR-NK) cells (Fig. 1).

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**FIG. 2** ICON and L-ICON are the first- and second-generation tissue factor (TF)-targeting immunoconjugates. (A and B) Diagrams and molecular weight of L-ICON1 and ICON. (C) L-ICON1 is free of coagulation activity (under detectable). FVIIa: positive control (100%); FVIIa-FFR: active site inhibited FVIIa (FVIIa-FFR) as negative control (~5%); and ICON(K341A) has an active site mutation (K341A) (5%), whereas ICON (WT) has a wild-type sequence of FVII (56%). All p values were significant except for ns (no significance). Modified from Hu Z, Shen R, Campbell A, et al. Targeting tissue factor for immunotherapy of triple-negative breast cancer using a second-generation ICON. Cancer Immunol Res 2018;6(6):671–684. https://doi.org/10.1158/2326-6066.CIR-17-0343.
Using CAR-NK cells in combination with antibody therapy to overcome NK cell impairment

Here, we will use breast cancer, specifically triple-negative breast cancer as an example, to discuss the idea of using CAR-NK cells in combination with antibody therapy to overcome NK cell impairment (Fig. 1). Triple-negative breast cancer (TNBC) is an unmet clinical problem worldwide [46], a subtype of breast cancer, accounting for ~20% of breast cancers. In the United States, one in every eight women will develop breast cancer, making it the most frequently diagnosed cancer (estimated nearly 280,000 new cases in 2020) and the second leading cause of cancer death among women [47]. In 2018, an estimated 2.1 million breast cancer patients were diagnosed globally. Due to the lack of targetable markers (ER, PR, HER2), most cases of TNBC are considered incurable with a shorter life expectancy (18-month from diagnosis) compared to other breast cancer subtypes [46, 48, 49]. Thus, there is an urgent need to discover novel surface targets, and then to develop corresponding targeted immunotherapy for TNBC.

To address the unmet need in TNBC, our group reported, for the first time to our knowledge, that tissue factor is a new surface target in TNBC [45]. Tissue factor (TF), also known as CD142 [50, 51], is the only membrane-bound coagulation factor (factor III), cofactor, and surface receptor for the coagulation factor VII (fVII)/activated fVII (fVIIa) [52–54]. Its best-known function involves the initiation of blood coagulation upon the disruption of vessel wall integrity [53, 54]. Under physiological conditions, TF is not expressed on peripheral blood lymphocytes (PBL) or quiescent vascular endothelial cells (VEC; the inner layer) of normal blood vessels in normal tissues and organs [55–58]. Besides its role as the primary initiator of coagulation, TF is also a modulator of pathological angiogenesis [59–61]. We and our collaborators along with other groups have identified TF as an angiogenesis-specific receptor on the VEGF-stimulated angiogenic microvascular endothelial models in vitro as well as in vivo in angioinvasive vascular endothelial cells of the pathological neovascularure of endometriosis, wet form of age-related macular degeneration (AMD) and solid cancers, including melanoma [39, 40], lung cancer [62] and breast cancer [62], and from tumor xenografts in mice and breast cancer tissues from patients [45, 63].

Through our own and others’ work, TF is highly expressed on the cancer cells in many types of solid cancers [45, 58, 64–66], acute myeloid and lymphoblastic leukemia (AML and ALL) and sarcoma [58, 66] as well as in Hodgkin’s lymphoma [67] and multiple myeloma [68]. Patients with TF-positive tumors often have metastases in the liver, lung, and brain [69], which are the major cause of most of breast cancer deaths. However, it was unknown if TF is expressed in the patient’s TNBC, and if so, to what extent. We investigated TF expression by immunohistochemistry (IHC) in tumor tissue and matched normal breast control tissue from 161 cases of TNBC. The findings demonstrated that TF is expressed on the TNBC cells in up to 85% of patients when using whole tumor tissues (n = 14) and ~50% when using tissue microarray (TMA) (n = 147) [45] (Fig. 3). In addition to the TNBC cells, our laboratory discovered that TF is commonly yet selectively expressed by the tumor vascular endothelial cells (TVECs) [45, 70] (Fig. 4) and the cancer stem cells (CSCs) [71] (Fig. 4) in the TNBC tumor microenvironment (TME), whereas TF is negative in normal adjacent breast tissues [45] (Fig. 3) and normal vascular endothelial cells [45, 63]. Taken together, we believe we have provided an evidence supporting the idea that TF is commonly yet

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Tissue factor (TF) is a new surface target in 50%–85% of TNBC patients. TF expression in 161 patients’ TNBC was examined by immunohistochemical (IHC) staining on tissue microarray (TMA) slides with TNBC tissues ($n = 147$) (A, B, D) and matched normal breast tissues (A, C) and whole tumor tissues ($n = 14$) (D). *The scores for TF expression were graded as follows: negative (−), moderately positive (+), positive (++), strongly positive (+ +), and very strongly positive (+++). Positive percentages included all cases graded from moderately positive through very strongly positive. **Fisher’s exact test was used to test IHC score percentage difference between whole tumor tissues and TMA tissues, there is a significant difference with $P = 0.0002474$. Modified from Hu Z, Shen R, Campbell A, et al. Targeting tissue factor for immunotherapy of triple-negative breast cancer using a second-generation ICON. Cancer Immunol Res 2018;6(6):671–684. https://doi.org/10.1158/2326-6066.CIR-17-0343.

### FIG. 3

Tissue factor (TF) is a new surface target in 50%–85% of TNBC patients. TF expression in 161 patients’ TNBC was examined by immunohistochemical (IHC) staining on tissue microarray (TMA) slides with TNBC tissues ($n = 147$) (A, B, D) and matched normal breast tissues (A, C) and whole tumor tissues ($n = 14$) (D). *The scores for TF expression were graded as follows: negative (−), moderately positive (+), positive (++), strongly positive (+ +), and very strongly positive (+++). Positive percentages included all cases graded from moderately positive through very strongly positive. **Fisher’s exact test was used to test IHC score percentage difference between whole tumor tissues and TMA tissues, there is a significant difference with $P = 0.0002474$. Modified from Hu Z, Shen R, Campbell A, et al. Targeting tissue factor for immunotherapy of triple-negative breast cancer using a second-generation ICON. Cancer Immunol Res 2018;6(6):671–684. https://doi.org/10.1158/2326-6066.CIR-17-0343.

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selectively expressed by the TNBC cells [45] (Figs. 3 and 4), tumor VECs [45, 70] (Fig. 4), and CSCs [71] (Fig. 4) in patients’ TNBC.

p0120 TF has been validated as a target under preclinical and clinical investigations in other solid cancer [11, 39–41] and noncancer indications (wet form AMD [72, 73] and endometriosis [74, 75]). The first TF-targeted immunoconjugate agent in human trials was the Hu & Garen coinvented ICON (Fig. 2A) [11, 39–41], which was tested via Iconic Therapeutics in patients with AMD (NCT01485588 and NCT02358889) [76] and in patients with ocular melanoma (NCT02771340). Another anti-TF agent in human trials was an ADC (Tisotumab vedotin).


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which was recently evaluated in patients with a variety of solid cancers (bladder, esophageal, cervical, ovarian, endometrial, prostate, nonsmall cell lung cancer, and squamous cell carcinoma of the head and neck) [77]. However, ICON has relatively large MW (210 kDa; Fig. 2A) [11, 45] and possesses a residual (5%) pro-coagulation activity (Fig. 2B) [41, 45], whereas the limitations of ADC include exhibiting significant toxicity due to toxic payloads [77–79], existing as heterogeneous mixtures, and requiring sophisticated site-specific conjugation technologies [80].

To target TF for TNBC and to address the limitations in our first-generation TF-targeting ICON, our laboratory recently improved it by removing the procoagulant heavy chain of fVII from ICON. As a result, we invented a second and then a third-generation TF-targeted ICONs called L-ICON1 [45] (Fig. 2A; GenBank accession no. KY760097) and L-ICON3 (GenBank accession no. KY223609; patent pending), respectively. We call these new fVII light chain ICONs as L-ICON (L-ICON1 with an IgG1 Fc and L-ICON3 with an IgG3Fc). L-ICON1 is a fusion protein of human (or murine) fVII light chain (for binding to TF) to a human IgG1 Fc [45] (Fig. 2A), whereas L-ICON3 is composed of human fVII light chain fused to a modified human IgG3 Fc (patent pending). The new L-ICONs (only with fVII light chain) have several advantages over ICON (with fVII light chain followed by an active-site mutated heavy chain). First, the MW of L-ICON is reduced more than 50% compared to ICON (Fig. 2A). Second, L-ICON has no detectable coagulation activity (Fig. 2B), whereas the coagulation activities in ICON (K341A) and ICON (WT) are about 5% and 58% of that for FVIIa, respectively. L-ICON may, therefore, be a safer product than ICON. Third, human L-ICON binds both murine and human TF [45], whereas human ICON bound strongly to human TF, but weakly to murine TF [41]. These features of L-ICON will allow us to test the same L-ICON agent in preclinical mouse models of human and murine cancers and translate the findings directly to clinical trials. Lastly, L-ICON is more effective than ICON in treating TNBC tumor xenografts in an orthotopic mouse model and in vivo studies demonstrated that L-ICON1 immunotherapy was effective and safe for the treatment of human TNBC (MDA-MB-231 with BRCA2 mutation) and murine TNBC (4T1) [81] in cell line-derived xenograft (CDX) mouse models [45]. L-ICON can also arrest the tumor growth of TNBC patient-derived xenograft (PDX) (with BRCA1 mutation) in an orthotopic mouse model [45].

We further demonstrated that the mechanism of action of L-ICON1 is mediated by ADCC (Fig. 5). First, we showed that L-ICON1 protein binds to TNBC cells (MDA-MB-231 and BT20) as well as non-TNBC chemoresistant cells (MCF7/MDR/TF as a TF-positive control) (Fig. 5A). Using an ADCC Effector Assay (Promega), we showed that L-ICON1 can induce ADCC to those TNBC and non-TNBC cells (Fig. 5B), whereas the human IgG isotype, as a negative control, has no binding (Fig. 5A) and has no ADCC effect (Fig. 5B) on those breast cancer cells.

To test the idea of using CAR-NK cells to enhance the effects for antibody immunotherapy (Fig. 1), we recently constructed TF-recognizing CAR constructs [5]. The TF-targeting CARs we constructed consist of human fVII light chain (as the TF-targeting domain) without or with a hinge region of human IgG1, human CD28 transmembrane and cytoplasmic domains and human cytoplasmic domains of 4-1BB and CD3ζ (Fig. 6), named TF-targeting CAR1 monomer and dimer (TF-CAR1).

After having verified the expression of CAR1 and CD16 in TF CAR-NK cells, we examined their cytotoxicity via CAR and the ability to mediate L-ICON ADCC via CD16 against human
and murine TNBC cells (MDA-MB-231 and 4T1) [5]. We previously reported that TF expression was detected on both TNBC lines and hFVII light chain in L-ICON could bind both human and murine TNBC lines [45]. The results in Fig. 7 suggest that while TF CAR-NK cells can kill MDA-MB-231 cells directly via CAR, they also can serve as effector cells to mediate L-ICON ADCC against MDA-MB-231 cells and achieve stronger effect than TF CAR-NK cell alone.

FIG. 5  L-ICON1 can mediate ADCC against TNBC and non-TNBC cells. (A) L-ICON1 binding to TNBC (MD-MB-231 and BT20) and non-TNBC cells (MCF7/MDR/TF). hlgG: Human IgG isotype control. (B) L-ICON1 mediating ADCC against TNBC and non-TNBC cells. A and B: P values were analyzed by ANOVA model. Adapted from Hu Z, Shen R, Campbell A, et al. Targeting tissue factor for immunotherapy of triple-negative breast cancer using a second-generation ICON. Cancer Immunol Res 2018;6(6):671–684. https://doi.org/10.1158/2326-6066.CIR-17-0343.

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FIG. 6  Diagram of TF-targeting CAR constructs and generation of stable TF CAR-NK cells. (A and B) Lentivirus encoding CAR1 monomer and dimer (GenBank accession no. MF806378 and MF806379). fVIII: factor VII light chain. Hinge: hinge region of human IgG1. GFP: green fluorescence protein. Puro: puromycin resistant gene. The only difference between CAR1 monomer and dimer is that the dimer construct contains an IgG1 hinge region, in which cysteine residues can form disulfide bonds to form a homologous dimer. (C) Control lentivirus encodes GFP without encoding CAR (Lenti-GFP). MCS: multicloning sites. (D) Generation of stable TF-targeting CAR-NK cell lines. NK-CAR1 monomer and dimer: lentivirus encoding CAR1 monomer and dimer-transduced NK92MI/CD16 stable cell lines under selection of puromycin. NK-GFP: lentivirus encoding GFP (Lenti-GFP)-transduced NK92MI/CD16 cells were generated and used as a lentivirus-transduced control. Untransduced parental NK92MI cells did not express GFP and were used as a GFP-negative untransduced control. Photos were taken using bright field and green channels and were merged under Zeo Cell Imager (Bio-Rad). Scale bars: 25 μm. Adapted from Hu Z. Tissue factor as a new target for CAR-NK cell immunotherapy of triple-negative breast cancer. Sci Rep 2020;10(1):2815. https://doi.org/10.1038/s41598-020-59736-3.

FIG. 7  TF CAR-NK cells (CAR1 dimer) can mediate L-ICON1-ADCC against human TNBC cells (MDA-MB-231), as determined in vitro by CytoTox 96 cytotoxicity assay (Promega). Control was the untransduced NK92MI control cells (CAR negative and CD16 negative). Modified from Hu Z. Tissue factor as a new target for CAR-NK cell immunotherapy of triple-negative breast cancer. Sci Rep 2020;10(1):2815. https://doi.org/10.1038/s41598-020-59736-3.
Taken together, we believe that we have established the proof of concept that CAR-NK cells can serve as effector cells to mediate ADCC against cancer cells [5]. We have also provided an evidence supporting the idea that tissue factor (CD142) is a new and useful therapeutic target in TNBC patients [5, 45]. At the time of writing this book chapter, there were no other published studies on combination therapy of antibody and CAR-NK cells except for our own study [5] that is discussed above (Fig. 7).

One of the major challenges in immune checkpoint blockade therapy: Lack of PD-1-positive immune cell infiltration in the TME

Immune checkpoint blockade therapy (ICBT) is another recent promising cancer treatment approach. One of the major breakthroughs in cancer immunotherapy in the past decades was the discovery of immune checkpoint molecules, notably cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) [82] and the programmed cell death-1 (PD-1, also known as CD279) T-cell receptor [83] and its ligands [programmed death-ligand 1 (PD-L1, also known as B7-H1 or CD274)] [84] and PD-L2 (also known as B7-DC or CD273) [85]. PD-1 is expressed on activated CD4 and CD8 T cells, NK cells, B cells, activated monocytes, and dendritic cells [86]. Recent clinical trials with anti-PD-1 and PD-L1 monoclonal antibodies as immune checkpoint blockades have shown unprecedented durable responses in some patients with a variety of cancers. However, the resistance to ICBT is commonly observed in most cancer patients [87–89] and consequently, ICBT generally has response rates <20% [87–89]. While the mechanisms of innate and acquired resistance to ICBT are yet to be fully understood, it is well documented that both the lack of intra-tumoral PD-1-positive immune cells [90, 91], such as T and NK cells [91–93], and accumulation/recruitment of suppressor cells, such as myeloid-derived suppressor cells (MDSC) [94, 95] (and regulatory T cells, Treg) in the TME, including breast cancer patients [96, 97], can contribute to the host resistance [98–100]. MDSC is a population of immature myeloid cells defined by their suppressive actions on immune cells such as T cells, dendritic cells, and NK cells [101–103]. Aberrantly elevated levels of MDSCs have been described in tumor-bearing mice and cancer patients [104], and thus dampening MDSC actions may improve therapeutic outcomes [95, 101, 105, 106]. Current strategies for targeting MDSC involve differentiating MDSCs into mature myeloid cells, inhibiting MDSC development and infiltration, interfering with MDSC activation, or depleting MDSCs using chemotherapy [107, 108]. However, these strategies are not designed to specifically eradicate MDSCs. Therefore, there is an urgent need to develop novel strategy to specifically eliminate MDSCs to improve the clinical outcome for cancer patients, particularly for patients with TNBC. Due to the scope of this review, we will focus on addressing the lack of PD-1-positive immune cell infiltration in the TME.

Clinical trial data [90, 91] demonstrated that patients (responders) with preexisting infiltration of NK cells (and CD4 and CD8 T cells) may benefit from anti-PD-1 therapy, whereas nonresponding patients lack of or have significantly lower numbers of immune cells in the TME. Consistent with the findings in the TME, a group demonstrated, using mass cytometry (CyTOF), that the number and activity of NK cells in peripheral blood were also notably elevated in nonsmall cell lung cancer (NSCLC) patients who responded to immunotherapy.

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compared with patients who did not respond, suggesting that the overall activity or number of peripheral blood NK cells may serve as a biomarker to predict immunotherapy response in patients with NSCLC [109]. These observations not only indicate the importance of immune cell filtration in the ICBT efficacy, but also suggest that any combination therapy, if it can increase infiltration of T and/or NK cells in the TME, may enhance the ICBT efficacy.

Based on the data from a phase 3 (IMpassion130) clinical trial [110], the FDA approved atezolizumab, a PD-L1-targeted antibody (Ab), in 2019 in combination with chemotherapy (nab-paclitaxel) for patients with locally advanced or metastatic TNBC expressing PD-L1. This is exciting because the combination is the first immunotherapy regimen for any type of breast cancer [111]. Despite these advances, there are a number of potential limitations of ICBT for TNBC. First, the efficacy was correlated with PD-L1 expression [111] yet PD-L1 expression on tumor cells and infiltrating immune cells is low in TNBC patients, ranging from 19% (20 of 105) [112] or 22% (24 of 108) [113] on tumor cells to 41% (18 of 44) on tumor-infiltrating lymphocytes [114]. Second, the phase 3 study did not suggest a significant overall survival benefit from the addition of atezolizumab to nab-paclitaxel in the intention-to-treat patients (median overall survival prolonged for 2.3 months, \( p = 0.078 \)) [115]. Third, ICBT generally has response rates <20% [87–89], as discussed above, largely due to the accumulation of suppressor cells (for example, MDSC and Treg) [94, 95], and the lack of immune cell infiltration (for example, T and NK cells) [91–93], in the TME [90, 91]. Lastly, ICBT could cause serious autoimmune toxicity due to immune-related adverse events [116–118]. Thus, there is an urgent need to develop new therapies that are not only effective and safe as monotherapies for TNBC, but also can enhance the ICBT efficacy in combination therapy so that ICBT can benefit more TNBC patients.

Using CAR-NK cells to increase PD-1-positive immune filtration in the TME and overcome the resistance to ICBT

To increase immune cell infiltration in the TME, we previously discussed two potential strategies [119]: one strategy is to use vaccines, cytokines, and other immune therapy that could indirectly augment local or systemic immunity. Another strategy is to systemically administer CAR-T and -NK cells to increase CAR-T and CAR-NK cell infiltration in the TME. This review will focus on the discussion of the latter strategy with an emphasis on the use of CAR-NK cells, for the reasons below.

Here, we propose to use CAR-NK cells to address the lack of PD-1-positive immune cell infiltration in the TME during immune checkpoint blockade therapy. This is because PD-1 is expressed on T and NK cells, but the role of NK cell in ICBT is much less explored [120, 121] than T cells. More importantly, despite that CAR T-cell therapy has shown remarkable clinical efficacy in B-cell cancers, CAR T cells can induce substantial toxic effects, and the manufacture of the cells is complex. CAR-NK cells have several advantages over CAR-T cells to overcome these limitations [98, 122–125]. First, clinical trials revealed that CAR-NK cell therapy is effective yet generally safer than CAR-T cell therapy [98, 122, 123]. Phase 1 and 2 clinical trials of HLA-mismatched CD19 CAR-NK cell derived from cord blood (encoding anti-CD19 CAR, interleukin-15, and inducible caspase 9 as a safety switch) demonstrated that CAR-NK cells
were effective in patients with CD19-positive cancers (non-Hodgkin’s lymphoma or chronic lymphocytic leukemia) [98] and were safer than CAR-T cell [98, 123, 126]. The administration of CAR-NK cells was not associated with the development of cytokine release syndrome, neurotoxicity, or graft-versus-host disease, which were often observed in many CAR-T clinical trials. The infused CAR-NK cells can persist at low levels for at least 12 months [98]. Second, unlike CAR-T cells, CAR-NK cells retain an intrinsic capacity to recognize and target tumor cells through their native receptors, making the escape of tumor cells through downregulation of the CAR target antigen less likely. Lastly, NK cells do not require strict HLA matching and lack the potential to cause graft-versus-host disease, an important risk imposed by CAR-T cell immunotherapy, which makes it possible for CAR-NK cells to be an off-the-shelf allogeneic therapeutic.

Besides CD142 (TF)-targeted CAR-NK cells [5] that we recently developed, CAR-NK cells targeting other tumor antigens, including but not limited to, CD4 [127], CD5 [128], CD7 [129], CD19 [130–132], CD20 [132], CD22 [133], CD24 [134], CD33 [135], CD123 [136], HER2 [137], EpCAM (epithelial cell adhesion molecule) [138, 139], FMS-like tyrosine kinase 3 (FLT3) [140], gypian-3 (GPC3) [141], CS1 [142] (also known as CD319 [143]), EGFR [144–146], and mesothelin [147], have been tested under preclinical and clinical investigation [148, 149].

Currently, there is no published study on the combination regimen of CAR-NK cells with immune checkpoint inhibitors for cancer treatment. However, the preclinical studies of NK cells and PD-1/PD-L1 blockade [150, 151] demonstrated the importance of PD-1/PD-L1 axis in inhibiting NK activity against cancer in vivo and also established the proof of concept of using NK cells, in addition to T cells, to enhance the ICBT efficacy. These results support the idea that we propose here for the use of CAR-NK cells in combination with anti-PD-1/PD-L1 agents to overcome the lack of PD-1-positive immune cell filtration in the TME during ICBT.

Beside PD-1, NK cells also express other checkpoint molecules, including but not limited to, CTLA-4 [152], LAG-3 [153], CD96 [154], and TIGIT [154]. Therefore, CAR-NK cell therapy may also be used in combination with other immune checkpoint inhibitors blocking currently known checkpoint receptors.

Conclusion

CAR-NK cells, therapeutic antibody, and immune checkpoint blockade immunotherapies are promising cancer immunotherapeutic approaches. For the development of novel targeted immunotherapy (such as therapeutic antibodies and CAR-NK/CAR-T cell therapy), it is important to identify new tumor antigens that, ideally, are commonly yet selectively expressed on multiple tumor compartments, whereas they are minimally or restricted expressed in normal cells. Such targeted immunotherapy can not only improve clinical outcomes (by targeting multiple tumor compartments), but also may overcome drug resistance [71], prevent local recurrence and metastasis (by targeting CSC and tumor VEC) [155]. Toward this goal, we provide an evidence from our own work demonstrating that tissue factor (CD142) is a useful therapeutic target in patients with TNBC [5, 45] and is commonly yet selectively expressed on the cancer cells [45], cancer stem cells [71], and tumor vascular endothelial cells [45, 70]. In addition, it is worth noting that TF is also expressed on the cancer cells in a variety of other
solid cancers [45, 58, 64–66], acute leukemia and sarcoma [58, 66], Hodgkin’s lymphoma [67], and multiple myeloma [68].

Here, we also aim to promote the idea of using CAR-NK cells to overcome host resistance to therapeutic antibody and immune checkpoint blockade immunotherapies in the current clinical practice. CAR-NK cells, antibodies, and ICBT can work in different ways against cancer but the latter two may encounter different host resistances in cancer patients. By combining CAR-NK cells with therapeutic antibody and ICBT, CAR-NK cells not only can kill cancer cells, or ideally, multiple tumor compartments (for example, simultaneously targeting the cancer cells, cancer stem cells, and tumor vascular endothelial cells by targeting TF), via the CAR mechanism. They also may overcome the host resistance to antibody and ICBT, via serving as effector cells and increasing immune cell infiltration in the TME, respectively, so that an optimal clinical outcome or even a cure may be achieved for cancer patients through novel combinational treatment regimens.

Acknowledgment

This work was supported in part by a grant R21CA216697 from the National Cancer Institute, an Accelerator Award from The Ohio State University Corporate Engagement Office, and a Translational Research Grant from The International Myeloma Society and Paula and Rodger Riney Foundation.

References


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References


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[58] Hu Z. Therapeutic antibody-like immunoconjugates against tissue factor with the potential to treat angiogenesis-dependent as well as macrophage-associated human diseases. Antibodies (Basel) 2018;7(1).


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CAR-NK cell immunotherapy: Development and challenges toward an off-the-shelf product

Anthony G. Mansour, Kun-Yu Teng, Ting Lu, Tasha Barr, and Jianhua Yu

Abstract

Chimeric antigen receptor (CAR)-T cells have emerged as a novel immunotherapy that has redefined cancer treatment. Despite its clinical success, this therapy has many limitations that have redirected scientists to investigate other immune cells for CAR therapy. Hence, natural killer (NK) cells have drawn increased attention as being superior to CAR-T cells due to the advantages they offer such as (1) better safety profile and less life-threatening complications such as cytokine release syndrome and neurotoxicity, (2) reduced cost of manufacturing, (3) maintaining cytotoxic activity independent of CAR, and (4) high potential for an off-the-shelf product. In this chapter, we focus on the sources, development, advantages, and challenges of CAR-NK cell immunotherapy and its future promise as a novel immunotherapy in cancer treatment.

Abbreviations

- CAR: chimeric antigen receptor
- CB: cord blood
- CRS: cytokine release syndrome
- DLBCL: diffuse large B-cell lymphoma
- EGFR: epidermal growth factor receptor
Introduction

Cellular therapies have gained a great deal of attention following their success in clinical settings with a tremendous increase in the variety of cell types being utilized and prepared for exogenous administration. Immunotherapy, which is the manufacturing of the different types of immune cells to boost the body’s natural defense, has provided additional therapeutic resources for cancer patients and given them new hopes that can make this disease an imperfect, almost curable one. In the past several years, we have seen significant growth in engineering immune effector cells with a chimeric antigen receptor (CAR), which specifically target cancer cells. CAR-T cells have gained very positive clinical recognition for their capability to achieve remarkable responses in patients with relapsed or refractory B-cell malignancies by targeting the CD19 antigen [1–3]. As a result, the FDA has approved three CAR-T-cell therapy agents Kymriah, Tecartus, and Yescarta against leukemia, diffuse large B-cell lymphoma (DLBCL), and mantle cell lymphoma, respectively. Hence, an increased need for new therapies against solid tumors remains high. Despite all of the clinical breakthroughs in CAR-T-based immunotherapy, further development to enhance the efficacy, to reduce toxicity, and to reduce costs of such strategies is essential. CAR-T cells have several limitations due to their restrictive use with autologous CAR-T-cell manufacturing for each individual patient, expensive manufacturing costs, prolonged time and, thus, missing the best window of time for the treatment of patients with advanced disease, inadequate efficacy against solid tumors, and the limited number of T cells in patients who are lymphopenic in the setting of advanced disease or pretreated with chemotherapy. Natural killer (NK) cells can provide an alternative to T cells for CAR engineering due to the lack of graft-versus-host disease (GvHD) and open the opportunity for “off-the-shelf” CAR-based therapy for immediate clinical use [4]. Hence, the process of engineering NK cells with a CAR has been previously described to improve the killing of solid tumors [5–7]. In this review, we summarize the recent advances
and current state of engineering CAR-NK cells and the challenges for the development of an off-the-shelf product for the treatment of cancer.

**What are NK cells?**

Natural killer (NK) cells comprise an imperative part of the immune system and play an essential role in cancer immune surveillance by killing malignant cells and suppressing tumor growth [8, 9]. NK cells were first described for their ability to spontaneously lyse target cells without any prior priming and were not restricted to target cells expressing major histocompatibility (MHC) molecules [10–12]. In addition to their ability to specifically recognize and lyse cells with downregulated self-MHC molecules, which often occurs in malignancy, NK cells do not require human leukocyte antigen (HLA) matching [13, 14]. NK cells hold both activating and inhibitory receptors that engage with target cells and provide a balance for their activation and inhibition [14, 15]. Upon activation, NK cell function is mediated directly by the secretion of cytotoxic granules containing granzyme B and perforin, and indirectly through the production of proinflammatory cytokines such as interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), and granulocyte macrophage colony-stimulating factor (GM-CSF) that regulate the adaptive or other innate immune responses [15, 16].

**Sources of NK cells**

Unlike T cells, NK cells with less immunogenicity and promising antitumor capacity represent as ideal off-the-shelf allogeneic cell therapy products. NK-92 cells, an NK cell-derived lymphoma cell line, display potent antitumor capacity in several hematological and solid tumors [17, 18] and can be effectively expanded in a clinical-grade manufacturing platform to meet the required quality and quantity [19]. In our previous work, we have demonstrated that CAR-NK cells expressing epidermal growth factor receptor (EGFR) and engineered from NK-92 can target both wild-type and mutant EGFR to effectively reduce tumor growth and prolong survival in a mouse glioblastoma model [6, 20]. Another study reported a combinational therapy of EGFR-CAR NK-92 cells and oncolytic herpes simplex virus 1 significantly reduced breast cancer brain metastases [21]. Currently, several CAR-engineered NK-92 cells targeting other different tumor-specific antigens, such as CS1, CD19, Her2, CD33, and αFR, have been reported [6, 22–26]. However, being a tumor cell line with unrestricted cell growth, lack of CD16, NKp44, and NKp46 expressions, and the positive expression of EBV cause concern for the use of NK-92 cells in clinical settings.

Different from NK-92 cells, primary NK cells can be engineered into CAR-NK cells from several various sources. Autologous NK cells from patients may reduce the potential of graft-versus-host reaction caused by unexpected T-cell contamination, but they have limited cytotoxicity against autologous tumors [27–29]. Peripheral blood (PB) NK cells from healthy donors represent a convenient and cost-effective source for CAR-NK cells, with approximately 5%–15% of circulating lymphocytes in healthy adults [30]. Due to the lack of GvHD, PB NK cells can be enriched from either HLA-matched or HLA-mismatched donors.
providing more choices for possible donors [31, 32]. Similar to PB, umbilical cord blood (UCB) can also provide a rich source of primary human NK cells in the same manner. Considering a large number of units of UCB now stored in public cord blood (CB) banks since the 1990s, over 600,000 in 2015, and additional private profit-driven CB banks worldwide [33], UCB provides a readily available donor source with certain HLA types and specific NK receptor profiles. Moreover, UCB units are available as an off-the-shelf frozen product and can generate large numbers of functional NK cells ex vivo [34]. Although there is a higher percentage of NK cells in UCB compared to PB, the major obstacle to obtaining sufficient UCB NK cells remains due to the limited volume of a single UCB unit [35]. Additionally, compared to PB NK cells, UCB NK cells are immature and less cytotoxic against tumor cells [36]. The UCB CAR-NK cells were successfully designed with an anti-CD19 antigen and tested in hematological malignancies in a preclinical study [37]. Recently, a clinical trial that incorporated interleukin (IL)-15 in CD19-CAR-NK cells showed impressive outcomes [38].

CD34+ hematopoietic progenitor cells (HPCs) provide another source for obtaining large numbers of functional NK cells for clinical application [39]. CD34+ HPC-derived NK cells usually have a mature phenotype and highly express activating NK receptors, which are mostly similar to PB NK cells [40]. Due to their unlimited proliferative capacity, induced pluripotent stem cells (iPSCs) have become another attractive source of CAR-NK cells [41]. Recently, iPSC-derived CAR-NK cells targeting mesothelin displayed superior antiovarian cancer cytotoxicity in vivo [42]. Compared to PB NK cells, iPSC-derived NK cells are usually characterized by an immature phenotype, which are similar to UCB NK cells, with lower expression of killer cell immunoglobulin-like receptors (KIRs) [43], and therefore they may serve as a universal “off-the-shelf” NK cell source for many recipients [44, 45].

### Chimeric antigen receptor: Construct development

A CAR is an engineered transmembrane receptor that is expressed on effector cells such as NK or T cells and plays a critical role in their activation upon binding to the surface antigen. A typical CAR is composed of a single-chain antibody variable fragment (scFv), hinge-transmembrane domain, and intracellular signaling domains [46]. scFv is derived from a tumor-specific antibody and can target any antigen expressed on the tumor cell surface [47]. The scFv determines the specificity of the CAR and is designed by combining the variable regions of an antibody with the constant region of a T-cell receptor (TCR) through a linker, grafting an immune cell with the specificity for tumor-associated antigens expressed on tumor cells. The hinge-transmembrane domain is designed to bridge the extracellular and intracellular domain with enhanced transport efficiency and stability of CAR molecules on the cell membrane surface [48]. An intracellular stimulation domain is included in a CAR construct to provide activation signals to the cells, thereby promoting cell survival, proliferation, and cytotoxicity.

A first generation of CAR in T cells was developed with only CD3ζ molecules for the intracellular signaling domain, which resulted in activation-induced cell death and limited expansion capacity. Indeed, early clinical trials showed little antitumor efficacy in patients [49]. The second generation of CAR-T was improved based on the first-generation CAR by adding
a costimulation signal to the intracellular signaling domain. Earlier studies showed that the second-generation CARs, achieved by fusing the intracellular region of CD28 or 4-1BB to CD3ζ, have much longer persistence and therapeutic efficacy compared to the first-generation CARs [50, 51]. The third generation of CAR-T possesses multiple costimulation domains including CD28, 4-1BB, or OX40 [52]. Some preclinical studies demonstrated its therapeutic potential that is superior to the second-generation CARs by preventing cell exhaustion and inducing less IL-10 secretion [53, 54], but others showed inconsistent data [55]. The fourth generation of CAR-T, also called “TRUCKs” (T-cells redirected for universal cytokine-mediated killing) or “armed CARs” combines the direct antitumor ability of CARs and delivery of immune-modulating cytokines such as IL-7, IL-12, IL-15, IL-18, IL-23, etc. [56, 57]. These cytokines could directly enhance CAR-T functions in an autocrine manner or indirectly stimulate other surrounding immune cells in a paracrine manner.

For CAR NK cells, the intracellular signaling domain such as CD28, 4-1BB, and CD3ζ are commonly shared with CAR-T cells. However, some studies also demonstrated NK functions could be enhanced by fusing NK-specific signaling domains (e.g., DAP10, DAP12, and 2B4) to CARs. DAP10 is an adapter protein that noncovalently binds to NKG2D on the NK cell surface, mediating NK activation and cytotoxicity [58]. Unlike DAP12 that contains an immunoreceptor tyrosine-based activation motif (ITAM), DAP10 has the YxxM motif to mediate phosphatidylinositol-3 kinase (PI3K) signaling. Studies showed that primary NK cells transduced with CD19 CAR-DAP10 enhance NK functions against leukemic cells, although weaker than CD3ζ [59]. DAP12 mediates activating signaling such as NKp44, NKG2C, and KIRs in NK cells. It has been shown that primary NK cells transduced with an epidermal growth factor variant III (EGFRvIII) CAR or a prostate stem cell Ag (PSCA) CAR fused with DAP-12 yet without CD3ζ, had a better survival outcome than control NK cells in animal models [60, 61]. Several studies have shown that CAR NK cells including 2B4 and CD3ζ in the costimulation domain had superior antitumor functions against T-cell malignancies [62, 63]. Of note, a recent study showed improved mobility of the second generation of CD19 CAR NK cells in vitro to the bone marrow stromal cells by coexpressing CXCR4 with CD19 CAR [64]. The transgenically augmented CAR NK cells (TRACKs) expressing CXCR4 are expected to have higher efficiency to combat persistent tumors in the tumor niche environment; however, this concept has not been validated in vivo [64]. Other than the enhanced chemotaxis activity, a recent phase I/II clinical trial using umbilical cord NK cells armored with CD19 CAR and secreting soluble IL-15 showed promising therapeutic benefits against lymphoid tumors and no major toxic effects were found [64].

Advantages of CAR-NK cells

The recent FDA approval of CAR-T cells for DLBCL, leukemia, and mantle cell lymphoma is a milestone in the field of immunotherapy. However, this newly available, promising therapy comes at a cost with the development of severe side effects, long intervals for manufacturing, and a huge cost for treatment. Unlike CAR-T cells, both NK and CAR-NK cells have a higher safety profile in the clinic using haploidentical NK cells in pre- and posttransplant settings with a reduced risk for GvHD [32, 38, 65, 66], thus opening the door for opportunities for an off-the-shelf product. Compared to allogeneic CAR-T cells that are available as an
off-the-shelf product, NK cells still offer many advantages for cancer immunotherapy (Table 1). In addition, CAR-NK cells maintain their function and intrinsic capacity for killing tumor cells independent of the presence of the CAR-specific tumor antigen and may aid in complete remission of disease, unlike CAR-T cells which face potential tumor escape due to their dependency on the CAR for activation [67]. Furthermore, NK cells after infusion lack the capability of clonal expansion unlike T cells and thus are less likely to have cytokine release syndrome (CRS) resulting in the development of life-threatening complications such as neurotoxicity due to the release of IL-6, IFN-γ, and IL-1 [68]. The development of different grades of CRS in the context of CAR-T therapy can lead to additional complications occurring later and include cardiotoxicity, pulmonary toxicity, prolonged cytopenia, renal abnormalities, and many more that are absent with CAR-NK cell therapies [69]. There are currently several ongoing clinical trials with CAR-NK cells and are summarized in Table 2. While CAR-NK cell therapy is still in its infancy with a few clinical trials so far, it will require more time before we can further understand the toxicities related to CAR-NK therapies.

**TABLE 1** Comparison of CAR-NK with Allogenic CAR-T.

<table>
<thead>
<tr>
<th>Source</th>
<th>CAR-NK</th>
<th>Allogenic CAR-T cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>CB, PB, iPSCs</td>
<td>Large-scale production and cryopreservation from a single donor for an off-the-shelf product readily available for patient treatment</td>
<td>Large-scale production and cryopreservation from a single donor for an off-the-shelf product readily available for patient treatment</td>
</tr>
<tr>
<td>Clinical indications</td>
<td>Ongoing trials for hematological and solid malignancies</td>
<td>Ongoing trials for hematological and solid malignancies</td>
</tr>
<tr>
<td>Main issues/ risks</td>
<td>No toxicity; freeze/thaw protocol needs to be established and tested clinically for an off-the-shelf product</td>
<td>Cytokine release syndrome, GVDH; rejection of allogenic cells, toxicity in case of severe lymphodepletion</td>
</tr>
<tr>
<td>Persistence</td>
<td>Short without IL-15, up to a year with IL-15</td>
<td>Short to intermediate (weeks to months)</td>
</tr>
<tr>
<td>Redosing</td>
<td>Not limited by cell number</td>
<td>Not limited by cell number but risk of alloimmunization</td>
</tr>
<tr>
<td>Cost</td>
<td>Low</td>
<td>Moderate</td>
</tr>
</tbody>
</table>

Challenges for CAR-NK therapy

**CAR-NK cell persistence**

One of the limitations of NK cell therapy is its short lifespan in the absence of cytokines such as IL-2 and IL-15, which restricts its therapeutic efficacy. For example, the short lifespan of NK cells was seen after in vivo infusion of activated NK cells into immunodeficient mice in which they were undetectable after 1 week, but persisted up to a month with the addition of exogenous IL-2 [70]. Hence, translation of NK cell therapy into the clinic requires supplementation of IL-2 to promote the survival, proliferation, and activation of NK cells [71]. Despite its benefits for NK survival, IL-2 has not been widely accepted as an immunotherapeutic drug due to its short half-life, severe side effects at the therapeutic dosage, and its capability of...
<table>
<thead>
<tr>
<th>NCT</th>
<th>Clinical trial phase</th>
<th>Cancer type</th>
<th>Antigen target</th>
<th>CAR vehicle</th>
<th>Cell source</th>
<th>Dose</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCT03692767</td>
<td>Early phase I</td>
<td>Refractory B-cell lymphoma</td>
<td>CD22</td>
<td>Unknown</td>
<td>Unknown</td>
<td>50–600 × 10⁷/kg</td>
<td>Not yet recruiting</td>
</tr>
<tr>
<td>NCT03690310</td>
<td>Early phase I</td>
<td>Refractory B-cell lymphoma</td>
<td>CD19</td>
<td>Unknown</td>
<td>Unknown</td>
<td>50–600 × 10⁷/kg</td>
<td>Not yet recruiting</td>
</tr>
<tr>
<td>NCT02892695</td>
<td>Phase I/II</td>
<td>Refractory B-cell lymphoma</td>
<td>CD19/CD22</td>
<td>Unknown</td>
<td>Unknown</td>
<td>50–600 × 10⁷/kg</td>
<td>Not yet recruiting</td>
</tr>
<tr>
<td>NCT02892695</td>
<td>Phase I/II</td>
<td>ALL, CLL, NHL, mantle cell lymphoma, B-PLL, DLBCL</td>
<td>CD19</td>
<td>NK-2 cell line</td>
<td>Cord blood</td>
<td>CD19</td>
<td>Complete</td>
</tr>
<tr>
<td>NCT03056339</td>
<td>Phase I/II</td>
<td>ALL, CLL, NHL</td>
<td>CD19</td>
<td>CD19</td>
<td>Haploidentical donor</td>
<td>3 dose levels: 10⁷/kg, 10²⁷/kg, 10³⁷/kg</td>
<td>Phase I portion completed, Phase II recruiting</td>
</tr>
<tr>
<td>NCT0305639</td>
<td>Phase I</td>
<td>B-ALL</td>
<td>CD19</td>
<td>CD19</td>
<td>Haploidentical donor</td>
<td>0.5–5 × 10⁶/kg and up to 1 × 10⁷/kg</td>
<td>Suspended for interim review</td>
</tr>
<tr>
<td>NCT01974479</td>
<td>Phase I</td>
<td>B-ALL</td>
<td>CD19</td>
<td>CD19</td>
<td>Haploidentical donor</td>
<td>Unknown</td>
<td>Completed</td>
</tr>
<tr>
<td>NCT00995137</td>
<td>Phase I</td>
<td>B-ALL</td>
<td>CD19</td>
<td>iPS-derived NK cell</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Recruiting</td>
</tr>
</tbody>
</table>

**TABLE 2** Ongoing Clinical Trials for CAR-NK.
## TABLE 2  Ongoing Clinical Trials for CAR-NK—Cont’d

<table>
<thead>
<tr>
<th>CAR vehicle</th>
<th>NCT identifier</th>
<th>Clinical trial phase</th>
<th>Cancer type</th>
<th>Antigen target</th>
<th>Cell source</th>
<th>Construct/method</th>
<th>Dose</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCT03940833</td>
<td>Early phase I/II</td>
<td>Multiple myeloma</td>
<td>CD33</td>
<td>NK-92 cell line</td>
<td>CAR.CD33-CD28-41BB-CD3ζ</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Recruiting</td>
</tr>
<tr>
<td>NCT02944162</td>
<td>Early phase I/II</td>
<td>AML</td>
<td>CD33</td>
<td>NK-92 cell line</td>
<td>CAR.CD33-CD28-41BB-CD3ζ</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Recruiting</td>
</tr>
<tr>
<td>NCT02839954</td>
<td>Early phase I/II</td>
<td>Hepatocellular carcinoma, nonsmall-cell lung cancer, pancreatic carcinoma, triple-negative breast cancer, glioblastoma, colorectal cancer, gastric carcinoma</td>
<td>MUC1</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
<td>0.5–3 × 10^6/kg</td>
<td>Not yet recruiting</td>
</tr>
<tr>
<td>NCT03692663</td>
<td>Early phase I/II</td>
<td>Castration-resistant prostate cancer</td>
<td>PSMA</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
<td>0.5–3 × 10^6/kg</td>
<td>Not yet recruiting</td>
</tr>
<tr>
<td>NCT03692637</td>
<td>Early phase I/II</td>
<td>Epithelial ovarian cancer</td>
<td>Mesothelin</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Autologous or allogenic NK</td>
<td>0.5–3 × 10^6/kg</td>
<td>Not yet recruiting</td>
</tr>
<tr>
<td>NCT03415100</td>
<td>Phase I</td>
<td>Solid tumors</td>
<td>NKG2D ligands</td>
<td>Autologous orallogenic NK</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Recruiting</td>
<td></td>
</tr>
<tr>
<td>NCT03940820</td>
<td>Phase I</td>
<td>Solid tumors</td>
<td>ROBO1</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Recruiting</td>
<td></td>
</tr>
<tr>
<td>NCT number</td>
<td>Phase</td>
<td>Disease Type</td>
<td>Treatment</td>
<td>Study Arm</td>
<td>Dose Levels</td>
<td>Status</td>
<td></td>
<td></td>
</tr>
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<td>------------</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>NCT03941457</td>
<td>I/II</td>
<td>Pancreatic cancer</td>
<td>ROBO1</td>
<td>Recruiting</td>
<td>$1 \times 10^7$ intracranial infusion</td>
<td>Recruiting</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCT0388978</td>
<td>I</td>
<td>Glioblastoma</td>
<td>HER2</td>
<td>Recruiting</td>
<td>NK-92</td>
<td>Recruiting</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCT04639739</td>
<td>Early phase I</td>
<td>Relapsed or refractory B-cell lymphoma</td>
<td>CD19</td>
<td>Recruiting</td>
<td>Unknown</td>
<td>Not yet recruiting</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>NCT number</th>
<th>Phase</th>
<th>Disease Type</th>
<th>Treatment</th>
<th>Study Arm</th>
<th>Dose Levels</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCT03941457</td>
<td>I/II</td>
<td>Pancreatic cancer</td>
<td>ROBO1</td>
<td>Recruiting</td>
<td>$1 \times 10^7$ intracranial infusion</td>
<td>Recruiting</td>
</tr>
<tr>
<td>NCT0388978</td>
<td>I</td>
<td>Glioblastoma</td>
<td>HER2</td>
<td>Recruiting</td>
<td>NK-92</td>
<td>Recruiting</td>
</tr>
<tr>
<td>NCT04639739</td>
<td>Early phase I</td>
<td>Relapsed or refractory B-cell lymphoma</td>
<td>CD19</td>
<td>Recruiting</td>
<td>Unknown</td>
<td>Not yet recruiting</td>
</tr>
</tbody>
</table>

**Abbreviations:**
- ALL: acute lymphoblastic leukemia
- AML: acute myeloid leukemia
- BCMA: B-cell maturation antigen
- CLL: chronic lymphocytic leukemia
- DLBCL: diffuse large B-cell lymphoma
- hnCD16: high affinity, noncleavable CD16
- IL15RF: IL15 receptor alpha
- NHL: non-Hodgkin lymphoma
- PLL: prolymphocytic leukemia
- PTCL: prolymphocytic T-cell leukemia
- PSMA: prostate-specific membrane antigen
- T-LGL: T-cell large granular leukemia

Adapted from https://doi.org/10.1158/2159-8290.CD-20-0556.
inducing proliferation of immune suppressor cells such as regulatory T cells that express the IL-2 receptor alpha [72]. Therefore, IL-15 has emerged as a new immunotherapy drug that is superior to IL-2 due to its limited toxicities and capability of enhancing the function of both NK and T cells to control tumor growth [73]. Another form of IL-15, the IL-15:IL-15Rα complex has been shown to be more effective than soluble IL-15 due to its prolonged half-life and enhanced therapeutic effect in various murine cancer models and is mediated by the activation of NK or T cells [74–76].

To improve the persistence of NK and T cells in vivo, IL-15 has been identified as a critical component and genetic modification of immune cells and CAR-cells by transducing these cells to express the different forms of IL-15 and include the secreting soluble IL-15 (sIL-15), or IL-15/IL-15Rα and expressing membrane-bound IL-15 (mIL-15) or membrane-bound IL-15/IL-15Rα [37, 38, 77]. To date, only the sIL-15 secreted by CAR cell therapy has been tested clinically in patients and has shown to increase the persistence and proliferation of NK cells that were detectable up to 1-year postinfusion without causing any toxicities or increased secretion of IL-15 [38]. Similarly, CAR-T cells have been modified to coexpress IL-15 that has been shown to enhance the proliferation, persistence, and antitumor effects of CAR-T cells in vivo [78, 79]. Despite the benefits of IL-15 for the persistence of both CAR-NK and CAR-T cells, the challenge for therapy failure remains with the loss of tumor antigens that can no longer be recognized by the CAR, highlighting the need for further development of identifying different targets on tumors to optimize the function of CAR-therapy. The modification of CAR therapy to secrete soluble cytokines raises the concern for safety and requires the incorporation of suicide genes or safety switches. The question remains whether sIL-15 is the best approach for its expression in CAR therapy and whether other forms of expression can have a better outcome. Even though the IL-15/IL-15Rα complex is superior to IL-15, it is still unknown if it will provide a superior effect with CAR therapy or will promote further exhaustion and limited function due to the cells being exposed to high concentrations of IL-15/IL-15Rα in vitro during expansion and in vivo after infusion. Hence, additional preclinical and clinical studies are required to get a better understanding and further insights into the role of the different forms of IL-15 in the context of CAR therapies.

Other approaches to improve the persistence of CAR therapy other than secreting cytokines with better safety profiles are to express an active cytokine receptor in CAR immune cells or to generate memory or memory-like cells. For example, GD-2 CAR-T cells were genetically modified to coexpress an active IL-7 receptor that has further enhanced the antitumor capacity, proliferation, and expansion of CAR-T cells [80]. CAR-NK cells have been stimulated with cytokines (IL-12, IL-15, and IL-18) to induce memory-like NK cells that have an amplified persistence and response to resistant NK cell lymphoma [81].

### Trafficking to tumor sites

Whether solid or liquid tumors, one of the critical factors for the efficacy of cellular therapy is homing to the tumor site that can be achieved by the interaction between chemokines expressed or secreted by NK cells and the tumor cells [82]. Since the receptors for some of these chemokines are lost in NK cells upon activation, a straightforward approach to overcome the trafficking barrier is to reexpress the receptors such as CXCR1, CXCR2, and CXCR4 by genetic modification [64, 83, 84]. The overexpression of CXCR2 on NK cells led to enhanced migration of NK cells toward their respective ligands in the tumor environment and resulted

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**IV. CAR-NK cells in immunotherapy**
in increased killing of the target cells [83]. Overexpression of CXCR4 on NK cells facilitates the migration in vitro toward bone marrow stromal cells that might boost the capacity of CAR-NK cells of clearing persistent tumor cells that remain in the bone marrow, but this concept has not yet been validated in vivo [64]. Furthermore, CAR-NK cells targeting EGFR and overexpressing CXCR4 have improved migration of CAR-NK cells toward CXCL12/SDF-1α-secreting tumor cells in a glioblastoma mouse model and led to complete tumor remission and prolonged survival [60]. Furthermore, the overexpression of CXCR1 with NKG2D CAR was capable of improving CAR-NK migration toward the tumor and enhancing NK cell cytotoxicity against peritoneal ovarian cancer [84]. These developments improved the efficacy of CAR-NK cells for migration toward tumor sites and can be part of the trajectory toward implementing CAR-NK cells therapy for solid tumors which remain a challenge with CAR-T therapy.

**Tumor microenvironment**

Different from the exciting results of CAR-NK cell therapy in liquid tumors [38], the main challenge of CAR-NK cell therapy in solid tumors is how to overcome the immunosuppressive properties of the tumor microenvironment (TME) which supports tumor growth, metastasis, and suppresses the immune system [85]. Transforming growth factor beta (TGF-β), one of the immunosuppressive molecules, is increased in the plasma of advanced cancer patients and correlated with worse progression of patients [86–88]. Several mechanisms by which TGF-β impairs NK cell function have been reported, such as decreasing the expression of NKG2D on NK cells, inhibiting the transcription of NKG2D ligands on tumor cells, and reducing human NK cell IFN-γ production through SMAD3 [89–93], although recent studies indicate that in mice SMAD4 is a positive regulator of NK cell cytotoxicity [94, 95]. Several strategies aiming at overcoming the inhibitor effect of TGF-β on NK cells have been reported. Blockade of TGF-β signaling or neutralization of TGF-β such as galunisertib and fresolimumab have obtained acceptable tolerability and safety results in treating patients with solid tumors in clinical trials [96, 97]. A stable SMAD3-silencing NK-92 cell line shows enhanced tumor-killing activity and cytokine production and can be used in clinical cancer immunotherapy [98]. NK cells expressing dominant-negative TGF-β receptor II (DNRII) maintain their perforin expression and NKG2D/DNMA1 expression in the presence of TGF-β [99].

Tumor cells often express the high level of ligands of immune checkpoints to escape from immune recognition and elimination [100]. Recently, the mAbs against immune checkpoint receptors or ligands have resulted in remarkable clinical benefits, such as anti-PD-1 mAb (pembrolizumab) in nonsmall-cell lung cancer [101] and anti-CTLA mAb (ipilimumab) in melanoma [102]. Additionally, the TME suffering from hypoxia and nutrient depletion also can suppress immune response [103]. Hypoxia supports tumor growth and downregulates the expression of NK cell-activating receptors [104]. Blockade of CD73, which can induce immunosuppressive adenosine in the hypoxic microenvironment of solid tumors, improved the intra-tumoral homing of CAR-NK cells against CD73+ lung cancer in vivo.

**Cell manufacturing**

The associated risk of GvHD and toxicity with CAR-T therapy makes CAR-NK therapy an attraction for large-scale manufacturing and offers a great opportunity for an off-the-shelf product. Therefore, CAR-NK cell therapy is currently being further developed to become
an inexpensive and readily available alternative therapy compared to CAR-T cells to achieve the goal of treating several patients with multiple doses from a single expanded donor. This growth in CAR-NK cell therapy has been faced with several challenges revolving around its manufacturing and freezing processes that are still being addressed. There are several parameters in the manufacturing process that should be considered to optimize the production of a large batch of NK cells including the cell source, the initial number of cells for expansion, the seeding density, the type of culture vessel used, the freezing process, etc. NK cells, unlike other immune cells and CAR-T, when activated and expanded in culture with cytokines are more vulnerable to the freezing and thawing process, and only moderate progress is being made on this end [105]. It remains that the survival, cytotoxicity, and persistence of NK cells are significantly reduced after thawing [106]. For example, the day of the harvest of NK cells postexpansion, stimulation of NK cells, cell seeding density, the time needed to freeze the cells are only a few among the various factors that can impact the cryopreservation of NK cells. As a result, further exploration for the strategies for optimal cryopreservation is required to take CAR-NK cells to the next level. Frozen CAR-NK products are yet to be tested for their potency in the clinical setting after the freeze and thaw process, which will be absolutely essential before the goal of an off-the-shelf product can be achieved.

On the other hand, manufacturing platforms are required for off-the-shelf CAR-T or CAR-NK cells. After gene transfer, a large-scale expansion of CAR-NK and CAR-T should be achieved to meet the clinical requirements and a variety of methods have been used from G-rex flasks, rocking bioreactors, static culture bags, or T-flasks [107–109]. Among the available automated, closed system in the market, the Miltenyi CliniMACS prodigy is one of the most utilized platforms for the manufacturing of CAR-T cells [110]. It provides a unique set of applications that allow the selection and activation of T or NK cells, transduction, expansion, and harvesting cells at a number at that is feasible for clinical application. Moreover, it removes the requirement of clean rooms for manufacturing, reduces labor costs, and reduces the risk of contamination. While we see several benefits in this closed system, one limitation is the number of cells capable of expanding in the closed system that prevents the production of a large batch of an off-the-shelf product. Hence, other automated cell manufacturing platforms are required to meet and secure the demand of providing a large batch of an off-the-shelf product.

Furthermore, NK cell numbers are limited from PB or UCB and comprise approximately 10%–20% of the total number of cells. In that regard, several approaches have been made to further expand NK cells to generate a large batch of CAR-NK cells, and the most commonly used method is the coculture of NK cells with irradiated K562 feeder cells expressing membrane-bound IL-21 and CD137 ligand [111]. This methodology can result in an expansion of greater than 10,000-fold in a 3-week timeline. On the other hand, other cell lines have been utilized such as the human B-lymphoblastoid cell-line 721.221 for the expansion of NK cells, and have been shown to be superior to the conventional K562 with membrane-bound IL-21 [112]. One of the major challenges is the expansion rate of these cells decreases beyond the 3-week duration and cell exhaustion can be a concerning factor. On a further note, the goal of generating a large batch of CAR-NK cells still requires further optimization of the protocols and machinery required to handle such a large number of cells for production and freezing for an off-the-shelf product of high quality. In addition, the NK cell yields and expansion capacity from PB and UCB are donor-dependent and make standardization for an off-the-shelf product more difficult [36, 65].
One of the other challenges in CAR-NK cells is the transduction of NK cells to express CAR with a high transduction efficiency. To engineer NK cells, a viral-based delivery is more established than a nonviral delivery system in the current field. However, the concerns of mutagenesis caused by the virus have driven the nonviral method, such as CRISPR/Cas, mRNA electroporation, and nucleofection to overcome the concerns regarding viral transduction [113–115]. Nevertheless, the efficiency of transducing primary NK cells remains challenging. Unlike CAR-T cells that can be engineered by either lentiv- or retrovirus, primary NK cells are difficult to be engineered with lentivirus. However, some studies have shown that lentivirus pseudo-typed with VSVG envelope could infect NK cells with an efficiency of about 20% [81]. Of note, it has been shown that lentivirus packaged with the baboon envelope could improve NK transduction rate up to fourfold when compared to VSVG enveloped lentivirus on primary NK cells [116]. Another way to approach the engineering of NK cells is through retrovirus. Retrovirus may achieve a better transduction rate; however, this could only be obtained in the proliferating or cytokine-primed NK cells as retrovirus could only cross the nuclear membrane when cells are dividing [38, 117].

On the other hand, other alternatives to generate CAR-NK cells have been investigated and iPSCs have attracted much attention due to the new genetic editing technologies and their easy use. It also provides further editing of the genome and replenishable sources for NK cells without repetitive genome modifications. NK cells derived from iPSCs raise the main concern for manufacturing as there are currently no closed culture systems for the entire process as well as the long duration of the culture required to reach the final step of CAR-NK cells. As a result, this increases the risk of contamination during the process and increases the variability which may have an impact on the consistency and the quality of the final product produced.

The recent advancements of CAR-NK and CAR-T cells have paved the way for immunotherapy treatments and further development into an off-the-shelf product. Clinical trials utilizing CAR-NK therapy have demonstrated impressive and promising results which suggest further improvement and research are required. Therefore, some strategies to further improve CAR-NK therapy are currently under development. Despite the initial results for treatment with CAR-T showing a rapid and high response rate, there is a partial or complete loss of tumor-specific antigen that causes relapse of cancer. Hence, the multitarget CARs have become an attraction for development and CAR-T cells have been modified to target both CD19/CD20 by transducing cells with multiple CAR constructs or alternatively developing bi-specific antibodies that show similar clinical efficacy in B-cell malignancies [118, 119]. Furthermore, various engineering strategies have been developed to further improve the trafficking of CAR-T and CAR-NK cells into solid tumors and such include the overexpression of chemokines that mediate cell trafficking that can lead to further enhancement of CAR-therapy with solid tumors [120]. On the other hand, the effector to target ratio after CAR-NK cell infusions can play a critical role in a better response rate to CAR therapy. Hence, treatment with chemotherapy prior to CAR-NK cell infusion can reduce the tumor burden and enhance the functionality of CAR-NK cells to eradicate tumor cells [121]. This strategy leads to further...
investigation for the combination of chemotherapy with CAR-NK cells for different tumors. As NK cell function can be boosted with a CAR, another potential strategy to improve its efficacy can be achieved by combinational treatment with antibodies that exerts an ADCC-mediated tumor killing via CD16 expression on NK cells [122]. CAR-NK cells will continue to evolve and have a great impact on cancer patient survival for those who have had previous failed treatments with limited options including CAR T cells.

References


IV. CAR-NK cells in immunotherapy
References


[40] Cany J, et al. Combined IL-15 and IL-12 drives the generation of CD34(+) derived natural killer cells with superior maturation and alloreactivity potential following adoptive transfer. Onco Targets Ther 2015;8(7), e1017701.


IV. CAR-NK cells in immunotherapy
12. CAR-NK cell immunotherapy


[56] Dragon AC, et al. CAR-T cells and TRUCKs that recognize an EBNA-3C-derived epitope presented on HLA-B


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References


IV. CAR-NK cells in immunotherapy


PART V

Targeting NK cells
Regulation of NKG2D by RKIP: Implications on NK-mediated cytotoxicity and cytokine production

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Abstract

RKIP (Raf kinase inhibitory protein) is a metastatic suppressor and a chemo-immuno-sensitizer. The deficiency or loss of RKIP has been linked with different cancer types, resulting in the activation of the MAPK pathway, as a result of Raf protein activation, which eventually leads to cell growth. Studies have suggested that RKIP can be used therapeutically to augment the proapoptotic tendencies in tumor cells, regulate the epithelial-mesenchymal transition (EMT), and decrease metastasis. NKG2D is a C-lectin-type receptor of the NK cell. It binds to a large variety of different surface proteins or ligands that are commonly expressed in virally infected and transformed cells and triggers the cytotoxic activity and production of cytokines. Accordingly, the regulation mechanisms underlying the dysregulated MAPK pathway by RKIP expression and NKG2D expression in NK cells are important for the NK cytotoxic anticancer activity. Hypothesizing that there may be a biochemical link between the two, we examined the potential cross talks of signaling pathways between NKG2D and RKIP. To this end, we examined whether the expression and activity of the NKG2D receptor are, in part, regulated by RKIP. Our findings indicate that there is a cross talk between the expression of RKIP and NKG2D in NK cells and an inverse correlation was established. These findings were corroborated by bioinformatic analyses using Oncomine, TCGA, and HPA data. We stipulate that overexpressing NKG2D by targeting RKIP in NK cells could potentiate their antitumor activity.

Abbreviations

\begin{tabular}{ll}
\textbf{Raf} & rapidly accelerated fibrosarcoma \\
\textbf{RKIP} & Raf kinase inhibitory protein \\
\textbf{PEBP} & phosphatidylethanolamine-binding protein
\end{tabular}
Conflict of interest

No potential conflicts of interest were disclosed.

Introduction: Natural killer cells

General properties and functions

Natural killer (NK) cells are a type of cytotoxic lymphocytes critical to the innate immune system, sharing a common progenitor with both B and T cells. NK cells were first noticed in 1960 when scientists conducted cytotoxic T-cell assays and unintentionally identified a novel cytotoxic cell with both adaptive and innate properties [1]. Traditionally, NK cells were thought to arise from common lymphoid progenitors in the bone marrow, but recent evidence suggested that they can also develop in secondary lymphoid organs such as the spleen, tonsils, and lymph nodes. NK cells are thought to constitute anywhere between 5% and 20% of the lymphocytes present in the human circulation [2]. This unique white blood cell can perform its cytotoxic effector functions through apoptotic pathways such as perforin/granzyme, Fas/FasL, or TRAIL. Through these pathways, NK cells can kill virally infected or cancer cells and secrete cytokines [3]. Unlike CD8+ cytotoxic T lymphocytes, NK cells do not require cross-presentation by antigen-presenting cells or previous exposure to antigens in order to

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perform their functions. Consequently, they generally migrate to the infection (or cancer) site rapidly in comparison to CD8+ T cells that require antigen-presenting cells and T-helper cells for their activation [4]. In humans, NK cells do not express T-cell receptors (TCRs), a product of DNA rearrangements, or the protein marker CD3; however, they are identified through the presence of the neural-cell adhesion molecule, CD56, as well as other pattern recognition receptors [5]. Other markers that can be utilized in the identification of the NK cell include NK1.1, CD16, and NCR1 (Nkp46/CD335). Due to the fact that NK cells are involved in complex pathways through which they mediate their activation (i.e., cytokine production and cytotoxicity), genetic studies can help produce better immunotherapies using NK cells as effectors.

There is continuing controversy among scientists regarding the development and maturation pathways that hematopoietic stem cells (HSCs) undergo to eventually become mature NK cells. In vitro studies have supported a linear model in which common lymphocyte progenitors (CLPs) downregulate the CD34 marker while subsequently upregulating CD16 (Fig. 1). One of the first linear developmental models was outlined by Freud and Caligiuri who presented the process in a multistaged process. In the first stage, HSCs give rise to novel progenitors that gain CD10 and CD45RA while maintaining the CD34 marker. Stage two is marked by the downregulation of CD10 and the subsequent acquisition of CD117. In stage three, CD34 begins its gradual downregulation process while LFA-1 is acquired by the progenitors. Stage four is marked by the progenitors beginning to express CD94. Finally, stage five includes the acquisition of the killer immunoglobulin-like receptors (KIR) and CD16 while CD94 is downregulated.

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who presented the multistaged process [6]. In stage one, HSCs give rise to novel progenitors that gain CD10 and CD45RA while maintaining the CD34 marker. Stage two is marked by the downregulation of CD10 and the subsequent acquisition of CD117. In stage three, CD34 begins its gradual downregulation process while LFA-1 is expressed. Stage four is marked by the progenitors beginning to express high levels of CD94. Finally, stage five includes the acquisition of killer immunoglobulin-like receptors (KIRs) and CD16, while CD94 is downregulated [7]. In vitro studies and flow cytometry have validated the linear development model; however, the results are contingent upon the type of cytokines tested. Recent studies suggest that NK development could undergo a branched pathway rather than a linear progression, highlighting their heterogeneous nature. Mass cytometry studies examining peripheral blood NK cells from dozens of unrelated human donors yielded results that indicated the presence of 6000–30,000 distinct NK phenotypes [8]. Research regarding the distinct populations of NK cells and how the phenotypes shift as individuals age is prevalent and still ongoing as scientists attempt to shed light on the developmental pathways for NK cells.

**NK cell receptors**

NK cells contain a balance of germline-encoded inhibitory and activating receptors that transduce antagonistic signals and dictate whether the NK cell will be activated to perform its effector functions or remain inactive. Inhibitory receptors are involved in NK cell “education,” specifically in self-tolerance and functionality, as they interact with the major histocompatibility complex (MHC)-class I molecules on the surface of cells, informing the NK cell that the host cell is healthy and does not need to be lysed [9]. The most common type of inhibitory NK cell receptors is the killer cell Ig-like receptors (KIRs), which signal through an immunoreceptor tyrosine-based inhibitory motif sequence (ITIM) in the cytoplasmic domain of this transmembrane protein. When these inhibitory NK cell receptors bind the MHC-I of a healthy cell, the ITIM region of the KIR becomes phosphorylated, which in turn recruits SHP-1 and SHP-2 phosphatases that inhibit the NK cell from performing its effector functions [10].

The NK cell activation receptors, including NKG2D, NCR1, NKG2C, and CD244, are responsible for binding and recognizing self-antigens that are commonly found on virally infected and malignant tumor cells. For example, the FcγRIII receptor (CD16) is an activating receptor present on NK cells that mediates antibody-dependent cell-mediated cytotoxicity (ADCC) by binding the constant region of antibodies that is bound on the surface of the target cells.

Additionally, natural cytotoxicity receptors (NCRs), such as NKP30, NKP46, and NKP80, are another common family of NK cell activating receptors that can bind viral hemagglutinins and tumor-associated ligands such as BAG6, a common protein product of transformed cell lines, subsequently activating the effector function of the NK cell [11].

The natural-killer group 2 member D (NKG2D) is the most heavily studied NK cell activating receptor and will be covered in more detail in the next section, as it binds ligands that are commonly produced by cancer cells but not by healthy ones [12]. Like the activating
receptors, NKG2D triggers signaling through the cytoplasmic domain, called the immunoreceptor tyrosine-based activation motif (ITAM), which is phosphorylated by Scr kinases. These receptors are essential in the identification and cellular destruction of transformed cells. In healthy adult cells, the expression of NKG2D ligands is under strict regulation in order to prevent autoimmune diseases, as a breach in this regulatory mechanism is suggested to result in pathologies such as type I diabetes [10]. The regulation of the expression for these receptors in NK cells is closely tied up with the production of gamma-chain cytokines, which will be discussed in more detail in a later section. The combination of a missing MHC-I to engage with the inhibitory NK cell receptor and the binding of a self-antigen to the activating receptor subsequently result in the initiation of the effector function of the NK cell.

**Activation of NK cells and cytotoxicity mechanisms**

Most virally infected cells are eventually lysed through the binding of a cytotoxic T cell to the MHC-I molecule on the surface of the host cell, which presents endogenous antigens to antigen presenting cells (APCs) and CD8+ T cells. However, many viruses have evolved mechanisms to decrease the expression of MHC molecules as a way to evade the cytotoxic effector function of CD8+ T cells [10]. A decreased MHC-I expression on the host cells can result in NK cell activation due to the absence of the inhibitory ligand, given that there is also interaction at the NK-activating receptor by a host-cell ligand. A similar process occurs in malignant tumor formation in cancerous tissue. As a tumor proliferates and divides uncontrollably, the newly synthesized cells tend to express less MHC-I molecules, a change that NK cells detect. Additionally, these transformed cells tend to express a variety of different proteins that are prevalent in stress-induced situations and can bind to activation receptors such as NKG2D.

Once the NK cell is activated, it produces its effector functions through two distinct pathways. The primary pathway is the perforin/granzyme pathway. Perforins (PRF) are pore-forming proteins, also known as granule toxins, whereas the family of granzymes (GZM) is composed of various serine proteases stored within cytotoxic granules. In humans, there are granzymes (GZMA, GZMB, GZMH, GZMK, and GZMM), among which GZMA and GZMB are the most abundant and best studied. The perforin/granzyme pathway features the exocytosis of perforins by the NK cell into the junction between it and the target cell. This is initiated through a combination of signals transduced from the NK cell’s receptors, resulting in the NK cell forming an intracellular junction or immunological synapse between it and the target cell through cytoskeletal rearrangement in its actin fibers [13]. The lysosome containing the soluble and cytotoxic perforins/granzymes then attaches to the polarized microtubule organizing center (MTOC) through granule convergence. This granule polarization then directs the secretory lysosome toward the plasma membrane of the target cell, with which it can fuse and undergo degranulation. The perforins are then endocytosed by the infected or transformed cell, and they form porous structures in its membrane, which facilitates the internalization of granzymes by the target cells and eventually leads to their apoptosis [13].
Secondary pathways that similarly lead target cells to apoptosis operate through binding of NK cell ligands to death receptors on the surface of target cells, resulting in apoptosis. NK cells express Fas ligands (FasL) on their surface that can bind the Fas cell death surface receptors on the target cell, which activates caspases [14]. Caspases are protease enzymes that can initiate apoptosis and cleave distinct cellular proteins that are necessary for the cell’s function, ultimately resulting in cell death. The tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) pathway is similar; however, it functions through the binding of TRAIL, which is present on the surface of NK cells, to trimeric death receptors (DR4 and DR5) on the target cell. This binding results in the formation of the death-inducing signaling complex (DISC) which contains an adaptor protein, FADD, that can bind caspase-8 activating it [15].

The last apoptotic pathway is through the secretion of the cytokine tumor necrosis factor alpha (TNFα) by activated NK cells, and its subsequent binding to the TNF receptor on the host cell. The secretion of cytokines by NK cells and dendritic cells has a profound effect not only on the target cell, but also on the effector function of the NK cell, as we will discuss in the next section.

Cytokines shape the NK immune response

Cytokines can also prime, sensitize, and attract NK cells. Dendritic cells are primarily responsible for the release of cytokines such as IL-12, IL-15, and IL-23, which can shape the immune response through modulating NK effector functions [16]. The NK cell itself also engages in cross talk directed back at the dendritic cell and macrophages through the secretion of interferon-gamma (IFN-γ) and TNF-α upon activation, in order to scale the immune response. IFN-γ has been proven to be a very influential effector cytokine that increases the expression of caspases, FasL, and TRAIL, ultimately causing a spike in antitumor and antiviral immunities through the sensitization of NK cells to cell death receptors [17]. Additionally, an increase of IFN-γ secretion has been found in NK cells in which the activating receptor NKG2D has formed an association with its ligand, suggesting its role in promoting NK effector cytotoxicity and scaling the immune response.

IL-12, which is produced by dendritic cells, can also stimulate NK cells into producing more IFN-γ. Further cytokines produced by dendritic cells, including IL-18, IL-12, and IL-10, can stimulate NK cell proliferation and inhibit tumor metastasis [17].

IL-15, which is a homeostatic and proliferation modulator of the NK cell, has been found to activate the mammalian target of rapamycin (mTOR) pathway in NK cells resulting in increased nutrient uptake and subsequent growth in the NK cell [18]. The mTOR pathway is important in increasing cellular metabolism and respiration through increased expression of genes associated with the two processes. While TNF-α sensitzes NK cells and guides their effector function, TNF-β inhibits IFN-γ and TNF-α, providing a negative feedback regulation loop for modulating NK cell functions. TNF-β is also involved in the inhibition of IL-15 from associating with mTOR, which arrests NK cell proliferation and development [19].

Due to these diverse properties, NK cells have been broadly investigated in their immunotherapeutic use against cancer and viral infections. A primary field of research is on genetically engineered NK cells with chimeric antigen receptors (CARs) that are specific to antigens found on tumors and virally infected cells. However, this form of therapy is still being
evaluated for its safety and effectiveness. Alternatively, other studies focus on the induction of cytokines to sensitize NK cells against their target cells. Ultimately, the use of NK cells is a promising therapeutic tool.

### NKG2D

#### General properties and structure

NKG2D is a well-studied activating NK receptor belonging to the C-lectins. It is expressed in all NK cell types, as well as in specific invariant natural killer T (iNKT) cells and cytotoxic T cells. This receptor is encoded by the NK gene complex and its secondary structure includes two β-sheets, two α-helices, and four disulfide bonds [20]. NKG2D bypasses signals from the negative receptor and initiates the effector function or sensitization of the NK cell upon contact with specific ligands (NKG2DLs), a function that has been coined as “master switch” capability. NKG2D can bind to a large variety of different surface proteins that are commonly expressed in stressed cells, such as virally infected and transformed cells, rendering it a good target for immunotherapy. Upon binding to a particular surface protein, the subsequent association of NKG2D with the DNAX-activating protein (DAP10) is followed by phosphorylation of the Tyr-X-X-Met (YXXM) motif and further transduces the signal by recruiting tyrosine kinases [21]. Phosphatidylinositol 3-kinase (PI3K) is the main tyrosine kinase that is recruited and its activation after the association between NKG2D and its ligand NKG2DL is crucial to phosphorylate Akt. Additionally, this association is linked to the activation of ERK1/2 and signaling through the MAPK pathway [20].

Studies show that the MHC-I chain-related proteins A/B (MICA/MICB) and the six distinct UL16 binding proteins are the most common self-antigens associating with NKG2D. NKG2D ligands are either structural homologs of MICA/MICB or of the HLA-I molecule, which are UL16-binding proteins and have been found to be commonly expressed in primary leukemia, melanoma, and glioma tumor cells among others [12]. Genetic analysis points to over 100 alleles for MICA, 40 for MICB, and under 20 for ULBPs [21]. This wide variety in expression of NKG2D ligands not only suggests that there are different signals that can be transduced from NKG2D binding, but also that the coevolution through thousands of years of host and virus interactions has resulted in the genetic polymorphism of stress antigens that can activate various functions of the NK cell. The preliminary signals offered by the binding of these various stress-induced antigens to the activating NKG2D receptor sensitize the NK cell. Therefore, if additional signals are transduced by inhibitory receptors that fail to bind to a healthy MHC-I molecule or CD16 binding an antibody, the NK cell can expedite its effector immune response. It has been noted that some viruses and transformed cells downregulate the production of NKG2D ligands as a method of decreasing the activity of NK cells. UL16 and UL142, both of which are CMV and HIV encoded proteins, have been shown to induce intracellular retention of NKG2D ligands [22]. This retention decreases the overall expression of NKG2D ligands, and as a result, the receptor and the signal transduction are attenuated, desensitizing these NK cells toward the infected cells and allowing for evasion of the cytotoxic immune effector response. Most commonly, the DNA damage response (DDR) pathway is what is commonly accepted as the primary synthesizer of NKG2D ligands [23].
In transformed cancer cells, mitosis proceeds with little regard for cellular checkpoints, resulting in nucleotide addition mistakes during the S-phase, as well as lesions in the structure. This results in the activation of the DDR cellular pathway, which is responsible for the identification and correction of that damage. Because the activation of the DDR presents a stressful situation for the cell, this, in turn, induces the production of NKG2D ligands that can then sensitize the NK cell toward its effector function through association with the NKG2D receptor [24].

The overwhelming evidence points to the fact that reduced expression of NKG2D or its ligands is the primary way in which viruses and tumors evade host immune surveillance systems. This highlights that studying the regulatory pathways that modulate both the receptor and the stress-induced surface proteins that bind to it might be effective in developing promising therapeutic drugs through NK-cell sensitization. Contrary to the normal tissue, the decreased expression of NKG2D and increased soluble ligand expression are both markers for poor prognosis in metastasis [21].

Regulation of NKG2D through multiple pathways

NKG2D regulation occurs through the calculated integration of multiple pathways with cross talks through various nuclear transcription factors driving the expression of downstream genes. As previously discussed, NKG2D is able to transduce signals through the adapter proteins DAP10 and DAP12, which upon phosphorylation can result in the recruitment of growth factors and other signaling proteins. Depending on which protein molecule binds the adapter protein, various downstream enzymes and signaling molecules can also interact. The interactions that occur at the adaptor of the NKG2D receptor largely affect which downstream pathway as well as what type of effector immune response is initiated. In the next section, the various pathways that take place and overlap with NKG2D regulation will be discussed.

STAT family

The signal transducer and activator of transcription (STAT) family of transcription factors is known to regulate the expression of the NKG2D receptor through stimulation or inhibition by various cytokines, such as IL-2, IL-10, IL-12, IL-21, and IL-15. Upon phosphorylation by the janus kinase (JAK), STAT3 molecules undergo dimerization and move to the nucleus where they bind and induce specific genes involved in cellular growth and proliferation. According to Zhu and colleagues, STAT3 inhibition using the inhibitory molecules JSI-123 and S3I-201 resulted in a significant downregulation of the NKG2D regulation, primarily through the decrease in IL-21 expression [25]. However, when the cell culture lines were induced with cytokines, STAT3 phosphorylation correspondingly increased, which in turn caused an increase in NKG2D expression. When the cell lines were cultured with IL-21 and IL-10 without STAT3, NKG2D expression dramatically decreased, indicating that the regulatory process is contingent on the presence of STAT3 [25]. Further immunoprecipitation studies showed that phosphorylated STAT3 interacts with a target-binding site in a region of the genome that is proximal to the NKG2D gene, and that interaction is augmented through IL-10 and IL-21 stimulation. This ultimately suggests that the phosphorylated STAT3 transcription factor is
a pretranscription regulatory factor of NKG2D. To expand on those findings, flow cytometry experiments were performed on NK cells received from metastatic melanoma patients before and after IL-2 induction. The results suggested that IL-2 not only increased NKG2D and pSTAT1/pSTAT5, but also the release of perforin [25], suggesting that this signaling pathway could be used immunotherapeutically to sensitize NK cells.

Apart from its well-characterized role in gastrointestinal homeostasis and increased production of B-1a cells, IL-15 has been linked to the proliferation of NK cells, as well as a possible growth signal. Ghosh and colleagues [26] conducted an experiment in which they cultured B-1a cells which produce IgM and IgA and can express the NKG2D receptor, with or without IL-15, and then observed the expression of the receptor through RT-PCR analysis. The results showed that incubation of cell lines with IL-15 significantly increased the expression of NKG2D. Specifically, the endogenous DAP10 adaptor which forms a transmembrane association with NKG2D was upregulated, while the expression of the secondary DAP12’s adaptor was not altered by the presence of IL-15 [26]. While further analyzing the signaling mechanism pathway, it was discerned that IL-15 incubation resulted in a time-dependent increase in the phosphorylated STAT5 transcription factor. Because phosphorylated STAT5 typically leads to nuclear localization and the transcription of the antiapoptotic survival genes Bcl-2 and Bcl-xL, flow cytometry was utilized to test these two proteins [27]. The results showed that Bcl-xL expression was augmented by IL-15; however, Bcl-2 expression was unchanged. Overall, the binding of STAT results in decisive chromatin remodeling that induces target gene expression, most commonly c-Myc and BCL-2.

**MAPK and NF-κB**

The regulation of the NKG2D receptor and its signaling is also largely affected by both MAPK and NF-κB pathways. The activation motif present on the DAP10 adaptor molecule has the ability to interact with the p-85 subunit of PI3K, which can consequently activate molecules such as Akt, Erk, JNK, and p38, all of which are various components of either growth pathway. Additional experimentation by Ghosh and colleagues [26] also suggested that IL-15 incubation resulted in a significant increase in the phosphorylation of the MAPK pathway’s transcription factor p38. This finding is interesting because p38 is a regulator of the NF-κB pathway, which is a mechanism that is known to have various proinflammatory roles including cell growth, proliferation, and apoptosis [28]. Through immunofluorescence techniques, the researchers showed that the p65 component of the NF-κB family translocates to the nucleus at a significantly higher rate with IL-15, as opposed to the control. These findings hint that NF-κB activation could be related to the increase in NKG2D and DAP10 expressions.

An interesting study conducted by Han and colleagues [29] featured increases in the concentration of sRAE-1, a transcript for NKG2D ligands, in a stepwise manner and examined the effect on PI3k/Akt activation and downstream activation of JNK, Erk, and p38. As expected, an 8-h stimulation with sRAE-1 greatly increased the expression and stimulation of PI3K-p85 and Akt/pAkt. Additionally, all three components of the MAPK pathway showed higher protein levels upon stimulation. JNK was shown to be significantly more phosphorylated in 8-h cultures with sRAE-1. Phosphorylated Erk and p38 also showed a significant increase. The transcription factors p50, p65, and p-p65 were also analyzed to

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see if NF-κB is involved in the signaling cross talks. The results were similar to the molecules tested for the MAPK pathway, as sRAE-1 promoted phosphorylation and increased expression for all the molecules [29].

**Downregulation pathways**

TGF-β is one of the most prominent immunosuppressive cytokines known to decrease the expression of NKG2D and Nkp30 and to inhibit the secretion of IFN-γ from NK cells [30]. TGF-β transcription is regulated by AP-1 and SP-1, which stabilize the transcript. Chen and colleagues attempted to shed some light on which transcription factor’s expression is affected the most by TGF-β induction in NK cells, as well as the corresponding effect on various receptors, including NKG2D. The results indicated that TGF-β most clearly affects the expression of c-Myb, which has various functions in eukaryotic cell growth. This was further explored through the addition of a c-Myb inhibitor, celastrol, which resulted in complete inhibition of the NK cell cytotoxicity and downregulation of NKG2D [31]. The c-Myb gene is a common binding target of the STAT family, which adds to the apparent complexity of the STAT pathway regulation for NKG2D. These findings not only suggest that c-Myb has an essential role in the effector response of NK cells, but also that it may be involved in the systematic regulation of the expression of NKG2D.

Alternative studies analyzed TGF-β’s impact on the expression of NKG2D in patients with endometriosis. The disease is characterized by inflammation on the outside of the uterine cavity and has long been associated with impaired NK functioning. Guo and colleagues [32] were able to establish that woman patients suffering from endometriosis not only featured an increase in TGF-β and a subsequent decrease in NKG2D expression, but also an overproduction of NKG2D soluble ligands that were shed from the inflamed cells. These results are not only consistent with the observation that TGF-β downregulates NKG2D, but also the earlier point that soluble NKG2D ligands result in the downregulation of the receptor [32]. Further reports hint that TGF-β may also have a distinct role in upregulating specific ULBPs, which act as ligands for the NKG2D receptor. This will be discussed in more detail in the next section [31].

**NKG2D: Regulation of the receptor’s diverse ligands**

Recent analysis of the effect of NKG2D ligand expression on NKG2D expression has shed some new light on the complicated cross talk pathways. Studies culturing MICA and ULBP2 with human NK cells have surprisingly found that although intermittent exposure to these NKG2D ligands results in NK cell sensitization, overexposure to these ligands over an extended period results in NKG2D receptor downregulation and reduced expression. MICA was found to be able to downregulate NKG2D receptor expression at a higher rate at similar concentrations in comparison to ULBP2 in vivo [33]. Although research in this particular field is still ongoing, these results are partially understood through the physiological function of the NKG2D receptor. Upon association of NKG2D with one of its ligands, the clathrin-dependent endocytosis of the receptor is promoted. In this process, the endocytosed receptor is passed on to a lysosomal compartment that facilitates its degradation. This process is dependent on the ubiquitination of DAP10 and the identity of the ligand, as some ligands may facilitate different types and rates of endocytosis [34]. However, before the degradation is complete, the internalized receptor can send signals to activate the effector function of the NK cell through the activation of the extracellular signal-regulated kinases 1 and 2 (ERK1/2),
which also serve as proteins in the MAPK pathway. Although the NKG2D/NKG2DL association does promote the downregulation of NKG2D through the receptor endocytosis pathway, studies utilizing NK cells that had a defective receptor internalization found that the process is still necessary in cytokine production and effector function. Furthermore, apart from ligand-induced regulation, cytokines can directly affect NKG2D expression through transcriptional inhibition. Specifically, TGF-β can interfere with DAP10 transcription, resulting in decreased NKG2D expression. In contrast, IL-15, IL-10, IL-2, IL-21, and IL-12 have all been found to increase the expression of both NKG2D and DAP10 [34].

Although the expression of the NKG2D receptor has a tremendous effect on sensitizing NK cells toward their effector function, the regulation of NKG2D ligand expression can also have profound effects. Chromatin immunoprecipitation studies found that when NK cells are exposed to a heat shock, the heat shock transcription factor 1 (HST1) binds to the MICA gene promoter, upregulating the transcription of the ligand. Further studies focused on the transcription factor p53, whose induction in NK cell cultures resulted in an increase in the transcription of ULBP1 and ULBP2 [20]. Transcription factors specific to the NF-κB pathway were found to have binding sites upstream of MICA as well, with induction of TNF-α in NK cultures causing upregulation of the NF-κB pathway and a small subsequent increase in the MICA ligand [35]. Finally, recent studies regarding the Sp family of transcription factors indicated that there may be putative binding sites not only in MICA and MICB, but also in ULBP1 as well. Specifically, Sp3 was found to closely interact with the ULBP1 promoter; however, the effects are still being investigated.

Both ULBP1 and ULBP2 have been found to increase in serum samples as a result of TGF-β induction. This upregulation is thought to occur indirectly, through the activation of SP1, which is responsible for the production of a wide range of ligands identified by NKG2D. The role of TGF-β is seemingly paradoxical, as it has roles in downregulating the production of the receptor itself, while the ligands themselves can be upregulated or downregulated, depending on the type of tumor and its stage. Chen and colleagues attempted to gain insight in the molecular pathway that TGF-β induces relative to NKG2D ligand production in PC3 and HepG2 cancer cells. Flow cytometry and western blotting indicated increased protein levels for MICA/B, ULBP1, and ULBP2 in both cell lines when TGF-β was added [31]. Another finding was that SP1 was absolutely essential in TGF-β’s upregulation of NKG2DLs and that TGF-β regulated SP1 posttranscriptionally by aiding in SP1’s localization to the nucleus. This promotion of SP1 protein expression through TGF-β was found to occur through the PI3K/AKT signaling pathway, as TGF-β inhibits the association between SP1 and GSK-3β [31].

On a posttranscriptional level, there are other factors that mediate the complex regulation of NKG2D ligands. The regulation that occurs at the mRNA level is increasingly important because the cell must maintain the stability of the transcript despite various stressors. The DDR pathway was commonly thought to participate in the induction of NKG2D ligands, however, recent reports have found that the DDR pathway actually functions in the stabilization of the easily altered mRNA transcripts of the NKG2D ligands, rather than the production of the ligands themselves [36]. This was found through studies on the murine NK ligand, Retinoic acid early inducible 1 (RAE-1), which demonstrated that induction of stress agents into cell culture lines to induce the DDR pathway did not result in any RAE-1 transcript increase. However, when comparing between cell cultures with and without activation of the DDR pathway, those with an increased DDR pathway activation had more stable RAE-1

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transcripts that degrade at a lower level [36]. Multiple reports suggest that miRNAs also have an important role in regulating the expression of MICA, MICB, and ULBP3 transcripts. The most prominent miRNAs are miR-17-5p, miR-20a, miR-93, miR-106b, miR-373, and miR-520 [37]. These studies suggest that miRNAs are constitutively expressed in normal cells; however, in stressful situations, p53 induces the downregulation of these miRNAs allowing for NKG2D ligands to be expressed. Another interesting finding is that IFN-γ can upregulate the expression of miR-520b, which regulates MICA. This is consistent with the fact that IFN-γ downregulates NKG2D ligands as discussed earlier (Table 1).

### Table 1

<table>
<thead>
<tr>
<th>Pathways</th>
<th>Proposed mechanism</th>
<th>Mechanism</th>
<th>Molecules that induce or inhibit the process</th>
<th>Overall effect(s)</th>
</tr>
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<tbody>
<tr>
<td>STAT protein family</td>
<td>Phosphorylated form is known to interact with a region proximal to NKG2D promoter</td>
<td>Cytokine mediated</td>
<td>IL-2, IL-12, IFN-α are all known inducers of the receptor dimerization and pSTAT upregulation</td>
<td>Phosphorylated STAT5 results in upregulation of NKG2D receptor expression</td>
</tr>
<tr>
<td>NF-kB</td>
<td>The DAP10 adaptor directly interacts through its activation domain with PI3K</td>
<td>PI3K can initiate downstream activation of molecules integral to the pathway</td>
<td>P50 and P65 are both transcription factors that operate in the pathway and are phosphorylated when the pathway is activated</td>
<td>Activation of the NF-kB through NKG2D signaling can result in cell survival, cytokine production, and growth that can affect NKG2D regulation</td>
</tr>
<tr>
<td>PI3K</td>
<td>PI3K’s p85 can associate with the activating domain of NKG2D’s DAP10</td>
<td>This is the major intersection for many of the pathways involved in NKG2D signaling.</td>
<td>The activation and association of PI3K with DAP10 results in the recruitment of transcription factors</td>
<td>Various pathways can be triggered downstream from PI3K activation depending on which transcription factors are recruited.</td>
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<tr>
<td>MAPK</td>
<td>Through interaction with Akt after PI3K signaling, the MAPK pathway is involved in NKG2D signaling</td>
<td>PI3K activation by NKG2D stimulation results in Akt phosphorylation which can then feed into the MAPK pathway</td>
<td>JNK, Erk, p38, and multiple other molecules in the pathway must be activated for the pathway to proceed</td>
<td>The MAPK pathway stimulates cell growth, proliferation, and differentiation and can expand NK cell count</td>
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Raf kinase inhibitor protein (RKIP)

**General properties**

*Raf* (rapidly accelerated fibrosarcoma) is an oncogene that has been linked to various types of cancer since its primary identification in 1983. There are three main protein isoform
products arising from alternative splicing of the Raf gene, and all serve as effectors of the Ras protein and activators of the MEK protein in the MAPK pathway [38]. The MAPK pathway has been a prevalent area of focus for contemporary research, as mutations and dysfunctions in different proteins of the cascade area are associated with tumorigenesis and metastasis [39]. When operating normally, the MAPK pathway is responsible for cell growth, proliferation, survival, and differentiation [40]. Mutations or dysfunctions with the Raf protein kinases, as well as the upstream Raf protein, can result in failure of the proper regulation of the cell cycle at the G0/G1 checkpoint. Over 15% of human cancers include a mutation in Ras, while 30% of human cancers can be traced to constitutively active ERK, which is found just two proteins downstream of Raf, and stimulates transcription factors that regulate cellular proliferation [40]. Originally, scientists only considered the Raf protein in the context of Ras and concluded that its involvement in cancer was primarily due to its downstream position relative to Ras; however, recent discoveries showed that B-Raf mutations exist in 30%–60% of melanoma cancers, 30%–50% of thyroid cancers, and about 30% of ovarian cancers [41]. These discoveries in the early 2000s led to increased research on the characterization of the Raf protein in more detail.

All three Raf protein isoforms (A-Raf, B-Raf, and C-Raf) are tyrosine kinase receptors that must be phosphorylated to reach the activated state, where interactions with the Ras protein allow for the signaling transduction pathway to proceed toward downstream effectors [42]. Transgenic knockout mice studies have allowed scientists to isolate the three different isoforms and gain insight on the individual and combined functions through a series of experimentation. Knockouts in the B-Raf isoform of the protein showed mice having severe neuronal dysfunctionality and eventually dying in the womb during embryogenesis [42]. Knockouts in the C-Raf (Raf-1) isoform displayed mice that also died during embryogenesis, while showing little to no development in the liver organ, placenta, and hematopoietic organs. Interestingly, scientists observed that the MAPK pathway during embryogenesis continued to function with little to no abnormalities despite the C-Raf knockout, pointing to the fact that C-Raf may have a role in other regulatory pathways [42]. Finally, A-Raf knockouts in mice did not cause the mice to die in the womb; however, most progenies were incapable of surviving past 21 days and showed severe intestinal and neurological defects [42]. These findings pointed to the fact that all three Raf isoforms are essential in sustaining life in animal models and could not be knocked out to stop metastasis, which directed scientists toward finding other methods to target mutated Raf. This resulted in a shift of focus to a newly characterized allosteric inhibitor of the Raf protein: the Raf-kinase inhibitor protein (RKIP).

RKIP is a small protein (21 kDa) that belongs to the phosphatidylethanolamine binding protein (PEBP) family and has been proven to bind directly to the Raf protein, thus stopping the MAPK pathway by inhibiting the phosphorylation of Raf through changing its conformation. RKIP is an inhibitor not only of the MAPK pathway, but also of alternate signaling pathways such as the β-adrenergic NFκB signaling pathways [43]. Deficiency or loss of RKIP in humans and animals alike has been linked with many different forms of cancer due to the overactive Raf protein stimulating the MAPK pathway and thus promoting constant cell growth and proliferation that bypass the cellular checkpoint system. Studies have suggested that RKIP can be used therapeutically to augment proapoptotic tendencies in tumor cells, regulate EMT, and decrease metastasis [44]. What is even more fascinating is that through genetically altering the expression of RKIP in tumor cells, they become more sensitized and less resistant to anticancer therapeutics. The pairing of RKIP expression as an immune therapeutic
with conventional methods has great utility, as some tumor cells have evolved an ability to evade apoptosis when subjected to traditional chemotherapy and immunotherapy [38].

The function of RKIP as an allosteric inhibitor of the Raf protein is largely driven by its structure and, primarily, its phosphorylation state [45]. RKIP functionality can be attributed to a three-state model, in which the protein assumes three distinct roles through its structure. In its unphosphorylated state, RKIP binds Raf and suppresses the MAPK pathway by downregulating ERK. However, through phosphorylation of serine-153 by protein kinase C, RKIP binds and inhibits the G-protein coupled receptor kinase 2 (GRK2). The third structural state of Raf involves its association with protein kinase C to allow for further stabilization through phosphorylation at multiple sites [45]. The exact function of the third RKIP-kinase association is still being explored; however, there are hints of the phosphorylation state being involved in regulation of RKIP activity.

The majority of RKIP’s function is dictated by its two-part structure consisting of: a globular high surface area region that induces protein-protein interaction, and a flexible pocket loop, which facilitates ionic ligand interactions with multiple partners through a dynamic shape. This flexible pocket loop contains a highly conserved amino acid region, P74L, which has been shown to bind multiple anions, such as α-phosphorylethanolamine (PE), acetate, and cacodylate through several X-ray crystallography studies [46]. Mutations in the flexible pocket loop P74L region result in the induction of RKIP toward its phosphorylation through the increased association with PKC and, thus, an upregulation of the MAPK pathway. These mutations are found in a variety of different cancer forms [46]. The model hints that RKIP’s phosphorylation state, and thus its function, is primarily mediated by which protein is interacting with its flexible pocket loop domain, whether it be the Raf protein, protein kinase C, or GRK2. Nuclear magnetic resonance (NMR) and mass spectrometry studies have shown that the pocket loop domain is capable of binding not only proteins but also small ligand phospholipids, such as 1,2-dihexanoylsn-glycero-3phosphoethanolamine DHPE, through its exceptionally dynamic pocket, allowing for further regulation of RKIP [47].

Expression in normal and malignant tissues

Because RKIP deficiencies or dysfunctionalities have been identified in a wide range of cancers, scientists have focused their efforts on the utilization of RKIP expression as an anticancer therapeutic. While the concentration of RKIP as an allosteric inhibitor has widely been noted as a cause of tumor formation and metastasis, the prevalence of RKIP in large quantities but in the phosphorylated, inactive form has been a new finding in cancer patients [47]. The function of RKIP as a metastatic suppressor has been chronicled and analyzed by different scientific studies since its primary identification. Regardless and independent of the patient’s age, gender, tumor depth, and site, RKIP expression in healthy versus metastatic tumor tissue shows a strong correlation indicating the molecule’s importance in regulating cellular proliferation and differentiation [48]. Studies involving samples from various cancerous tissues suggested that RKIP deficiency is a major contributor to diseases such as multiple myeloma, breast cancer, and prostate cancer [49]. RKIP is said to primarily function in inhibiting the EMT, intravasation, colonization, and local invasion [48]. The distinctions in RKIP expression as they relate to overall tissue health are well documented through immunohistochemical
staining studies. A recent study staining various breast tissues with RKIP antibodies uncovered that normal breast tissue cells and local benign tumors are 84.85% and 71.43% positive for the inhibitor protein, respectively, while out of a total 38 cases of malignant breast cancer only 10.53% of tissues appear to be positive for RKIP expression [50]. These results support the hypothesis that loss of RKIP protein results in a lack of proapoptotic tendencies, thus augmenting the possibility of tumor cells to metastasize throughout the body. The same study reported that an overexpression of RKIP in tumor breast cancer tissue resulted in a 32.5% decrease in the migration percentage of the corresponding cells, confounding RKIP’s antimetastatic properties, as well as hinting at its potential therapeutic use in the future, while downregulation in RKIP caused a 77% increase in migration of cancer cells [50].

RKIP’s function in metastasis inhibition is further supported by studies in prostate cancer. An immunohistochemical study examining the difference in positive RKIP expression in primary prostate cancer tissue and metastasized tissue found that primary tumor tissues expressed RKIP at a near similar percentage to healthy tissue, while a statistically significant small percentage of metastatic tissue showed positive RKIP expression [51]. When RKIP was passively injected into the tissue samples, the group found that metastatic tissue showed suppression in proliferation, while the primary tumors were largely unaffected by the same injection. These findings all support the premise that RKIP’s function is primarily focused on the suppression of tumor metastases rather than the initial formation of the cancer [51]. Thus, RKIP is thought to be an essential component to inhibiting the spread of malignant tissue through the modulation of EMT, motility, and invasion; however, there is no indication yet that it can be effective in quelling benign and initial tumor formation. Recent studies that attempted to expand on the knowledge of RKIP’s physiological role have found that cancer is not the only pathology that results from RKIP’s dysregulation. There are links relating RKIP expression directly to Alzheimer’s disease, diabetic nephropathy, and failure in spermatogenesis, which suggests its multifunctional and complex activity [52].

Alternative breast cancer studies have focused on examining the association of RKIP concentration and molecular factors associated with metastasis such as metalloproteases (MMP’s), miRNA molecules, migration factors, cytokines, and other biomarkers. In breast cancer patients, a strong inverse association was found between MMP-3, -10, -13, and RKIP [53]. Metalloproteases allow tumor migration and metastasis, and almost all tissues with high MMP concentrations in breast cancer patients showed very low RKIP expression [53]. Matrix metalloproteases are responsible for directly disassembling the extracellular matrix and the basement membrane of responding cells, allowing for the cellular mobility that is seen in tumor malignancies. RKIP antagonizes the transcriptional activation of the metalloproteases through the Erk2 pathway, thus inhibiting tumor dispersal throughout the body and acting as an overall metastatic suppressor [44]. Thus, it is intuitive that the majority of the breast cancer cell line samples show an inverse relationship between RKIP and metalloproteases, and the overall concentrations of both biomarkers are good prognostic measures for local tumor invasion and spread. These findings also suggest RKIP’s role as a regulatory factor in EMT, as underexpression of RKIP resulted in increased cancer cell extravasation [53]. Another well-noted prognostic marker for breast cancer is the presence of miRNAs that bind posttranscriptionally to the RKIP mRNA silencing its protein’s expression. miR-224 was specifically found to be at hypernormal concentration levels in highly invasive breast cancer cell lines, illustrating the drastic effect of RKIP miRNA regulation on tumor progression [54].
At moderate levels of miRNA expression, breast tissue cells appear largely normal and unaffected; however, high miRNA concentration is directly related to decreased RKIP activity, which promotes metastasis [54].

Additional studies regarding patients with primary colorectal cancer indicate that RKIP expression is one of the most valuable indicators of overall patient survival and can predict metastatic relapse and histopathological features associated with the disease. The primary method for these analyses included tissue microarrays probed with RKIP antibodies, which indicated the vast statistical differences between healthy tissue and colorectal cancer tissue [55]. RKIP also showed a very strong association with indicators of metastasis such as vascular invasion, tumor budding, and NFκB activation. In gastric cancer, which is the second most common cause of cancer related deaths currently, cell lines showed that RKIP was expressed at higher than normal levels in about 83% of nonneoplastic normal tissue cells, while it was practically lost in 90% of the samples that featured lymph node metastasis from the gastric tissue [56]. This also reaffirms the belief that RKIP is heavily associated in preventing EMT and overall spread of cancer cells throughout the body. The study also concluded that RKIP expression was involved in approximately two out of every three cases with gastric tumor locations [56]. In vitro experiments showed that gastric cancer samples transfected with RKIP exhibited proapoptotic behavior, delayed G1/S transition, and lack of migration [57].

RKIP activity is also comprehensively noted through research conducted in lung cancer. These studies allow for the examination of various expression levels in healthy tissue versus both small cell lung cancer and nonsmall cell lung cancer. Zhu and colleagues measured the expression of RKIP through RT-PCR in lung squamous cell carcinoma, and their findings showed that RKIP was expressed in 47.7% of squamous cell carcinomas, but in 76.7% of healthy nontumor cells [58]. In another study of 126 lung tumor tissue samples, RKIP was also expressed at a similar rate (41.9%). The difference in RKIP expression between lung cancer and healthy tissue was significant [59]. The results of the study reveal that lower RKIP mRNA levels in nonsmall cell lung cancer are more likely linked to poor cellular differentiation.

Although deregulated RKIP expression is a prognostic marker of metastasis, extravasation, and migration across different cancer types, it is differentially expressed among these cancerous tissues. Using data from The Cancer Genome Atlas (TCGA), a study utilized RNA-seq as a quantification method to compare mRNA expression across 37 different cancer types [43]. The findings suggested that, in almost all of the cancer types, RKIP levels are deficient in the cancerous tissue when compared with the healthy tissue. However, when comparing the different cancer types across each distinct tissue, the analysis showed acute myeloid leukemia, esophageal carcinoma, and stomach carcinoma samples to have the lowest RKIP mRNA levels. The highest RKIP expression levels were seen in thyroid carcinoma, liver hepatocellular carcinoma, and adrenocortical carcinoma [43]. These are valuable data that allow scientists to form conclusions on which molecules can serve as prognostic markers in therapeutic research.

More recent studies have shown that expression levels of RKIP and tumor metastasis might not exactly represent a perfectly inverse correlation. In patients with multiple myeloma, overexpression of RKIP was actually noted in several distinct cases [60]. However, although the RKIP protein concentration was relatively high in comparison to normal tissue, the majority of the RKIP molecules appeared in the phosphorylated and inactive form, thus they were incapable of forming an association with the Raf protein in order to inhibit its function. These results suggest that the relative concentration levels of RKIP are not the only aspect that should
be tested in cancer patients. Relatively high levels of RKIP were found in many patients suffering from nonmetastatic melanoma; however, the majority of those molecules were phosphorylated at serine-153, rendering them inactive [60]. A study concerned with nonsmall cell lung cancer utilized western blotting and tissue microarrays to compare differences in RKIP phosphorylation between normal and cancerous tissue, in order to see whether these could be used to predict the survival of lung cancer patients [61]. The results showed that phosphorylated RKIP can predict the survival of nonsmall cell lung cancer patients aged older than 65, as well as in stage one tumors for all patients. This, among many other studies, has highlighted the importance of analyzing phosphorylated RKIP levels along with the unphosphorylated, inactive RKIP, as both can be prognostic markers for patient survival.

### Regulation of cell signaling pathways and outcomes

There are multiple layers to RKIP regulation that factor into the regulation of its activity and synthesis, and furthermore, studies suggested that its low expression is a common trend in various cancer types [44]. The most common cause of pretranslational regulation of RKIP expression is due to the hypermethylation of the CpG region of RKIP’s promoter, which suppresses the protein’s expression [62]. Conversely, binding of the androgen receptor to a putative androgen responsive element (ARE) within the promoter for the RKIP gene augments the genetic transcription of the RKIP gene [63]. On a posttranslational level, the activity of RKIP is regulated through two primary methods, the first of them being the phosphorylation state of RKIP, which determines which protein it can subsequently bind to. In its unphosphorylated and active state, RKIP can bind directly to Raf-1 and inhibits it from forming an association with MEK and thus inducing the MAPK pathway. It has been noted that RKIP is only able to allosterically inhibit the Raf-1 isoform, by binding at the regions of phosphorylation, serine-338 and tyrosine-341. The B-Raf isoform is only affected by RKIP indirectly, through its inability to dimerize with C-Raf, which is inhibited by RKIP. Furthermore, once PKC phosphorylates RKIP, it can no longer allosterically inhibit Raf and the MAPK pathway can be activated downstream of Raf. A second posttranslational regulation of RKIP is through the binding of a phospholipid ligand, known as 2-dihexanoyl-sn-glycero-3-phosphoethanolamine (DHPE), to the auxiliary residues around the flexible loop pocket. The interaction of DHPE with RKIP results in the disassociation of Raf from RKIP [47].

When RKIP is phosphorylated by PKC, it disassociates from Raf-1 and no longer inhibits it. The phosphorylated RKIP protein is now capable of binding directly to GRK2, which inhibits RKIP activity by blocking the binding and internalization process, thus facilitating the transduction of the MAPK signaling pathway. GRK2 is involved in a variety of different regulatory signaling pathways across the body that have multiple functions involved in cell differentiation, proliferation, and overall growth. GRK2 specifically ensures successful regulation and transition of the G1/S and G2/M checkpoints in the cell cycle by modulating the migration of immune and epithelial cells [47]. Studies have found that GRK2 is one of the protein players necessary in the cytoskeletal reorganization process that allows for not only cell movement, but the mitotic spindle formation process that is part of the mitotic cycle [64]. When there are breaches in the regulation of RKIP molecules and their phosphorylation state, there are often drastic effects on cell proliferation rates which can lead to cancer.

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Furthermore, miRNAs can downregulate RKIP expression in human cells, and subsequently result in cancer. Studies have shown that triple-negative breast cancer, which is a more severe form of the disease, includes patients who are deficient in RKIP [49]. miRNA regulation works through its simple hairpin structure with a near complementary base pairing to 3′ untranslated regions (UTR) in target mRNAs. The binding of these noncoding RNA molecules results in the silencing of that mRNA, which in turn affects the translation and overall expression of a particular gene product [65]. There are multiple miRNA molecules that have been found to interact with RKIP mRNA posttranscriptionally and subsequently result in RKIP depletion including miR-224, -27a, -23a, and -543 [49]. Bioinformatics and RNA-seq were utilized in the identification of these miRNAs by testing their correlation to being associated with RKIP mRNA and finding several distinct types of miRNAs binding the amino acid coding sequence (CDS) located in the 3′ UTR of RKIP mRNA. It is hypothesized that scientists have not fully identified all of the different miRNAs that act in the expression of RKIP in healthy cells, and as a result, it is inferred that multiple miRNAs function together to effectively silence and regulate RKIP expression levels [65]. This binding, which results in the silencing of RKIP translation, has been identified as one of the primary sources of cancer. Studying miRNA regulation of RKIP might be a point of interest that is much brighter than others, as miRNA molecules have shown a much larger success rate in therapeutic drug targeting then other molecules such as kinases, transcription factors, or ligands [49].

The majority of the discussion about RKIP is concerned with its regulatory activity in the MAPK pathway; however, it has been proven that MAPK is not the only pathway in which RKIP is involved. Studies in the early 2000s demonstrated RKIP’s involvement in antagonizing the NF-κB pathway through interacting with kinases upstream of the NF-κB transcription factor. RKIP is stimulated by TNF-α and IL-1 to interact with the upstream kinases NF-κB kinase, lkB kinase alpha, lkB kinase beta, and transforming growth factor beta-activated kinase 1 (TAK1), which results in a loss of their activity, and subsequently inhibits NF-κB. The active form of NF-κB promotes the synthesis of metalloproteases, which aids in the migration process of metastases cells, as well as activates another identified oncogene known as Yin Yang 1 (YY1). Constitutively active YY1 has been found to be one of the markers associated with EMT in both prostate and breast cancer cell lines [44]. Through negatively regulating NF-κB, RKIP can also indirectly suppress YY1. RKIP has further been suggested to act as an inhibitor of the transcriptional repression of death receptors. Commonly, tumor cells will lose their death receptors in order to gain resistance from immune cells; however, RKIP overexpression in tissue has been found to stop overall tumor resistance to TRAIL and Fas-ligand apoptosis [66]. The NF-κB protein cascade accomplishes a variety of tasks and cellular processes including the control of DNA transcription, tumor angiogenesis, cell survival, and cytokine production. RKIP’s regulation of NF-κB appears to be very similar to its method of action in the MAPK pathway, which suggests that this could be a result of the continuous cross-modulation of two pathways with different physiological effects. STAT3 is a transcription factor that can result in cellular disregard for apoptotic signals, increased epithelial-mesenchymal transition, and cancer cell metastases when it is dysregulated and constitutively active. RKIP has been found to be an inhibitor of STAT3 activation, through blocking it from being phosphorylated by interacting with Janus kinase 1 and 2 (JAK1/2). These findings were confounded by the inverse correlation that was found when comparing phosphorylated STAT3 (active form) and RKIP expression in a variety of different cancerous tissues [44] (Fig. 2).
a. Pre-Transcriptional Level

- Androgen receptor binds in the promoter of the RKIP gene
- Hypermethylation at the promoter of the RKIP gene

b. Post-Transcriptional Level

- Translation
- miRNA binds to the 3' UTR of the RKIP mRNA

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Cross talks between RKIP and NKG2D

Data mining the Oncomine platform

The emergence of DNA microarray technology paired with the prevalence of next-generation sequencing (NGS) has allowed for rapid analysis of individual genomes and the ability to cross compare genetic profiles across thousands of patients. Databases such as ONCOMINE compile large amounts of cancer microarray results and can be used effectively for data mining and differential expression analyses. Through the platform, one can visualize differences between multiple cancer subtypes or genes through a selected analysis filter of thousands of microarray experiments that are chronicled on the database. For the purpose of this review, NKG2D (KLRK1) and RKIP (PEBP1) were analyzed across multiple datasets to determine if there are any trends in gene expression across distinct tissue types, both normal and cancerous. Additionally, this exploration was taken one step further through the systematic analysis of PEBP1 expression in both healthy and cancerous patient NK cell lines, through bioinformatic means. The four primary datasets that we examined were Roth Normal (Dataset 1), Su Normal 2 (Dataset 2), Critchley-Thorne Melanoma (Dataset 3), and Eckerle Lymphoma (Dataset 4). All four datasets featured experiments that utilized DNA microarrays and were either coexpression or differential analyses. An additional fifth dataset from the human protein atlas (HPA), which measured RNA transcript expression, was also analyzed.

The Roth Normal Dataset (Dataset 1) was selected because it examines 353 patient samples for over 19,574 genes and the p-value was set at ≤ 0.0004 [67]. Additionally, it screened 65 different tissues from organs in the body. This dataset only analyzed gene expression from patients who were completely healthy and did not have any form of cancer, and as a result, it gives insight on the normal expression levels of NKG2D and RKIP relative to each other. A marked and significant inverse correlation between the PEBP1 and KLRK1 genes is observed in the dataset across almost every tissue type. For example, the spleen is the tissue that exhibits the highest expression of KLRK1, and subsequently, for PEBP1 expression the spleen is one of the organs with the lowest intensity values. This is also seen in the liver, where increased KLRK1 expression met with one of the lowest intensity values for PEBP1 expression. Conversely, the adrenal cortex exhibited the highest PEBP1 expression across all tissue types; however, KLRK1 expression was markedly low in the adrenal cortex relative to other tissue types (Fig. 3).

The Su Normal 2 dataset (Dataset 2) included 158 patient samples tested for coexpression across 12,624 genes through the Human Genome U133A Array [68]. This dataset was selected because it analyzed PEB1 expression levels across 79 different tissue and cell types, including lymphocytes such as CD56+ NK cells in primarily healthy patients. The study’s results indicate that the expression of RKIP in peripheral blood NK cells was significantly lower than that in the cells of other tissue types in the body. Peripheral blood monocytes, CD4+ T cells, and CD8+ T cells all exhibited relatively low expression levels of RKIP as well. However, the median value for intensity was still higher than that of CD56+ NK cells. Interestingly, CD19+ B cells were among the cells with the highest RKIP expression among the 79 cell types analyzed in the study, with intensity values comparable to that of liver and kidney cells (Fig. 4).
Further data mining on the ONCOMINE platform led to the bioinformatic analysis of the Critchley-Thorne Melanoma dataset (Dataset 3), which was unique in that it considered expression levels of various genes in lymphocytes only [69]. The study included 46 samples that measured the expression levels of 16,179 genes. The findings showed moderate to high levels of RKIP expression across B cell, CD4+ T cells, and CD8+ T-cell lymphocytes for healthy patients. However, the expression levels for PEBP1 in NK cells were very low to nonexistent for healthy patients. When considering patients with melanoma in the study, expression levels for all four lymphocytes showed marked increases in RKIP expression, especially for both CD4+ and CD8+ T cells. The between PEBP1 expression levels across the four different lymphocytes were deemed statistically significant by the predetermined p-value of $\leq 0.0001$ (Fig. 5).

Eckerle Lymphoma (Dataset 4) was especially remarkable as it analyzed the expression levels of PEBP1 in NK cells and T cells of healthy patients and compared them directly to those of patients with various forms of lymphoma [70]. This is particularly interesting because it lends insight to the regulation of a wide range of gene products in an anaplastic large cell, classical Hodgkin’s, and primary cutaneous anaplastic large cell lymphomas. The study incorporated 64 patient samples and analyzed the expression of 19,574 genes using the Human Genome U133 Plus 2.0 Array. The results confirmed what was seen in other related

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experiments: *PEBP1* expression levels in NK cells were markedly lower than what is examined in T-cell lymphocytes. In all four lymphoma types that were examined, however, there was a significant increase in *PEBP1* expression compared with the healthy patients. Anaplastic large cell lymphoma (ALK+), in particular, showed a very high expression of *PEBP1* relative to the other cancer types. The Eckerle Lymphoma dataset was also utilized to examine NKG2D expression through its gene *KLRK1* across the same lymphocyte populations. The results showed that *KLRK1* expression is relatively high in healthy NK cells compared with the cell lines that have lymphoma. This significant drop in the expression levels of NKG2D from healthy patients to those with lymphoma was statistically significant. These findings not only indicate that NKG2D expression is a prognostic marker for various cancer pathologies, but also support our hypothesis that RKIP and NKG2D are anticorrelated, hinting that there are potential cross talks between these two biomolecules (Fig. 6).

Diving further into the Eckerle Lymphoma (Dataset 4), the expression levels of various NK Cell receptors such as NKP30, NKP46, and CD94 were compared with that of NKG2D in the lymphocytes of healthy patients [70]. The results from the dataset showed that CD94 expression is highest across all lymphocytes that were examined in the study. When comparing NKG2D to NKP30 and NKP46, however, it was clear that NKG2D expression is higher. Specifically, NKG2D is found in much higher levels in activated CD8+ T cells, while the Nkp family receptors are nonexistent in this lymphocytic population. These findings are aligned

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with the belief that NKG2D is one of the most prevalent and integral activating receptors of NK cell functioning (Fig. 7).

The last dataset that was explored (Dataset 5) was sourced from the Human Protein Atlas (version 19.3) and Ensembl (version 92.38). It analyzes six independent experiments on NK cells and their expression of various genes through measuring their RNA transcripts. The study utilized 109 blood cell samples and its findings suggest that \textit{KLRK1} expression is almost threefold compared with that of \textit{PEBP1}. This is well aligned with the results of the previous datasets that were analyzed from Oncomine (Fig. 8).

**Insights from other research studies**

Throughout the previous sections, it has been established that the RKIP protein exhibits multiple functions in cancer cells. It acts as a tumor suppressor, an inhibitor of metastases, cell viability, and cell proliferation. In addition, RKIP exhibits chemo-immuno-sensitizing activities against resistant cancer cells. Across most cancers, RKIP is underexpressed, while it

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FIG. 6 Bioinformatic analysis: Eckerle Lymphoma Analysis 1. Oncomine log2-mediated intensity graphs for the Eckerle Lymphoma datasets for the expression of PEBPI (gene for RKIP) and KLRK1 (gene for NKG2D) in the lymphocytes of healthy donors as well as in the lymphocytes of patients with four distinct lymphoma types. Columns 1–3 constitute healthy donor lymphocytes while columns 4–7 include patients with various lymphoma types.
may be completely absent in the majority of metastatic tumors. Through the analysis of the signaling pathways that regulate these functions of RKIP, it is clear that many of the molecular components are shared with the regulation of NKG2D. Both regulatory pathways include the modulation by NF-κB components, the association with STAT family proteins,

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and the interaction with TRAIL and Fas apoptosis ligands. The following section will explore potential links between NKG2D and RKIP. These findings highlight the specific agents that modulate the expression level of RKIP in NK cells and can potentially lead to the overexpression of the activating NKG2D receptor, which will subsequently enhance NK antitumor activity.

One of the most promising studies that analyzed RKIP, as well as numerous related regulatory gene products in multiple myeloma (MM) patients, was performed by Shvartsur and colleagues [60]. The expression levels of both RKIP and the mRNA of these gene products were analyzed through Oncomine and comparisons across the heterogenic field of patient samples were made. The findings reflected that RKIP is commonly underexpressed in malignant tumor forms or overexpressed in benign melanoma in multiple patient samples, but in its inactive and phosphorylated form, it is significantly overexpressed in comparison to other cancer types. As a result, the MAPK, NF-κB, YY1, and JAK/STAT3 pathways have a lack of inhibition, which allows for EMT and unchecked cellular growth in these MM patients which can ultimately result in anemia, bone marrow failure, or osteoporosis. Inversely, the PTEN pathway, as well as other proapoptotic factors such as TRAIL, is inhibited in many cases. The report tracked the expression levels for RKIP, AKT, Bcl-2, Bcl-6, CIAP1, DR5, E-cadherin, Fas, FasL, NF-κB, PTEN, SNAI1, SNAI2, TNF-α, TNFR-1, TNFR-2, TRAIL, XIAP, and YY1; however, we will only discuss the molecular agents common between both RKIP and NKG2D regulation [60].

The most interesting link was found through a significant inverse correlation between survival promotion and proapoptotic genes. For example, in patient samples that included overexpression of Fas and TNF-α, there is marked underexpression of YY1, and vice versa. This is largely intuitive as TNF-α is known to stimulate RKIP expression, while YY1 is a known repressor of the RKIP gene [71]. Additionally, this is related to NK cells and NKG2D expressions, as it is well known that transcription factors for the NF-κB pathway have upstream binding sites for NKG2D ligands such as MICA, and induction in NK cell cultures with TNF-α results in an increase in the MICA ligand [72]. Increasing the amount of MICA can, in

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turn, have effects on the expression of its receptor, NKG2D. In terms of NK cells, overexpression of Fas and TNF-α directly activates caspases that can trigger effector function through either the Fas/FasL or TRAIL pathways, respectively [15]. In the MM patient samples, Fas was found to be underexpressed, which is consistent with the constitutive activation of the NF-κB pathway and the YY1 repressor, as well as the overexpression of phosphorylated and inactive RKIP [60]. This is significant because it suggests that dysregulation in the RKIP loop can result in a decrease in the functioning of Fas-mediated apoptosis, a primary effector function of NK cells and a common cross talk pathway for NKG2D.

Another key finding in this particular study was centered around Bcl-2 which is regulated by the NF-κB pathway and by RKIP’s repressor, PTEN. When RKIP expression is low, such as in many instances of cancer, Bcl-2 is upregulated through NF-κB, promoting antiapoptotic cellular proliferation and survival. This occurs through Bcl-2 essentially blocking the release of cytochrome c in the mitochondria during standard apoptosis, thus inhibiting caspase 3 activation [73]. As expected, Bcl-2 was found in high concentration in MM patients in the study, which may account for the chemoresistance of MM tumors to many different types of therapies as well as tumor persistence and spread. This relates directly to NK cells because studies have indicated that the transcription of both Bcl-2 and the related Bcl-xL is induced by the presence of phosphorylated STAT [27]. As discussed in detail in the NKG2D section, the STAT family proteins upregulate NKG2D expression. The STAT family is not only known to stimulate the production of cytokines that upregulate NKG2D, but STAT3 also inhibits molecules such as JSI-123 and S3I-201 that downregulate NKG2D [73]. Additionally, immunoprecipitation studies have shown that after phosphorylated STAT3 dimerizes and localizes toward the nucleus, it binds in a region that is proximal to the NKG2D gene, hinting that it may be a potential pretranscriptional regulator of the receptor. Additions to that research by Zhu and colleagues have found that specifically in MM patients, phosphorylated STAT1/STAT5 upregulates not only NKG2D expression but also the mRNA levels of perforin, in the presence of IL-2, which is part of the cytotoxic pathway and effector functioning [25]. Clearly, there is an implication in the shared pathways for regulation of RKIP and NKG2D through Bcl-2, indirectly through the STAT family of proteins.

Another study conducted by Kaufhold and colleagues [74] related to YY1, the common RKIP gene repressor, and its association with multiple different cancer stem cell transcription factors, highlights alternative potential cross talks between NKG2D and RKIP regulation pathways. Through data mining from the proteomic tissue-based datasets that are publicly available on the Human Protein Atlas, expression patterns between YY1 and the cancer stem cell transcription factor, sex determining region Y-box 2 (SOX2) were clearly established [75]. SOX2 is known to induce cellular proliferation and possible tumorigenesis when unchecked and has been identified in the evasion of apoptosis by tumor cells in prostate, gastric, and nonsmall cell lung carcinoma cancer types. This is relevant to NKG2D regulation because the PI3K/Akt pathway was found to be active in prostate cancer cells and inactive in ovarian adenocarcinoma cells [72,76]. In the NKG2D section, PI3K’s role in associating directly with NKG2D’s DAP10 adaptor protein, and subsequently recruiting various transcription factors such as Akt in order to initiate downstream responses in NK cells, is thoroughly explained. The differing results in regard to SOX2’s induction of the PI3K/Akt pathway, YY1’s association with SOX2 and RKIP, as well as NKG2D’s reliance on the PI3K/Akt pathway to perform
its function, all indicate that this is an area that needs additional research as there is potential to discover direct cross talks.

Concluding remarks and future perspectives

This review reported on the interrelationship between the expression of RKIP in NK cells with cytotoxic function, particularly via the expression of the activating NKG2D receptor. The data that was presented was sourced from the analysis of different scientific studies on each gene product independently, and then the subsequent identification of the cross talks between these molecules. These cross talks established that there exists an inverse relationship between the expression of RKIP and that of NKG2D in NK cells. These findings were corroborated by bioinformatic analyses using Oncomine, TCGA, and HPA data.

The inverse relationship that was determined is the result of the interplay of various gene factors and signaling pathways (Fig. 9). The low expression of RKIP results in the weak inhibition of the NFkB pathway and its activation, as well as the activation of its downstream gene targets, YY1 and SNAIL. The activation of NF-kB results in the induction of NKG2D as well as in the activation of the RKIP suppressor SNAIL by YY1 (Fig. 9A). Hence, there is a loop by which the activation of NFkB and both YY1 and SNAIL represses RKIP, maintaining it at low levels. Furthermore, the low expression of RKIP, in addition to poor inhibition of NFkB, also poorly inhibits STAT3, and thus activated STAT3 positively regulates NKG2D expression (Fig. 9B).

These mechanisms imply that the activation of the cytotoxic trigger via NKG2D depends, in part, on the level of RKIP in NK cells. Thus, if the RKIP expression is altered in cancer patients and/or in the tumor microenvironment and RKIP is upregulated, this will lead to a poor antitumor cytotoxic response by the NK cells and will contribute to tumor escape from immune surveillance.

This supposition must be investigated in cancer patients to determine whether RKIP expression in the NK cells is elevated compared with the normal controls. If this were the case, further analyses should follow to determine the clinical significance and whether it contributes to tumor growth and survival.

Interestingly, the expression of RKIP in cancer cells plays an opposite role than the one found in NK cells. Most cancer cells express low levels of RKIP that correlate with tumor growth, metastasis, and chemo-immune resistance. The induction of RKIP overexpression abrogates these protumorigenic properties. These contrasting findings raise a therapeutic challenge. If we look for agents that can be administered to cancer patients who can reduce RKIP in NK cells to potentiate their antitumor cytotoxic activity, these same agents will also inhibit RKIP in cancer cells and potentiate tumor growth and, most likely, resistance to NK cytotoxicity. Hence, the regulation of RKIP in NK cells must be done ex vivo before their passive infusion in the patient. In addition, other agents that can induce RKIP expression in cancer cells must exert their anticancer effects and render cancer cells more sensitive to the administered low RKIP expressing NK cells. These approaches are challenging. Both anti- and pro-RKIP regulators may be agents that target various gene products diagrammed in Fig. 9. It will be of interest to determine whether clinical trials will be undertaken to test these hypotheses in cancer patients.

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FIG. 9 Regulation schematic for RKIP and NKG2D. These two schematics (A and B) provide a hypothesized regulation pathway that can contribute to the downregulation of RKIP and subsequent upregulation of NKG2D. Both RKIP and NKG2D are regulated in part by similar effectors including Snail, YY1, and components of the NF-kB pathway.

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13. Regulation of NKG2D by RKIP: Implications on NK-mediated cytotoxicity

Acknowledgments

The authors acknowledge the various authors whose studies were used in the present review. In addition, the authors apologize for any reports that were not referenced unintentionally. In addition, the Department of Microbiology, Immunology & Molecular Genetics and the David Geffen School of Medicine are acknowledged for their support.

References


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References


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13. Regulation of NKG2D by RKIP: Implications on NK-mediated cytotoxicity


[49] Castro J, Odeh H, Figg C, Yeung M, Trumbly R, Yeung KC. Regulation of RKIP expression in breast cancer cells by miRNA. Department of Cancer Biology, College of Medicine, University of Toledo, Health Science Campus, Toledo, Ohio; 2015.


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References


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Benjamin_Bonavida, 978-0-12-824375-6

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Checkpoin inhibition in the fight against cancer: NK cells have some to say in it

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Abstract

Immunotherapy has revolutionized the management of patients with cancer, achieving better clinical results with limited side effects compared with conventional therapies, but in a limited subset of patients. Among these approaches, immune checkpoint blockade (ICB) therapy directly counteracts cancer-associated immunosuppression, disrupting the negative signaling provided by checkpoints and allowing antitumor responses. Despite that the tumor eradication has been classically linked to the activity of CD8\textsuperscript{+} T cells and NK cells, the efficacy of ICB therapy is usually attributed to T lymphocytes. Herein, we discuss the state-of-the-art checkpoint modulators, highlighting their impact on the immune function, and whether NK cells play a relevant part in the clinical response of ICB therapy.

Abbreviations

- A2AR: adenosine A2a receptor
- ADCC: antibody-dependent cellular cytotoxicity
- allo-HSCT: allogenic hematopoietic stem cell transplantation

*These authors contributed equally.
### Checkpoint inhibition in the fight against cancer

<table>
<thead>
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<th>Definition</th>
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<tr>
<td>AML</td>
<td>acute myeloid leukemia</td>
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<tr>
<td>APCs</td>
<td>antigen-presenting cells</td>
</tr>
<tr>
<td>BTLA</td>
<td>B- and T-lymphocyte attenuator</td>
</tr>
<tr>
<td>CEACAM1</td>
<td>carcinoembryonic antigen cell adhesion molecule 1</td>
</tr>
<tr>
<td>CLL</td>
<td>chronic lymphocytic leukemia</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>cytotoxic T lymphocyte-associated antigen 4</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>FDA</td>
<td>US Food and Drug Administration</td>
</tr>
<tr>
<td>FGL1</td>
<td>fibrinogen like 1</td>
</tr>
<tr>
<td>Gal-3/9</td>
<td>galectin-3/9</td>
</tr>
<tr>
<td>GITR</td>
<td>glucocorticoid-induced TNFR-related protein</td>
</tr>
<tr>
<td>GITRL</td>
<td>glucocorticoid-induced TNFR-related protein ligand</td>
</tr>
<tr>
<td>GIST</td>
<td>gastrointestinal stromal tumor</td>
</tr>
<tr>
<td>HCC</td>
<td>hepatocellular carcinoma</td>
</tr>
<tr>
<td>HL</td>
<td>Hodgkin lymphoma</td>
</tr>
<tr>
<td>HLA-I/II</td>
<td>human leukocyte antigen I/II</td>
</tr>
<tr>
<td>HMGB1</td>
<td>high-mobility group protein B1</td>
</tr>
<tr>
<td>HSCT</td>
<td>hematopoietic stem cell transplantation</td>
</tr>
<tr>
<td>HSV gD</td>
<td>herpes simplex glycoprotein D</td>
</tr>
<tr>
<td>HVM</td>
<td>herpesvirus entry mediator</td>
</tr>
<tr>
<td>ICOS</td>
<td>inducible costimulator</td>
</tr>
<tr>
<td>ICOSL</td>
<td>inducible costimulator ligand</td>
</tr>
<tr>
<td>IDO1</td>
<td>indoleamine-2,3-dioxygenase 1</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>interferon-γ</td>
</tr>
<tr>
<td>IL-2/12</td>
<td>interleukin-2/12</td>
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<td>immunoglobulin-like transcript 2</td>
</tr>
<tr>
<td>ITAM</td>
<td>tyrosine-based activating motif</td>
</tr>
<tr>
<td>ITIM</td>
<td>immunoreceptor tyrosine-based inhibition motif</td>
</tr>
<tr>
<td>ITSM</td>
<td>tyrosine-based switch motif</td>
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<tr>
<td>KIR</td>
<td>killer cell Ig-like receptors</td>
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<td>LAG-3</td>
<td>lymphocyte-activation gene 3</td>
</tr>
<tr>
<td>LSECtin</td>
<td>liver sinusoidal endothelial cell lectin</td>
</tr>
<tr>
<td>LT-α</td>
<td>lymphotoxin-α</td>
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<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>MDCS</td>
<td>myeloid-derived suppressor cell</td>
</tr>
<tr>
<td>MGUS</td>
<td>monoclonal gammopathy of undetermined significance</td>
</tr>
<tr>
<td>MM</td>
<td>multiple myeloma</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>NOX</td>
<td>NADPH oxidases</td>
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<td>NSCLC</td>
<td>nonsmall-cell lung cancer</td>
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<td>OX40</td>
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<tr>
<td>OX40L</td>
<td>tumor necrosis factor receptor superfamily member 4 ligand</td>
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<td>programmed cell death 1</td>
</tr>
<tr>
<td>PD-L1/2</td>
<td>programmed cell death ligand 1 and 2</td>
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<td>PVR</td>
<td>poliovirus receptor</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<td>TACTILE</td>
<td>T cell activation, increased late expression</td>
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<td>T-cell acute lymphoid leukemia</td>
</tr>
<tr>
<td>TAM</td>
<td>tumor-associated macrophage</td>
</tr>
<tr>
<td>Th1/2</td>
<td>T helper 1/2</td>
</tr>
<tr>
<td>TIGIT</td>
<td>T-cell immunoreceptor with immunoglobulin and ITIM domains</td>
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V. Targeting NK cells
Cancer still remains one of the leading causes of mortality worldwide and its incidence is increasing year after year [1]. Major efforts are being aimed at developing new therapeutic strategies to fight cancer since most conventional treatments fail to achieve tumor remission or exhibit severe toxicity. Chemotherapy lacks selectivity, whereas targeted agents, despite taking advantage of intrinsic characteristics of the tumor, are commonly associated with acquired resistance at a clinical level. Thus, directly targeting the tumor results in heterogeneous response rates in patients with cancer, prompting the design of therapeutic approaches that rely on activating antitumor immune responses [2, 3]. Higher efficacy and specificity together with its lower side effects are increasing the interest in immunotherapy for the management of patients with cancer.

The immune system is strongly related to tumor establishment and progression and its activity can be cancer promoting or detrimental [4]. The vast majority of nascent tumors are eradicated by effector immune cells, mainly CD8+ T cells and natural killer (NK) cells, that recognize malignantly transformed cells [5]. NK cells are part of the innate branch of the immune system and display a strong cytotoxic function. NK cell activation is antigen-independent and strictly controlled through the integrated signals provided by a collection of activating (e.g., NKG2D, DNAM-1) and inhibitory (e.g., KIRs) surface receptors [6, 7]. Malignant transformation generally leads to the upregulation of ligands for NK cell-activating receptors and, subsequent to receptor engagement, NK cells release cytotoxic granules containing perforin and granzymes (reviewed in Refs. [5, 8]) that ultimately lyse the neoplastic cell. Thus, NK cells take an active part in the immune control of tumorigenesis and metastasis [9–11]. On the other hand, certain immune subsets contribute to tumor growth and progression and are normally recruited to the tumor niche. Proinflammatory cells, such as tumor-associated macrophages (TAMs), directly influence tumor survival, promoting angiogenesis [12], whereas myeloid-derived suppressor cells (MDSCs) or T regulatory (Treg) cells are primarily in charge of impairing antitumor responses [13, 14]. Immunosuppression can be achieved via several mechanisms, but aberrant expression of checkpoints is one of the most studied due to its therapeutic interest. Immune checkpoints are coinhibitory or stimulatory molecules that modulate lymphocyte functions. Malignant cells from a wide variety of cancers as well as tumor-promoting immune cells exhibit high levels of these inhibitory checkpoints or their ligands coupled to decreased levels of stimulatory checkpoints. Constant exposure to negative signaling induces a state of exhaustion in effector immune cells, allowing tumor immune escape. Hence, checkpoints have arisen as attractive targets for
cancer immunotherapy and their blockade aims to revert cancer immunosuppression and reinvigorate antitumor responses. Immune checkpoint blockade (ICB) therapy was initiated with the development of monoclonal antibodies (mAbs) targeting CTLA-4 and PD-1 that have demonstrated their efficacy at a clinical setting and have been approved for the treatment of advanced melanoma [15]. Up to date, a considerable number of inhibitory, but also stimulatory, checkpoints are under investigation to elucidate whether their modulation might help in the fight against cancer (Fig. 1).

Herein, we discuss the relevance of different checkpoints in the cross talk between cancer and the immune system as well as in the treatment of neoplastic malignancies, highlighting how modulation of each signaling pathway affects NK cell function and whether these effector immune cells might play a role in the effectiveness of ICB therapies at a preclinical and clinical level (Table 1).

**Inhibitory checkpoints**

**CTLA-4**

Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) was one of the first checkpoint molecules targeted for cancer immunotherapy [42]. This type I transmembrane protein is generally expressed on CD4+ and CD8+ T cells following activation, but it also appears in Treg cells and certain tumor-infiltrating NK cells. CTLA-4 is an inhibitory receptor with two
<table>
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<td>CD4+ and CD8+ T cells, T reg cells</td>
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<td>CD137/4-1BB</td>
<td>CD137L</td>
<td>CD4+ and CD8+ T cells, T reg cells, NK cells, B cells, monocytes, DCs</td>
<td>Increased IFN-γ production</td>
<td>[40, 41]</td>
</tr>
<tr>
<td>ICOS</td>
<td>ICOSL</td>
<td>CD4+ and CD8+ T cells, T reg cells, NK cells</td>
<td>No direct effect reported</td>
<td>–</td>
</tr>
<tr>
<td>OX40</td>
<td>OX40L</td>
<td>CD4+ and CD8+ T cells, T reg cells, NK cells, NKT cells, neutrophils</td>
<td>No direct effect reported</td>
<td>–</td>
</tr>
<tr>
<td>GITR</td>
<td>GITRL</td>
<td>CD4+ and CD8+ T cells, T reg cells, NK cells, B cells, macrophages</td>
<td>No direct effect reported</td>
<td>–</td>
</tr>
</tbody>
</table>

NK, natural killer; DC, dendritic cell; APC, antigen-presenting cell; MDSC, myeloid-derived suppressor cell; ADCC, antibody-dependent cellular cytotoxicity.
cognate ligands, CD80 (B7–1) and CD86 (B7–2), that can also bind to the costimulatory receptor CD28 with lower affinity [43]. The competition for ligand binding ultimately modulates the degree of cell activation. In cancer, CTLA-4 primarily contributes to tumor immune escape via inhibition of effector responses.

CTLA-4 has been largely studied due to its inhibitory activity on T cells, but little is known regarding its role in NK cell function. CTLA-4 expression was detected on mouse NK cells in response to interleukin (IL)-2 stimulation and receptor engagement led to reduced interferon (IFN)-γ secretion upon coculture with mature dendritic cells (DCs) [44]. Early work demonstrated that CD80+ tumor cells inhibited human NK cell proliferation in vitro, although no CTLA-4 expression was detected on this immune subset [45].

Despite the limited CTLA-4 presence on NK cells, blocking CTLA-4 might indirectly release the immunosuppressive hold on these immune cells, stimulating NK cell antitumor responses. Increased numbers of CTLA-4+ Treg cells correlated with attenuated NK cell cytotoxicity and poor prognosis in patients with head and neck cancer [16]. Treatment with the anti-CTLA-4 mAb ipilimumab led to Treg cell depletion and restored NK cell cytolytic activity ex vivo. In melanoma, ipilimumab prompted selective Treg cell elimination via engagement of CD16 on monocytes [46, 47]. Likewise, ipilimumab treatment triggered NK cell-mediated antibody-dependent cellular cytotoxicity (ADCC) of melanoma cells, together with increased tumor necrosis factor-α (TNF-α) release [18]. CTLA-4 antibodies lacking an Fc portion lose their antitumor properties, unraveling that anti-CTLA-4 therapeutic efficacy distinctly relies on inducing ADCC [48]. In addition, an increase in IL-2-producing effector cells was detected as a result of CTLA-4 blockade in melanoma-bearing mice [49], which might counteract NK cell inhibition in an indirect fashion given the immunostimulatory role of IL-2 [50, 51]. Indeed, NK cell, and CD8+ T lymphocyte, tumor infiltration was reported in melanoma mouse models following treatment with anti-CTLA-4 mAbs and combination with IL-2 decreased NK cell exhaustion [19]. Higher levels of IL-2Rα were detected in NK cells from patients with melanoma treated with ipilimumab [17]. NK cells obtained from patients with good clinical response exhibited superior response rates to IL-2 stimulation and cytotoxicity in vitro compared with nonresponders. Altogether, these data point out that NK cells play a relevant part in the antitumor efficacy of CTLA-4 blockade.

Ipilimumab is the only CTLA-4 blocking antibody approved for the treatment of metastatic melanoma [52]. Nonetheless, the upregulation of other immune checkpoints associated with treatment may be caused for concern. Surface levels of the inhibitory checkpoint V-domain Ig suppressor of T-cell activation (VISTA) were increased as a consequence of ipilimumab administration in patients with prostate cancer, providing a rationale for combination regimens employing different ICBs [53]. In line with this, combination therapy with the anti-PD-1 mAb nivolumab shows greater clinical benefits in advanced nonsmall-cell lung cancer (NSCLC), advanced renal cell carcinoma, advanced melanoma, and colorectal cancer compared with either treatment alone [54–57]. Several clinical trials are currently analyzing the efficacy of CTLA-4 blockade in combination with chemotherapy or as a treatment for refractory cancer.

Employing CTLA-4 blocking agents in the management of patients with cancer stands as an alternative to conventional therapeutic approaches and has proven beneficial so far, especially in high-risk malignancies, such as melanoma. Nonetheless, the impact of combining CTLA-4 blockade with other treatments needs to be fully determined, along with its therapeutic value in other types of cancer.

V. Targeting NK cells
PD-1/PD-L1

Programmed cell death 1 (PD-1) is a monomeric surface receptor that belongs to the immunoglobulin superfamily that binds to programmed cell death ligand 1 and 2 (PD-L1/PD-L2) and negatively regulates immune responses through its immunoreceptor tyrosine-based inhibition motif (ITIM) domain. PD-1 is predominantly expressed on T lymphocytes and is absent or expressed at low levels on NK cells. However, PD-1+ NK cells have been detected in a wide variety of solid tumors and hematological malignancies, including liver cancer, multiple myeloma (MM), and Hodgkin lymphoma (HL) [20, 58–62]. Furthermore, intracellular PD-1 has been detected in the Golgi apparatus of NK cells [63].

PD-1 blocking mAbs have classically thought to target CD8+ T lymphocytes. However, T-cell-mediated responses are antigen-presenting dependent, even in tumors with low HLA-I and -II expression, suggesting that other immune subsets might be involved in the antitumor effect of these treatments [64–66]. To assess whether PD-1 therapy efficacy relies on NK cells, HLA-I-deficient lymphoma murine models were employed; PD-1 expression was observed on NK cells and tumor-derived PD-L1-induced defective NK cell-mediated responses and more aggressive tumors, which was reversed with anti-PD-L1 therapy [67]. In MM, PD-1 expression was detected on NK cells and PD-L1 levels on plasma cells from patients with monoclonal gammapathy of undetermined significance (MGUS) correlated with an increased incidence of progression to symptomatic MM [68, 69]. Treatment with the immunomodulatory drug lenalidomide altered the phenotype of NK cells and tumor cells in vitro and in patients with MM, downregulating the expression of the checkpoint and its ligand [58, 68]. Blockade of PD-1/PD-L1 axis in these patients led to strengthened cytotoxic, migratory, and cytokine production capacity by NK cells [58]. Increased expression of PD-1 on peripheral and tumor-infiltrating NK cells was reported in patients with digestive cancer among other solid tumors, including head and neck and breast cancer; PD-1 blockade led to enhanced NK cell-mediated cytotoxicity [20, 21, 70]. Altogether, these data support that PD-1 blockade may reverse NK cell exhaustion and benefits obtained from this therapy could be, at least in part, due to the recovery of NK cell activity.

TIM-3

T-cell immunoglobulin and mucin-domain-containing protein 3 (TIM-3) belongs to the TIM family, consisting of Type I transmembrane proteins that typically bind to phosphatidylserine (PtdSer) [71]. In particular, TIM-3 also interacts with galectin-9 (Gal-9), high-mobility group protein B1 (HMGB1), and carcinoembryonic antigen cell adhesion molecule 1 (CEACAM1) [72]. The effect of each interaction varies depending on the cell type and settings, underscoring the complex role of this receptor in the regulation of the immune response. TIM-3 was initially identified as a surface molecule present on CD4+ T cells that suppressed effector Th1 responses [73]. Nonetheless, TIM-3 expression is not only limited to T cells, as it appears in innate immune subsets as well, including macrophages, DCs and NK cells, among others [74].

Although classically considered an inhibitory checkpoint similar to CTLA-4 or PD-1, several reports described an immune stimulatory role for TIM-3. For instance, Gal-9 administration led to enhanced granzyme B and perforin expressions by CD8+ T cells from sarcoma-bearing mice, which correlated with increased survival in this model [75].

V. Targeting NK cells
Likewise, TIM-3 is upregulated on the surface of activated NK cells and its interaction with Gal-9-expressing targets promoted IFN-γ production [76]. Despite the positive impact, it may exert on the immune function, TIM-3 mostly acts as a negative regulator of immunity. TIM-3 is essentially expressed on mature NK cells, in which it has been demonstrated to restrain NK cell cytotoxicity upon target cell encounter, therefore behaving as an inhibitory receptor that limits NK cell activity [74]. Consequently, this checkpoint has arisen as a marker of poor prognosis in patients with cancer and its expression is generally associated with immune cell exhaustion. NK cells with elevated TIM-3 levels, characterized by a functionally defective phenotype, have been detected in a variety of tumor types, such as gastric cancer, advanced melanoma, lung adenocarcinoma, gastrointestinal stromal tumor (GIST), glioma, or acute myeloid leukemia (AML) [22, 23, 77–80]. TIM-3 blockade translated into improved NK cell killing activity and cytokine production, together with increased CD107a expression, which correlated with attenuated tumor growth of hepatocellular carcinoma (HCC)-bearing T-cell-deficient nude mice [24]. In line with this, ex vivo treatment with anti-TIM-3 mAbs restored the cytotoxic capacity and IFN-γ production by NK cells from patients with advanced melanoma and lung adenocarcinoma [22, 23, 25]. Interestingly, the combination of TIM-3 and PD-1 blockade achieved higher NK cell-mediated cytotoxicity rates than individual treatments upon coculture with anaplastic thyroid cancer cells [26]. Coblockade of these two pathways rendered better results in preclinical models of certain cancers, arresting tumor growth in a superior fashion that PD-1 blockade alone and providing the rationale for the combination of these ICBs at a clinical level [81–84]. The encouraging data obtained in preclinical settings employing patient-derived samples as well as tumor models prompt the development of an array of TIM-3 blocking mAbs, most of which are currently undergoing clinical trials as monotherapy or in combination with PD-1 blocking agents (e.g., Sym023 [NCT02817633], MGB453 [NCT02608268], TSR-022 [NCT03311412], LY3321367 [NCT03099109]). Up to date, several of these antibodies have successfully finished phase I clinical trials and their administration is safe and well tolerated [85, 86]. Further, the combination of TSR-022 with the PD-1 blocking antibody TSR-042 achieved objective responses in patients with NSCLC and melanoma that were refractory to anti-PD-1 therapy [87].

The detrimental role of TIM-3 in antitumor immunity has turned this checkpoint into an interesting target for cancer immunotherapy. Even though clinical data concerning the efficacy of TIM-3 blockade still remain scarce, the promising results obtained from preclinical studies support the idea that employing these agents might provide a therapeutic advantage in cancer treatment, especially in combination with other ICBs.

**KIRs**

The killer cell Ig-like receptors (KIR/CD158) family is composed of a repertory of cell surface receptors harboring intracellular tyrosine-based activating motifs (ITAMs) or ITIMs. These receptors recognize and bind to canonical HLA class I molecules (HLA-A, HLA—B, HLA—C, and HLA-G).

Lirilumab is a pan-KIR2D blocking mAb for inhibitory KIRs (KIR2DL/DS-1, –2, and –3), which prevents KIR/HLA-C interaction. Altered expression of HLA-I molecules is a
recurrent feature in tumor cells leading to increased immune evasion by negatively regulating NK cells, among others. It has been described that mismatched adoptive NK cell transfer and allogeneic hematopoietic stem cell transplantation (allo-HSCT) lead to improved antitumor responses due to impaired recognition of HLA-I molecules by NK cells, reducing the risk of relapse in patients with MM, chronic lymphocytic leukemia (CLL), and AML [88–92]. In this context, using KIR-blocking antibodies might mimic the missing-self scenario, stimulating NK cell-mediated responses.

In vitro and ex vivo results show that lirilumab alone or in combination with lenalidomide, daratumumab, or rituximab increased the cytotoxic capacity of NK cells in samples from patients with MM or B-cell lymphoma mouse models [27–29]. Although data obtained in preclinical and phase I clinical trials were promising [93, 94], lirilumab as monotherapy failed to show effectiveness in patients with AML and MM, probably due to treatment-induced downregulation of KIRD2 expression by trogocytosis and lack of responsiveness of NK cells [95–97]. Future studies should be focused on establishing the combinatorial potential of lirilumab with other treatments such as immunomodulatory drugs, chemotherapy, or mAbs.

NKG2A

CD94/NKG2A heterodimer is a type II transmembrane receptor that belongs to the C-type lectin family containing a single intracellular ITIM domain that recognizes HLA-E, a nonclassical HLA-I molecule [98–101]. NKG2A participates in NK cell-mediated self-tolerance upon binding to HLA-E, which in turn has been related to tumor evasion in a wide variety of solid tumors and hematological malignancies, such as MM [30, 102–106]. NKG2A dampens NK cell cytotoxicity through binding to its ligand, and blockade of this interaction with a mAb has tipped the balance to favor stimulation of NK cell-mediated antitumor responses.

Monalizumab is a humanized IgG4 antibody that abrogates NKG2A inhibitory function by impeding its interaction with HLA-E. This mAb has already shown promising results in head and neck squamous cell carcinoma (in combination with cetuximab) and gynecological cancers in phase II clinical trials [107, 108]. In preclinical models of CLL, treatment with monalizumab in vitro enhanced NK cell direct killing of HLA-E-expressing target cells [30]. Likewise, a study using mouse models of different hematological malignancies [including CLL, T-cell acute lymphoid leukemia (T-ALL), AML, lymphoma, and MM] demonstrated the antileukemic capacity of anti-NKG2A antibodies [31].

HSCT still remains the only curative treatment option for many patients with hematological malignancies. Interestingly, the vast majority of reconstituted NK cells express NKG2A after allo-HSCT [109]. Since most of the malignant cells express HLA-E, NKG2A inhibitory signaling gains importance in NK cell function impairment. Moreover, increased expression of HLA-E has been observed after HSCT induced by IFN-γ-producing NK cells, promoting tumor escape [104]. In contrast to the KIR/CD158 family, and due to the limited polymorphism of HLA-E, mismatched allo-HSCT is not usually achieved. Therefore, the use of monalizumab after matched or mismatched HSCT may enhance the population of NK cells that exert antitumor effector responses [31, 106, 110]. Thereby, clinical trials using monalizumab in monotherapy or in combination with other therapeutic options are currently ongoing in different hematological malignancies and solid tumors [108].

V. Targeting NK cells
BTLA

B- and T-lymphocyte attenuator (BTLA/CD272) was first identified as a negative checkpoint regulator structurally related to CTLA-4 and PD-1, and BTLA-deficient murine models show enhanced T lymphocyte-mediated responses, including proliferation and cytokine production [111]. This checkpoint protein is mainly expressed on B cells, T lymphocytes, antigen-presenting cells (APCs), and, at low levels, on NK cells [112, 113]. Structurally, BTLA includes a unique extracellular domain, a transmembrane region, and a cytoplasmic tail with ITIM and immunoreceptor tyrosine-based switch motif (ITSM) domains. Upon binding to its ligand, BTLA recruits SHP-1 and SHP-2, leading to negative signaling on T cells [114].

On one hand, Herpesvirus entry mediator (HVEM/CD270), also known as TNFRSF14, represents the sole binding partner described for BTLA to date, as well as the first known case of cross talk between two superfamilies; while BTLA is a member of the immunoglobulins superfamily, HVEM belongs to the tumor necrosis factor (TNF/TNFR) superfamily [115]. On the other hand, HVEM was identified as a receptor for both, coinhibitory and costimulatory molecules, including CD160, LIGHT, lymphotoxin-α (LT-α), and herpes simplex glycoprotein D (HSV gD), displaying dual functions depending on the ligand: while HVEM engagement to BTLA and CD160 leads to T-cell inhibition, its binding to LIGHT induces T-cell stimulation [116]. Moreover, HVEM-mediated signaling results bidirectional, since BTLA and CD160 may directly activate the HVEM-dependent NF-κB pathway based on their cis or trans interactions with HVEM, leading to the formation of a complex signaling network [117].

In the context of cancer, the BTLA/HVEM axis is dysregulated in a vast number of solid tumors and hematological malignancies [118–124]. High expression of BTLA has been related to poor prognosis in NSCLC, gastric cancer, and HCC [120, 121, 125]. Furthermore, increased plasma levels of soluble BTLA and HVEM have been associated with aggressiveness, risk of disease progression, and tumor survival in different solid tumors, such as prostate cancer, pancreatic adenocarcinoma, clear cell renal cell cancer, and pancreatic cancer, among others [126–129].

Despite the increasing interest in interrupting BTLA/HVEM engagement as a potential therapeutic target in cancer, only one anti-BTLA mAb has been approved for clinical trials by FDA to date (TAB004/JS004, Junshi Biosciences). During allo-HSCT, BTLA blockade has been demonstrated to increase proliferation, cytokine production, and cytotoxicity of alloreactive T cells ex vivo [112]. Moreover, BTLA expressed on γδ T cell may play a role in lymphomagenesis by inducing decreased proliferation of this immune subset, thus promoting tumor escape [130]. In a recent study by Chen et al., BTLA blockade in monotherapy or in combination with chemotherapy demonstrated increased survival in murine models of epithelial ovarian carcinoma [124]. In melanoma, BTLA is highly expressed on tumor-specific CD8+ T lymphocytes and treatment with an anti-BTLA blocking mAb resulted in enhanced proliferative capability and cytokine production in vivo and in vitro [131, 132]. Interestingly, BTLA+ CD8+ tumor-infiltrating T lymphocytes showed improved IL-2 production and survival when compared with BTLA-counterpart in melanoma, suggesting that the complex signaling network composed by HVEM and its ligands needs to be fully elucidated [133–135].

To date, scarce data about the role of this checkpoint in NK cells are available, since most of the studies have focused on T lymphocytes. Nevertheless, given that BTLA blockade increases proliferation and cytokine production on T cells, the possibility that NK cells may directly or indirectly benefit from this treatment deserves further investigation.

V. Targeting NK cells
LAG-3

Lymphocyte-activation gene 3 (LAG-3) is an immune checkpoint receptor that belongs to the immunoglobulin superfamily with approximately 20% amino acid homology with CD4 that is expressed on activated and exhausted T lymphocytes and activated NK cells. LAG-3 is also expressed on B cells, DCs, and Treg cells [136–139]. Upon engagement with HLA-II molecules with higher affinity than CD4, LAG-3 negatively regulates T cells and NK cells through its KIEELE motif, thereby contributing to tumor escape, although the exact signaling pathway has not been fully elucidated [139–144]. HLA-II was the first ligand described for LAG-3, but three new ligands have been discovered to date, including galectin-3 (Gal-3), liver sinusoidal endothelial cell lectin (LSECtin), and, more recently, a liver-secreted protein known as fibrinogen like 1 (FGL1) [145–147].

LAG-3 expression has been observed to be dysregulated and related to prognosis in a wide variety of cancers, including those of hematological origin, such as CLL or AML, and solid tumors [148–157]. LAG-3 blockade has widely been studied on T cells, but little is known about its role in NK cells. LAG-3 impairs T-cell proliferation, cytokine production, and cytolytic function while enhancing Treg cell-mediated responses [138, 158–161]. This immune checkpoint is usually coexpressed with PD-1, giving a rationale for combinatorial blockade as a promising therapy in cancer [155, 162–165]. Indeed, dual LAG-3/PD-1 blockade showed encouraging results in vitro and in vivo in squamous cell carcinomas, CLL and ovarian cancer, among others [165–167]. There are several clinical trials currently ongoing, being relatlimab (BMS-986016) monotherapy or in combination with anti-PD-1 mAbs the most advanced to date [163]. In addition, bi-specific mAbs targeting LAG-3 and PD-1 have been developed owing to increased antitumor activity over monotherapy or the combination of LAG-3 and PD-1 blockade [163, 165, 168, 169].

Even though LAG-3 mediates exhaustion in NK cells and T lymphocytes, the role of this checkpoint in NK cells remains elusive, since LAG-3 blockade failed to increase NK cell-mediated cytotoxicity [170, 171]. Nonetheless, murine models of breast cancer demonstrated the loss of NK cell anti-metastatic activity due to increase expression of inhibitory checkpoints, such as LAG-3, during IL-12 treatment. Monotherapy with anti-LAG3 mAb failed to show any benefit, whereas its combination with IL-12 synergistically restored NK cell-mediated antitumor function that fully relied on NK cells [32]. Interestingly, treatment with IMP321, a soluble form of LAG-3 protein, led to increased activation and IFN-γ production by CD8+ T lymphocytes and NK cells in patients with advanced renal carcinoma [33, 34]. Altogether, these data highlight that the impact of LAG-3 on NK cell functions merits further investigation.

ILT2

Immunoglobulin-like transcript 2 (ILT2/CD85j/LILRB1) is a well-known inhibitory checkpoint for NK cells. Withal, ILT2 receptor is extensively expressed in different immune subsets, including B cells, T lymphocytes, monocytes, DCs, and MDSCs [172–178]. ILT2 ligands include classical HLA-I molecules, exhibiting higher affinity for HLA-G, which promotes negative signaling and immunosuppression upon engagement [175, 176]. This inhibitory activity depends on a cytoplasmic tail with four ITIMs that recruits SHP-1, SHP-2, or Src homology 2 domain-containing inositol phosphatase (SHIP) [175, 179].
Increased ILT2 expression has been described in patients with solid tumors and hematological malignancies [175, 179–184]. As stated above, HLA-G represents the main ligand for ILT2, and it is aberrantly expressed in several tumors. This molecule overexpression has been related to prognosis, tumor progression, and metastasis in solid tumors due to, at least in part, NK cell suppression [185–188]. Moreover, increased levels of soluble HLA-G in plasma have been detected in a broad variety of solid tumors, which leads to immunosuppression through ILT2 binding [189–194]. Contrastingly, the role of HLA-G and ILT2 in hematological malignancies remains controversial. Despite the abovementioned role of HLA-G in solid tumors, ILT2 expression on neoplastic cells may reduce proliferation in B-cell lymphomas; ILT2/HLA-G binding disruption with specific mAbs or siRNA in vitro in MM, B-cell lymphoma, and leukemia cell lines was able to restore tumor cell proliferation [195, 196]. Interestingly, our group did not detect increased proliferative capacity on leukemic cells upon ILT2 blockade ex vivo in patients with CLL, suggesting that the HLA-G/ILT2 axis role in hematological malignancies deserves further investigation [35, 36].

ILT2/HLA-G signaling dampens NK cell cytokine production (such as IFN-γ), proliferation, and cytotoxicity [197, 198]. Specifically, our group demonstrated ex vivo that ILT2 blockade alone or in combination with lenalidomide enhances NK cell-mediated responses and malignant B-cell depletion, thus promoting IFN-γ production and target cell killing in patients with CLL [35, 36]. Furthermore, a recent study published by Chen et al. evidenced that ILT2 blocking mAbs enhance NK cell-mediated cytotoxicity against cancer cells in vitro and in murine models of hematological malignancies and solid tumors [37]. Since NK cells play a key role in ADCC, cetuximab treatment was evaluated in vitro according to ILT2 expression levels in breast cancer; ILT2 increased expression on NK cells correlated with cetuximab inefficacy, providing the rationale for the combination of checkpoint blockade along with ADCC-promoting treatments in cancer [181]. To date, information regarding ILT2 blockade efficacy has been obtained from preclinical models. Nonetheless, Biond Biologics recently announced the first clinical trial in HLA-G-expressing tumors with BND-22 (a humanized IgG4 ILT2-blocking mAb) at the Annual Meeting of the American Association for Cancer Research 2020 (AACR) [199].

TIGIT

T-cell immunoreceptor with immunoglobulin and ITIM domains (TIGIT) is an immunoglobulin superfamily receptor present on the surface of NK cells and T lymphocytes and classified as an immune inhibitory checkpoint [200–202]. Decrease of IFN-γ production or loss of the cytotoxic potential is some of the main features associated with TIGIT stimulation on NK cells [202–205]. Poliovirus receptor (PVR, CD155) stands as its primary ligand, showing a high affinity for TIGIT binding [202], although the PVR-related proteins Nectin-2 (CD112) and Nectin-3 (CD113) can interact with TIGIT as well [202, 206]. Despite the stimulatory role of the interaction between DNAM-1 and PVR, TIGIT frequently wins the competition for PVR binding in the context of cancer, tipping the balance toward NK cell inhibition [207]. Further, in vitro assays unraveled that TIGIT blocks DNAM-1 dimerization, which might prevent NK cell stimulation through this activating receptor [208]. High TIGIT expression was detected on infiltrating NK cells from distinct tumor mouse models, which correlated with in vivo NK cell dysfunction [38]. Concomitantly, even though PVR is hardly expressed in...
healthy tissues—it mainly appears in endothelial cells [209]—aberrant levels of this ligand have been detected in a myriad of human malignancies [210–214]. Taken together, these data bring to light that TIGIT primarily contributes to tumor immune evasion by negatively regulating NK cell responses via PVR engagement.

NK cell-specific TIGIT deletion led to prolonged survival of B16 melanoma-injected mice and correlated with a higher proportion of DNAM-1-expressing NK cells [38]. Likewise, TIGIT blockade resulted in tumor growth arrest, even in the absence of the T-cell compartment, and increased the expression of NK cell activation markers, including CD107a or IFN-γ, in different tumor-bearing mouse models, thus supporting the connection between TIGIT expression and NK cell exhaustion in the tumor microenvironment [38]. Interestingly, TIGIT inhibition exerts synergistic effects in combination with other immunotherapeutic approaches. For instance, TIGIT blockade sensitizes trastuzumab-treated breast cancer cells to NK cell-mediated ADCC [39]. Coblockade of TIGIT with other checkpoints shows promising results as well. Combination with anti-PD-1 antibodies enhanced antitumor immunity in glioblastoma [215], whereas anti-TIGIT plus anti-PD-L1 strengthened T-cell responses in an NK cell-dependent manner [38]. TIGIT blockade, alone or in combination with other ICIs, has also proven effective in restoring T-cell immunity [216, 217]. Given the encouraging preclinical data, an array of mAbs targeting TIGIT is currently under clinical trials with no results generated to date. These studies are evaluating the efficacy of anti-TIGIT treatment in solid cancers as monotherapy (e.g., AB154 [NCT03628677], MK-7684 [NCT02964013], or BMS-986207 [NCT02913313]) as well as in combination with anti-PD-L1 or anti-PD-1 agents (e.g., Tiragolumab [NCT03563716] or BMS-986207 [NCT02913313]) [218].

TIGIT has recently arisen as an attractive target in cancer immunotherapy and its blockade might become a novel weapon in the fight against cancer, as suggested by the evidence collected in preclinical studies. Still, clinical data regarding the available agents targeting TIGIT are required to properly assess the therapeutic potential of this checkpoint in cancer.

**CD96/tactile**

CD96/Tactile (T-cell activation, increased late expression) belongs to the immunoglobulin superfamily and it is thought to negatively regulate T lymphocyte and NK cell activities through its ITIM domains [202]. Along with TIGIT, CD96 competes with DNAM-1 (CD226), a costimulatory receptor highly expressed on T cells and NK cells, in binding to PVR and Nectin-2 [219–221]. Upregulation of PVR, a glycoprotein that also belongs to the Ig superfamily and member of the Nectins/Nectin-like family, has been observed in diverse tumors, whereas this upregulation has been related to increased proliferation, survival, invasion, and migration capabilities of cancer cells [222]. CD112 expression has also been reported to be deeply dysregulated in various types of cancer, including solid tumors and those from the hematological origin [213, 221, 223–228].

The role of CD96 on NK cell-antitumor responses remains unclear. Even though results obtained in murine models revealed diminished IFN-γ production consequent to CD96/PVR binding, CD96−/− mice did not show augmented NK cell cytotoxicity [229]. In line with that, CD96-deficient mice and wild-type mice treated with anti-CD96 blocking mAb showed increased IFN-γ production. This increment played a protective role against metastasis since...
anti-IFN-γ mAb administration abrogated this protective effect. Moreover, DNAM-1 blockade also abolished the antitumor effect of CD96 blocking mAb, highlighting the importance of CD96/DNAM-I competition for PVR binding. However, no effect on NK cell-related cytotoxic activity was observed [229, 230]. Accordingly, data obtained in humans unraveled that CD96 blockade failed to boost NK cell-mediated killing against multiple targets, including MM and ovarian carcinoma cell lines [231, 232]. Further, the CD96/PVR axis promotes NK cell adhesion to target cells and triggers NK cell cytotoxicity in humans [220]. These contrasting results may be explained by the CD96 Tyr-X-X-Met activating motif contained on its cytoplasmic tail in humans, but not in mice. Considering that CD96 contains both, activating and inhibitory motifs, more studies are required in order to elucidate whether this checkpoint may be a potential target for immunotherapy [233].

### Activating checkpoints

#### CD137/4-1BB

Tumor necrosis factor receptor superfamily 9 (CD137/4-1BB) is an inducible costimulatory receptor mainly expressed on activated T lymphocytes and NK cells [234]. Thus, CD137 binding to its ligand (CD137L) enhances immune responses, leading to increased cytokine production and upregulation of antiapoptotic molecules in CD8+ T cells, as well as, strengthened NK cells cytotoxic capacity [235–237]. In vitro analysis revealed increased expression of CD137 and its ligand in non-Hodgkin lymphomas (diffuse large B-cell lymphoma, follicular lymphoma, and mantle cell lymphoma) and prognosis value in patients with AML [40, 238]. In vitro and in vivo analyses brought to light that treatment with CD137 agonist mAb increased NK cell activation and IFN-γ production, as well as delayed tumor growth in a mouse model of MM (in combination with daratumumab) and B-cell lymphomas upon treatment [40, 41].

Two agonist mAbs, urelumab (human IgG4 mAb) and utomilumab (humanized IgG2), are currently being tested in different clinical trials in hematological malignancies. The main differences between these mAbs lie in the immunoglobulin subclass, agonistic activity, ligand-blocking capacity, and safety profile. Urelumab activates CD137 signaling without impeding CD137L binding, while utomilumab recognizes a different epitope, acting as an agonist and blocking mAb. However, utomilumab has demonstrated milder agonistic activity than urelumab in vitro and in vivo [239]. Nonetheless, safety studies in patients with advanced solid cancers revealed that treatment with urelumab at doses ≥1 mg/kg causes severe liver damage, while utomilumab therapy showed a well-tolerated safety profile at doses of 10 mg/kg [240, 241].

Despite clinical development was halted due to transaminitis induced by urelumab observed during phase I/II, combinational therapy is currently ongoing in different clinical trials. Since CD137 expression is upregulated upon ligation of FcγRIII with the Fc region of immunoglobulins, there has been increasing interest in the combination of CD137 agonist mAbs and ADCC-dependent antibodies such as rituximab [242]. The combination of anti-CD137 and anti-CD20 antibodies resulted in a synergistic therapy, inducing tumor regression and prolonging survival in a mouse model of B-cell lymphoma [243]. Currently, there are

V. Targeting NK cells
different clinical trials exploring the effect of urelumab and utomilumab with already approved tumor-targeted mAbs in diverse hematological malignancies.

**ICOS**

Inducible costimulator (ICOS) is similar in structure and function to CD28. Engagement to its only known ligand, ICOS ligand (ICOSL, CD275, B7-H2), triggers CD8+ T-cell activation, contributing to tumor control and elimination [244]. Nonetheless, Treg cells also benefit from the stimulatory signaling provided by ICOS/ICOSL pathway, which might be detrimental for cancer immunosurveillance [245–247]. Apart from its role in T cells, ICOS stimulates NK cell function as well. ICOS-deficient mice displayed abnormal NK cell maturation and homeostasis, which led to defective NK cell effector responses [248]. Further, ICOS engagement resulted in enhanced NK cell cytotoxicity and IFN-γ production coupled to reduced tumor growth in mice injected with ICOSL-expressing tumor cells [249]. Thus, ICOS/ICOSL signaling modulates the immune system at different levels in the context of cancer.

Given its wide activity on immune cells, ICOS modulation appears as an interesting strategy in cancer immunotherapy. ICOS/ICOSL pathway can be subjected to dual regulation, using either antagonist or agonist agents. Several works studying the potential of anti-ICOS antagonist mAbs reported a decrease in tumor-infiltrating Treg cell numbers, which might indirectly promote NK cell antitumor responses [250, 251]. Conversely, treatment with ICOS agonists synergized with CTLA-4 blockade in tumor mouse models suggests that a combination of these treatments might prove beneficial at a clinical level [252, 253]. Preclinical studies have also demonstrated the antitumor effectiveness of ICOS agonists alone [254]. As a result of the encouraging preclinical data generated, three anti-ICOS agonist mAbs are currently under phase I/II clinical trials in advanced solid tumors (JTX-2011 [NCT02904226], GSK33359609 [NCT02723955], and BMS-986226 [NCT03251924]). ICOS engagement is being assessed alone or in combination with anti-PD-1 or anti-CTLA-4 blocking mAbs. The safety of their administration has been determined in two of these agents [255, 256]. Nonetheless, data regarding clinical and side effects have not been reported yet.

Thanks to its role as an antitumor immunity stimulator, ICOS arises as a new target in cancer immunotherapy, especially in combination with agents blocking coinhibitory molecules, such as CTLA-4. Still, the dual role of this molecule in the immune system might entail clinical disadvantages in the long term that remains to be evaluated.

**OX40**

Tumor necrosis factor receptor superfamily member 4 (TNFRSF4, OX40) works similar to ICOS, costimulating T-cell responses. This type I transmembrane protein belongs to the TNFR family and has one cognate ligand, OX40 ligand (OX40L, CD252). OX40 is upregulated following TCR ligation in T cells, including Treg cells, and it is also transiently expressed on neutrophils, NK cells, and NKT cells [257–259]. Even though OX40 is not likely to play a central role in NK cells, several studies have brought to light its costimulatory activity promoting cytokine secretion or proliferation in this immune subset [260, 261]. Coculture of OX40L-expressing DCs triggered IFN-γ production by NK cells and subsequent activation of
CD8+ T cells in melanoma-bearing mice, ultimately leading to tumor progression [259]. Further, OX40 cross-linking upregulated CD107a surface expression on human NK cells [262], suggesting that the OX40/OX40L pathway might strengthen NK cell tumor recognition and elimination. OX40L also has some say in NK cell activity. NKG2D engagement induced OX40L expression in IL-2-stimulated NK cells [263], which has been linked to enhanced cytokine production and cytotoxicity [264]. Indeed, disruption of OX40L signaling abrogated NK cell-mediated lysis of primary AML cells [264]. An early study reported cross talk between NK cells and CD4+ T cells based on OX40L-OX40 interaction that induces IFN-γ production by CD4+ T cells [263]. Altogether, these data bring to light that NK cells bearing OX40 or OX40L distinctly take part in antitumor immune responses either directly or via interaction with adaptive immune cells.

The therapeutic potential of stimulating OX40/OX40L signaling has largely been under investigation at a preclinical and clinical level. Noticeably, a great number of studies described tumor growth attenuation or even regression consequent to treatment with OX40 agonists in murine models of a wide range of primary cancers, such as colorectal carcinoma, melanoma, breast cancer, or sarcoma [265–268]. Compared with an OX40 agonist mAb, administration of an OX40L-Fc fusion protein accomplished complete remission in colorectal and renal cell carcinoma in vivo models, arising as a promising alternative for OX40 modulation [269]. CD8+ and CD4+ T-cell expansion was thought to be the main cause for the antitumor effect of these therapies [267, 270–272]. Nevertheless, OX40 agonist agents also bring about Treg cell depletion [273–276]. Impairment of Treg cell activity partially counteracts the immunosuppression exerted on effector immune populations, like NK cells, hence allowing antitumor responses. A phase I clinical trial evaluating an anti-OX40 agonist mAb (9B12) reported partial regression in patients with metastatic solid tumors with acceptable safety profiles, further supporting the use of OX40 agonists in the management of patients with cancer [277]. Still, despite the promising results highlighted so far, OX40 therapy did not achieve such impressive results in poorly immunogenic tumors, which tipped the balance toward combination strategies with a wide variety of available and novel anticancer agents in an effort to improve the antitumor activity [268, 278]. For instance, cotreatment with anti-CD20 and anti-OX40 mAbs produced tumor rejection in a synergistic fashion in B-cell lymphoma-bearing mice, an effect that was abrogated in the absence of NK cells [262]. Combination with PD-1 blockade resulted in increased survival in ovarian cancer models [279]. Similarly, diverse studies demonstrated synergistic antitumor responses after the combination of OX40 agonists with treatments targeting other checkpoints [280, 281], chemotherapy [282], radiotherapy [283], or even vaccination [284]. More complex combinations are being assessed in preclinical studies as well, such as the sum of a STING agonist, PD-L1 blockade, and OX40 activation that proved effective in tumor eradication through overcoming immune tolerance [285]. Clinical trials evaluating the anticancer potential of strategies based on OX40 stimulation alone or in combination are being conducted (e.g., BMS986178 [NCT03410901], MED10562 [NCT03336606], MOXR0916 [NCT02410512]), although no data concerning efficacy have been published yet.

Taking into consideration the disparate preclinical data available, the therapeutic advantages of employing OX40 agonists as monotherapy remains unclear. Still, the inclusion of these anticancer agents as adjuvants in established treatment regimens might prove useful in the fight against cancer.

V. Targeting NK cells
Glucocorticoid-induced TNFR-related protein (GITR) is a member of the TNFR family that exclusively binds to the GITR ligand (GITRL). This receptor is highly and constitutively expressed on Treg cells [286, 287] and can be induced in CD4+ and CD8+ T cells via TCR engagement [288]. Other immune cells, including NK cells, B cells, or macrophages, display GITR on their membrane as well. The GITR/GITRL axis exerts a complex role in the immune system and acts as a double-edged sword in cancer immunosurveillance. Stimulation of Treg cells via GITR blocks their suppressive function, breaking immune tolerance [286, 287], whereas its ligation leads to enhances proliferation and survival in effector T cells and mediates resistance to Treg cell inhibition [289–292]. Interaction with GITRL+ DCs boosted NK cell cytotoxicity and IFN-γ production in vitro [293]. However, GITR predominantly hampers NK cell activity, limiting proliferation and enhancing apoptosis [294]. In fact, GITR expression is a widespread strategy employed by tumor cells to evade NK cell surveillance, since signaling via the GITR/GITRL pathway impairs NK cell-mediated lysis, as found in CLL, AML, and gastrointestinal cancers [295–297]. Release of TGF-β as a consequence of tumor-derived GITRL ligation was detected in a battery of cancer cell lines in vitro [296], which might further repress NK cells [298]. The presence of soluble GITRL has been detected in patients with various malignancies, which correlated with NK cell dysfunction [297, 299]. Interestingly, an early study described an alternative mechanism acquired by tumor cells lacking GITRL, called pseudoexpression, that consists of coating themselves with GITRL+ platelets, thus avoiding NK cell-mediated killing [300]. Altogether, these data bring to light that GITR signaling is primarily detrimental for NK cell antitumor responses. These preliminary studies prompted the development of agents that modulate the GITR/GITRL pathway. GITR agonists exhibited antitumor activity in melanoma and sarcoma murine models that were associated with a decrease in Treg cell accumulation at the tumor site coupled to increased effector T-cell responses [301, 302]. Similar works reported diminished tumor growth in colon carcinoma and B-cell lymphoma in vivo following treatment with anti-GITR agonist mAbs alone or in combination with CpG vaccination, respectively [303, 304]. Combination with other therapies, such as anti-PD-1 mAbs [305, 306], anti-CTLA-4 mAbs [307], or vaccination [308–310], achieved positive results in preclinical investigations as well. Still, it is yet to be determined whether these antitumor strategies based on GITR ligation overcome the inhibitory effect this checkpoint exerts on NK cells.

At a clinical level, the efficacy of therapies targeting the GITR/GITRL axis is controversial. TRX518, an anti-GITR agonist mAb, was the first to enter clinical development, with limited effectiveness reported in advanced solid tumors at a phase I trial [311]. This study provided the rationale for its combination with PD-1 blockade to overcome resistance, though cotreatment with drugs promoting NK cell activation might prove advantageous due to the negative impact of GITR engagement on this immune subset. Likewise, administration of the agonist mAb BMS-986156 as monotherapy rendered no clinical response in a phase I/IIa clinical trial [312], while treatment with the agonist fusion protein MEDI1873 barely led to stable disease [313], raising the question of whether the acquisition of GITR resistance might be related to the suppression of NK cell antitumor activity. Up to date, an array of anti-GITR mAbs are undergoing clinical trials (e.g., INCAGN01876 [NCT03126110], GWN323 [NCT02740270], or ASP1951 [NCT03799003]), although their anticancer potential stays unclear.
The dual role of GITR in the immune system turns this checkpoint into a questionable target for cancer immunotherapy. Indeed, the opposing effects of GITR in antitumor effector immune subsets might account for the lack of response observed in clinical studies. Further research exploring new therapeutic combinations is needed in order to properly assess the interest of GITR in cancer.

Apart from the classical checkpoints, interest has arisen for a group of molecules that act as immune regulators, even though they take part in disparate pathways including metabolism or ROS production.

Indoleamine-2,3-dioxygenase 1 (IDO1) is a cytosolic enzyme that catalyzes the first step of the catabolism of tryptophan, ultimately rendering metabolites such as kynurenine or quinolinic acid. This enzyme is widely expressed in cancer—by tumor cells and tumor-promoting cells—and has been associated to immune evasion [314–320]. IDO1-expressing cells tamper with the immune function at different levels by controlling tryptophan availability or generating certain immunosuppressive metabolites. Tryptophan depletion negatively impacts T-cell proliferation [321–323], whereas accumulation of tryptophan metabolites essentially leads to Treg cell expansion and effector cell function inhibition [324–327]. For instance, coculture of NK cells with melanoma cells resulted in increased IDO1 levels in the latter, which, in turn, downregulated the expression of the NK cell-stimulating receptors NKp30, NKp40, and NKG2D, crucial for tumor recognition and activation [328, 329]. IDO1 knockdown in ovarian cells strengthened NK cell-mediated killing in vitro and enhanced NK cell tumor infiltration in vivo [330]. Similarly, IDO1 inhibition in a battery of tumor cell lines promoted NK and T-cell growth and reversed Treg cell suppression [331]. In light of these results, IDO1 emerges as an attractive target for cancer immunotherapy and several IDO1-inhibiting drugs, including natural compounds, have been developed and are under clinical investigation (e.g., BMS-986205 [NCT03792750], epacadostat [NCT03493945], or indoximod [NCT04049669]) [332, 333]. Results reported so far indicate limited antitumor activity as monotherapy for the inhibitors indoximod [334] and epacadostat [335], although combination strategies worked better in phase I trials [336, 337]. Nonetheless, termination of numerous trials due to the disappointing therapeutic effects observed with IDO1 inhibitors casts serious doubts regarding the potential of these treatments in cancer [338].

The adenosine pathway stands out as an immune regulator and engagement of its receptors, mainly adenosine A2a receptor (A2AR), brings about suppression and exhaustion in immune cells. NK cell activity and survival are largely hindered by adenosine signaling. Various studies described that A2AR interaction with adenosine restricted NK cell maturation, proliferation, cytokine secretion, and even cytotoxicity [339–344]. Cancer cells have been reported to release adenosine to the tumor microenvironment, which is largely promoted by enhanced activity of the ectonucleosidases CD39 and CD73, albeit other enzymes with ecto-activity collaborate in this task [345–351]. Tumor-promoting cells, such as stromal cells, TAMs or MDSCs, show CD73 and CD39 overexpression as well, further contributing to immune tolerance [352–355]. As a consequence, both ectonucleosidases together with A2AR are...
considered immune checkpoints and have been subjected to modulation as an anticancer strategy. Indeed, inhibition of CD39 arrests tumor growth and mitigates the immunosuppressive hold on cytotoxic immune cells [356, 357]. Similar effects have been observed upon CD73 inhibition, proving the value of these checkpoints in the management of cancer [358, 359]. Likewise, A2AR blockade not only diminishes tumor progression, but also favors antitumor immune responses, enhancing infiltration of effector immune subsets, NK cell cytotoxicity, and cytokine production [341, 360–362]. These encouraging data have prompted the development of agents that modulate the adenosine pathway, mainly mAbs against A2AR, CD73 and CD39, and A2AR antagonists. Up to date, anti-CD39 agents have yet to enter clinical trials, whereas several anti-CD73 treatments are under investigation (e.g., oleclumab [NCT02503774], CPI-006 [NCT03454451]). In this respect, a phase I trial reported encouraging the clinical activity of the combination of oleclumab and PD-L1 blockade [363]. The therapeutic potential of small molecules inhibiting A2AR is unclear, given that CPI-444 showed modest clinical activity [NCT02655822] [364], while N1R178 [NCT03207867] achieved clinical benefits with possible immune stimulation [365]. Data from clinical trials assessing the efficacy of other A2AR targeting therapies (e.g., AZD4635 [NCT02740985]) remain to be disclosed. Despite the importance of the adenosine pathway in the immune system, there is a long way to go to determine whether therapies targeting its components might provide clinical advantages in the field of cancer treatment.

The family of NADPH oxidases (NOX) takes a central part in the defense against infections, hence this type of protein appears as checkpoints that regulate the immune function. NOX proteins generate reactive oxygen species (ROS) within the cell in a controlled manner [366]. In cancer, oxidative conditions, characterized by high levels of ROS, are a typical feature of the tumor microenvironment and contribute to tumor development and progression and immune tolerance [367–369]. Tumor-associated myeloid cells stand as the main source of ROS [370–372]. In fact, myeloid-derived ROS have been proposed to promote NK cell exhaustion in certain malignancies, such as AML, in which downregulation of NK cell-activating receptors NKG2D and NKp46 was observed as a result of ROS production by phagocytes [373]. Exacerbated generation of ROS is caused by overexpression of NOX2 in these tumor-promoting myeloid cells, providing the rationale for the impairment of NOX2 enzymatic activity as a novel anticancer approach [370]. NOX2 inhibition in melanoma abrogated the immunosubversive activity of tumor-associated myeloid cells in NK cell cytotoxicity [374]. In vivo studies support the role of this enzyme, since NOX2-deficiency translates in improved NK and T-cell responses [371, 374]. Despite the interest in targeting NOX2 for immunotherapy, no specific inhibitors for NOX isoforms have been developed so far [375, 376]. Still, a phase I trial assessing GKT137831, a dual NOX1/4 inhibitor, the administration reported no toxicity (NCT02010242), although clinical anticancer efficacy has not been investigated yet. These results open a door for further analyzing the therapeutic potential of inhibiting NOX enzymes in cancer in a clinical setting.

The field of checkpoint modulation as immunotherapy is constantly growing and new names are frequently added to the long list of molecules that regulate antitumor immunity and might prove useful targets in cancer. As a consequence, a vast number of proteins—apart from the ones reviewed herein—such as VISTA, B7-H3, Siglec-15, CSFR1, or TLRs, are under preclinical and clinical research, aiming to find novel therapeutic approaches that might improve the management of patients with cancer.
Concluding remarks

Immunotherapy has supposed a major breakthrough in the fight against cancer, achieving encouraging results even in cancers with the poor outcome or metastatic tumors. Among immunotherapeutic agents, ICB therapies, such as nivolumab or ipilimumab, arose as a revolutionary milestone in the treatment of cancer. Withal, only a limited subset of patients responds to these therapies, highlighting the need for novel approaches or combination regimens that improve these response rates. Immune checkpoints such as ILT2, NKG2A, or TIGIT rise as promising alternatives in ICB, as discussed herein. Classically, ICB therapy has been focused on recovering T lymphocyte antitumor activity. Nonetheless, increasing evidence sustains that immune checkpoints also modulate NK cell-mediated responses, which play a key role in antitumor immunity. Interestingly, the development of certain tumors has been associated with NK cell dysfunction, further supporting the relevance of this immune subset as a target in the field of cancer treatment. Restoring and harnessing NK cell function have potential in antitumor therapy. This goal might be reached upon abrogation of the negative regulation exerted by checkpoints on this immune subset, opening the window for novel applications of ICBs in cancer immunotherapy.

Acknowledgments

This work was supported by the Spanish grant of Instituto de Salud Carlos III (PI19/01353) and FEDER European Union. CSB holds a Severo Ochoa grant (BP19-066).

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[150] Chen J, Chen Z. The effect of programmed microenvironment on the progression and prognosis of colorectal cancer. Med Oncol 2014;31:82.


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References


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14. Checkpoint inhibition in the fight against cancer


V. Targeting NK cells
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V. Targeting NK cells
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The dual role of Natural Killer cells during tumor progression and angiogenesis: Implications for tumor microenvironment-targeted immunotherapies

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Abstract

Immune cell polarization, as a consequence of their cellular plasticity has been described both for innate and adaptive immunity. This cell polarization is finely tuned in cancers by several molecular mechanisms that include cell-to-cell contact, interaction via soluble factors, and metabolic alterations/adaptations. The final readout is the generation of immune cells with reduction of targeting/killing of tumor cells, tolerogenic/immunosuppressive behavior, and acquisition of proangiogenic functions. Natural killer (NK) cells are effector lymphocytes involved in tumor immunosurveillance. However, in solid malignancies, tumor-associated NK cells (TANK cells, those in the peripheral blood) and tumor-infiltrating NK cells (TINK, those in tumor
of tissues) show an altered phenotype and functions. Here, we discussed current state of the art on the dual role of NK cells during tumor progression and angiogenesis, focusing on the tumor microenvironment-related factors orchestrating NK cell polarization. Finally, we summarized current approaches used to manipulate/repolarize NK cells for immunotherapy approaches.

Abbreviations

- ADCC: antibody-dependent cellular cytotoxicity
- A1R: adenosine receptor 1
- Arg1/2: arginase 1/2
- BMMSC: bone marrow mesenchymal stem cell
- cDC1: conventional type 1 dendritic cell
- circRNA: circular RNA
- CLIR: C-lectin-like inhibitory receptor
- CSC: cancer stem cell
- DNAM1: DNAX accessory molecule 1
- dNK: decidual NK cell
- GM-CSF: granulocyte macrophage-colony stimulating factor
- GVHD: graft versus host disease
- HIF-1α: hypoxia-inducible factor-1α
- HCST: hematopoietic cell signal transducer
- HLA-I: human leukocyte class I antigen
- HOX: heme-hoxigenase
- IDO: indoleamine dioxygenase
- IMID: immunomodulatory drug
- iPSC: induced pluripotent stem cells
- KIR: killer-immunoglobulin-like inhibitory receptors
- LFA-1: lymphocyte function activated-1
- LILR: leukocyte immunoglobulin-like receptor
- IncRNA: long noncoding RNAs
- MC: mast cell
- MDSC: myeloid-derived suppressor cell
- MM: multiple myeloma
- MSC: mesenchymal stromal cells
- NCR: natural cytotoxicity receptor
- ncRNA: noncoding RNA
- NGF: nerve growth factor
- NK: natural killer cell
- NKG2D: natural killer group 2 D
- NO: nitric oxide
- NSCLC: nonsmall cell lung cancer
- PBMC: peripheral blood mononuclear cells
- Pericyte
- PD-L1: programmed cell death-ligand 1
- PD-1: programmed death 1
- PGE2: prostaglandin E2
- piRNA: piwi RNA
- snoRNA: small nucleolar RNA
- TAF: tumor-associated fibroblast
- TAM: tumor-associated macrophage

V. Targeting NK cells
**Introduction**

Inflammation represents a host-dependent hallmark of cancers, as defined by Hanahan and Weinberg [1], that acts as a relevant player in both tumor onset and development [2, 3]. Inflammatory cells from innate and adaptive immunities are characterized by cellular plasticity, which refers to their ability to phenotypically and functionally adapt to the micro (tissue-local) and macro (peripheral blood) pathophysiological environments. In this scenario, “escape to immune cell elimination” and “tumor-promoting inflammation” represent key features in the immune cell polarization capability exerted by cancers [1, 3, 4]. This puzzle is further complicated by angiogenic switch, a phenomenon by which cancers cells instruct infiltrating immune cells to acquire a proangiogenic phenotype, necessary to support the generation of a tumor vasculature for nutrient and oxygen supplies and metastasization [1, 3, 4]. Immune cell polarization, as a consequence of their cellular plasticity, has been described for cells of both innate and adaptive immunities, as reviewed in refs. [5–7]. Cancer cells and other cell components of the tumor microenvironment (TME) can regulate immune cell polarization through cell-to-cell interactions, soluble factors, and metabolic alterations/adaptations [5–7]. Macrophages represent the typical prototype in the scenario of immune cell polarization [8–10] together with neutrophils and myeloid-derived suppressor cells [5–7, 11].

NK cells, large granular lymphocytes belonging to the innate arm of the immune system, are involved in the control of tumor growth by their cytotoxic activity [12, 13]. Recently, Lim et al. demonstrated that NK cells share the same innate lymphoid cell (ILC) progenitor [14]. Gao et al. identified the conversion of NK cells (CD49a− CD49b+ Eomes+) into the innate type 1 lymphoid cells, called ILC1 (CD49a+ CD49b− Eomesint), following TGF-β stimulation within the TME [15]. ILC1s are not able to control tumor growth. Based on this concept, the TME is able to support tumor immune-escape by converting cytolytic NKs into non-cytotoxic ILC-1 cells, in a TGF-β dependent manner [15].

Two major NK cell subsets have been characterized based on the surface expression of the neural cell adhesion molecule (NCAM/CD56) and the Fc-gamma-receptor III (CD16) molecule [12, 13]: CD56dimCD16+ NK cells, which account for 90%–95% of total circulating NKs, are endowed with cytotoxic activities via perforin and granzyme release and mediate...
antibody-dependent cellular cytotoxicity (ADCC) via CD16; the remaining 5%–10% of total circulating NKS are the CD56<sup>bright</sup>CD16<sup>-</sup> NKS that are able to produce Th1 cytokines, such as IFN-γ and TNF-α [12, 13].

Another NK subset has been characterized in the developing decidua, known as the decidual NK cell (dNK) phenotype, which accounts for 50% of the total decidual lymphocytes. dNKS have a tolerogenic and proangiogenic phenotype, identified as CD56<sup>superbright</sup>CD16<sup>-</sup>VEGF<sup>high</sup>PlGF<sup>high</sup>CXCL8<sup>+</sup> dNKS, and are a crucial orchestrator in the generation of the spiral artery during embryonic development [16, 17].

With the single-cells analysis era, this dichotomy of NK cell subsets has been completely revolutionized generating a large amount of data, including the transcriptional landscape, allowing the identification of multiple further NK cell subsets [18–20] that are pathophysiologically context-dependent.

As for many other immune cell types, NK cells have been found to be compromised in diverse cancer types, as a consequence of alteration of surface antigens, expression of tissue-resident antigens, decreased anticancer activities, and acquisition of protumor and proangiogenic functions [2, 3, 5, 12, 21–23]. Major alterations on NK cells in cancer patients have been attributed to decreased expression of the human natural cytotoxicity receptors (NCRs), reduced expression of the NKG2D activation marker, together with impaired degranulation capabilities (reduced CD107a) and release of IFN-γ and TNF-α [2, 3, 12, 21–26]. As for T cells, exhausted NK cells have also been found in cancer, characterized by increased expression of PD-1, TIGIT, CD96, and TIM3 [27–33]. Apart from the induction of exhausted, tolerogenic NK cells, the generation of proangiogenic NK cells in the TME has been recently characterized [3, 21, 34, 35]. Here we reviewed and discussed the current state of the art on the dual role of NK cells during tumor progression and angiogenesis, focusing on the TME-related factors orchestrating NK cell polarization and on the NK cell/TME cellular component interactions.

### TME-derived soluble mediators instructing the protumor/proangiogenic phenotype and functions in NK cells

The tumor micro (tissue-local) and macro (that in the peripheral blood) environments are enriched in different soluble factors acting on immune cells by blocking their effector functions or by sustaining the induction of protumor and proangiogenic phenotypes.

### Transforming growth factor-β

TGF-β represents the most abundant and characterized TME-factor acting on immune cells [36, 37]. TGF-β acts as a Janus cytokine: in the early tumor phase, it is a tumor suppressor molecule, while in the later stages of carcinogenesis, TGF-β drives relevant mechanisms of tumor progression, including induction of immunosuppression, angiogenesis, and epithelial mesenchymal transition (EMT) [38–40].

TGF-β has been largely reported to impair via multiple routes NK cell functions ranging from induction of functional anergy to decreased NK cell maturation and the induction of a proangiogenic phenotype [3, 25, 34, 41, 42]. TGF-β decreases the expression level of NKG2D,
one of the major NK cells activating receptors [36, 43, 44]. TGF-β acts as an immunosuppressive cytokine on NK cells, by reducing their cytolytic effector functions, such as the CD16-mediated ADCC, thus affecting the efficient induction of the immunological synapsis. TGF-β negatively impacts on NK cell functions by limiting their secretion of perforin and granzymes, all molecules relevant to induce the intrinsic and extrinsic apoptotic pathways in target cells. Also, TGF-β represses the capability of NK cells to release TNF-α and IFN-γ that affect tumor cells directly or indirectly, by activating dendritic cells and the Th1 response. Interestingly, TGF-β exerts its immunosuppressive activities, both as a free molecule and exosome-encapsulated factors; indeed, tumor-derived exosomes have been reported to be largely enriched in TGF-β both in preclinical models and in cancer patients [24, 45–47].

The serine and threonine kinase molecular target of rapamycin (mTOR) represents a crucial molecular signaling for NK cell responsiveness to IL-2 and IL-15 [48]. TGF-β has been reported to inhibit mTOR signaling in NK cells, thus limiting their ability to exert antitumor activities triggered by IL-2 and IL-15 [48].

Novel NK cell protumor activities, driven by TGF-β, have been described by Bruno A. and coworkers. In this context, TGF-β has been reported to sustain the angiogenic switch of cytolytic NK cells [3, 34]. TGF-β increases the proportion of CD56<sup>bright</sup>CD16<sup>+</sup> NK cells, a peculiar subset occurring within the developing decidua that is necessary for the correct embryo developments [3, 34]. These decidual-like NKs have been found in patients with nonsmall cell lung cancer (NSCLC) [34], colorectal cancer [21] and in the pleural effusions of patients with metastatic tumors [26]. dNK-like cells in cancer patients have been reported to functionally sustain angiogenesis in vitro via soluble factors in a STAT5/STAT3-dependent manner [21, 25]. Further studies found that TGF-β is also able to increase the expression of other relevant decidual-like markers, such as CD9 (tetraspanin) and CD49a (α1-integrin) [22]. In this view, TGF-β parallels the NK cells occurring during the decidualization to those in cancer patients [49].

**Prostaglandin E2**

Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) is a small lipid molecule that regulates diverse biological processes, ranging from reproduction to neuronal and metabolic functions, cancers. In cancers, PGE<sub>2</sub> acts as a potent regulator of inflammation and cells of both innate and adaptive immunities are functionally affected by PGE<sub>2</sub>, resulting in immune escape in malignancy. PGE<sub>2</sub> is released by several cellular components of the TME, including cancer cells, tumor-associated macrophages, and stromal cells, as a strategy to induce immunosuppression [50–52]. PGE<sub>2</sub>-producing tumors have been found to have reduced NK cell infiltration, together with NK cell functional impairment [53]. PGE<sub>2</sub> affects NK cell cytotoxicity, by downregulating the expression of NCRs (NKp46, NKp44, NKp30) and NKG2D, through the binding to E-prostanoid 2 (EP2) and EP4 receptors on NK cells [54], via a common cAMP-PKA signaling [55, 56]. Also, PGE<sub>2</sub> downregulates IL2-activated LAK cell cytotoxicity, including NK cells, via EP2 receptors [57, 58]. In patients with gastric cancer, PGE<sub>2</sub> has been found to be critical in inducing NK cell dysfunctions by reducing their proliferation and inducing apoptosis. PGE<sub>2</sub> has been observed to contribute to the thyroid cancer immune escape, by suppressing NK cell cytotoxicity and differentiation [59]. Another mechanism by which PGE<sub>2</sub> dampens NK cell antitumor activities includes the blocking of the production of cytokines/chemokines necessary for conventional type 1 dendritic cells (cDC1s) recruitment into tumors [60]. This PGE<sub>2</sub>-mediated
evasion of the NK cell-cDC1 axis blocks a relevant NK/DC cross talk that generates potent antitumor activities, since cDC1s are critical for antitumor immunity, and their abundance within tumors is associated with immune-mediated rejection and the success of immunotherapy [60]. Another mechanism by which PGE₂ dampens NK cell antitumor activities includes the blocking of the production of cytokines/chemokines necessary for cDC1 recruitment into tumors [60]. This PGE₂-mediated evasion of the NK cell-cDC1 axis blocks is a relevant NK/DC cross talk that generates potent antitumor activities, since cDC1s are critical for antitumor immunity, and their abundance within tumors is associated with immune-mediated rejection and the success of immunotherapy [60].

**Leptin**

Adipose tissue represents an abundant source of secreted soluble factors endowed with proinflammatory functions [61–63]. Also, adipocytes are present in the tumor microenvironment and secrete large amounts of adipokine that have “remote” effects in carcinogenesis both at tissue/local and systemic levels [63].

The link between obesity and cancer has been largely demonstrated by the contribution of adipocytes as well in sustaining diverse cancer types [64, 65]. Adipocytes produce large amounts of leptin [64, 65]. Leptin has been reported to be strongly involved in breast cancer, by contributing to the generation of a proinflammatory environment, particularly in obese patients, that are characterized by increased metastases [66, 67]. Several studies demonstrated that leptin exerts relevant immunomodulatory functions in NK cells [68–70]. Short-term exposure of NK cells to leptin results in increased expression of the CD69 activation marker, together with increased IFN-γ secretion and NK cytotoxicity [70]. Conversely, long-term exposure of NK cells to leptin impairs NK cell proliferation [70].

Leptin has been reported to promote breast cancer resistance in vitro when cocultured with NK92 cells by upregulating the peroxisome proliferator activated receptor coactivator-1α [71]. Reduced level numbers of leptin receptor expressing NK cells have been found in the livers of obese subjects, suggesting that obesity-associated alterations of immune cell numbers vary in human organs and can differ in their contribution to cancers. However, NK cell dysfunctions might represent a relevant link to the elevated cancer risk and the higher susceptibility for viral infections and cancers of obese individuals.

**Interleukin-10**

Interleukin (IL)-10, a pleiotropic cytokine produced by diverse immune cells and cancer cells, has been described to exert a controversial role in tumor immunology [72], ranging from antitumor [73, 74] to protumor activities [75]. A brilliant paper by Mocellin S. and colleagues explored the IL-10 stimulatory effects on human NK cells by gene expression profiling. They found that NK cells exposed to IL-10 increased the expression of genes related to migratory activities L-selectin, VEGF-R1, uPA-R, and lipoxin A₄ receptor [72]. In this setting, IL-10 did not show any significant effect on NK proliferation [72]. Conversely, NK cells stimulated with IL-10 exhibited increased mRNA levels of cell activation/cytotoxicity-related genes, such as secretogranin, TIA-1, HMG-1, interferon-inducible genes, that were not upregulated by IL-2 [72]. Another report documented that IL-10 synergizes with NK cells in increasing NK cell cytolytic activities [76]. Increased levels of IL-10 producing NK cells have been detected in the peripheral blood of NK cells in patients with pancreatic ductal adenocarcinoma; these NK cells...
cells were characterized by reduced cytotoxic activities. Together, these results outline the controversial role of the IL-10/NK cell interactions in the TME.

**Lactate**

Lactate represents a molecule largely generated within the TME, as a consequence of the Warburg effect, a relevant metabolic hallmark of cancers [77, 78]. The generation of lactate results in the induction of an acidic TME, creating an unfavorable soil for efficient tumor cell elimination both from innate and adaptive immunities. Acidosis is a major hurdle in conferring anergy in NK cells [79–81]. Acidification via efflux of lactic acid has been reported to affect NK cells activity via downregulation of the NKp46 receptor, impairing their ability to produce IFN-γ. Mechanistically, it has been found that lactate-induced anergy is mediated by the inhibition of nuclear factor of activated T cells (NFAT), which is necessary for IFN-γ transcription [82]. Depletion of LDH in murine models results in improved cytolytic function in vivo. Lactate can also indirectly affect NK cell functions by increasing the infiltration of MDSCs [83] that inhibit NK cell cytotoxicity via release of immunosuppressive factors, such as TGF-β, nitric oxide (NO), and IDO-1 [84–86].

**HLA-G**

Firstly characterized in the decidual trophoblast [87, 88], HLA-G is an immunoregulatory class I MHC molecule that has been found to be expressed also in diverse cancers, such as NSCLC, ovarian, breast, colorectal, esophageal, gastric, hepatocellular, and endometrial cancers [89–92]. HLA-G induces NK cell anergy not only by interacting with the immunoglobulin-like transcripts ILT-2, ILT-4 and killer Ig-like immunoglobulin receptor (KIR) 2DL4 [93–95], but also by inducing senescence in NK cells [96]. HLA-G has been also found to reduce CCR2 and CXCR3 expressions in the CD56^{bright} and CD56^{dim} NK cells, suggesting that HLA-G limits efficient NK cell recruitment and infiltration into tumors [97].

**Adenosine**

Adenosine represents an immunosuppressive molecule within the TME that is generated from ATP degradation by the ectonucleotidase CD39 and CD73 [98], as a consequence of hypoxia [99] and extracellular stress [100, 101]. Four adenosine receptors, A1, A2A, A2B, and A3, have been characterized. Following release in the extracellular TME, adenosine acts on NK cells by reducing their ability to produce IL-2-induced TNFα and suppressing perforin and Fas ligand (FasL)-mediated cytotoxic activities by NK cells. These effects are largely mediated by the induction of the cyclic AMP/protein kinase A pathway, after the binding of adenosine to A2A receptors on NK cells [24]. Adenosine has been demonstrated to mediate inhibition of cytotoxic activity and cytokine production by IL-2/NKp46-activated NK cells [24]. Also, it has been reported that the peripheral blood CD56^{bright}CD16^{−} NK cell subset can produce adenosine [102]. Adenosine has been also reported to dampen NK cell activation, induced by IL-2, IL-12, and IL-15, by downregulating the activation receptors NKG2D and the NCR NKp30 molecules [103].

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Adenosine can participate in the inhibition of NK cell maturation via engagement of A2AR [104]. Also, overall and NK cell-specific conditional deletion of A2AR results in augmented frequency of NK cells with a terminally mature phenotype [104].

Nanovesicles/exosomes

Several reports have indicated that NK cells can produce exosomes and that these nanovesicles can be involved in NK cell-mediated cytotoxicity (Fig. 1) [105–107]. Indeed, exosomes are a class of extracellular vesicles that resemble the cell from which they are derived; thus, NK cell exosomes express at their cell surface the relevant activating molecules usually expressed on the NK cell membrane [105–107]. It has been reported that NK cell-derived exosomes from healthy donors can bear detectable amounts of NKp30, NKp46, NKG2D, CD16, and DNAM1 activating NK cell receptors [107]. The level of expression of these molecules was not strong [108]. Indeed, some of these receptors were barely detectable (NKp30, NKp46, and CD16), whereas others were weakly positive (NKG2D and DNAM1). Importantly, NK cell exosomes expressed adhesion molecules involved in target cell recognition, such as lymphocyte function activated (LFA)-1, or involved in cell-cell or cell-protein extracellular matrix interaction such as CD56. The level of expression of the abovementioned molecules is usually lower than that present on NK cells [108]. This fact can be related to the methods used for the detection of a specific antigen on exosomes [108, 109]. NK cell-derived exosomes can be produced in a consistent amount such as 5.3 ± 3 μg/10^6 cells or 0.62 ± 0.2 μg /10^6 cells [108, 109]. These differences can be related to the presence of serum

FIG. 1 Cross talk between mesenchymal stromal cells and NK cells in the tumor microenvironment. Mesenchymal stromal cells (MSCs) can influence the TME (see the text) and can interact with NK cells leading to the inhibition of their antitumor activities such as killing of tumor cells. Also, MSCs can downregulate NK cell functions when they are differentiated into adipocytes or tumor-associated fibroblasts (TAF/CAF) or pericytes. Pericytes are relevant in endothelial-mesenchymal cross talk, and as stellate cells in the liver they can interact directly with NK cells. On the other hand, NK cells can influence the TME by killing MSCs. Both NK cells and pericytes can influence the angiogenesis in healthy and tumor-affected tissues. Importantly, immunomodulatory drugs (IMiDs) can trigger NK cell-mediated killing of tumor cells influencing the behavior of MSCs. Also, NK cells can release exosomes and microvesicles that in turn may play a role in regulating the surrounding microenvironment; thus, the influence of NK cells can be exerted by direct cell-to-cell interaction and through nanovesicles.

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exosomes in the NK cell culture supernatant from which exosomes are isolated. Indeed, in some experimental settings, cell culture contains serum [108], whereas when NK cells are cultured in serum-free medium, it yielded less amounts of exosomes [109]. Importantly, NK cell-derived exosomes expressed high amounts of FasL, granzyme B, and detectable amounts of granzyme A and perforins, suggesting that these nanoparticles can exert a cytotoxic effect on tumor target cells [106, 108, 109]. It is of note that NK cell-derived exosomes were able to trigger tumor cell target killing of hematopoietic cell lines such as Jurkat T cells, K562 erythroleukemia, Daudi, and NALM-18 B cell lymphomas, whereas the breast cancer cell line SKBR3 and metastatic melanoma Mel501 appeared to be resistant. It is worth noting that the amount of NK cell-derived exosomes that can induce a strong killing of tumor target cells was about 50–60 μg, while at lower concentrations such as 5 μg the killing was not so evident. This implies that hematological malignancies are mainly sensitive to NK cell exosomes and the amounts of exosomes should be derived from several millions of NK cells against several thousands of tumor target cells [106, 108, 109]. Thus, the ratio between NK cells and tumor target cells should be at 1000:1. This ratio is not consistent with the actual situation that usually has been reported for several different solid tumors where the presence of infiltrating NK cells is not so evident [110, 111]. It is not clear whether NK cell exosomes can be concentrated at the tumor site and whether a few NK cells can physiologically produce μg of exosomes in vivo during tumor growth and recognition. However, although NK cell exosomes express cytolytic molecules and bear NK cell-activating receptors, they do not have a cytotoxic effect on healthy peripheral blood mononuclear cells. More recently, the association of NK cell exosomes with their biomimetic core-shell nanoparticles [112] has been used for therapy of neuroblastoma. Indeed, the successful development of a hybrid composed of NK cell exosomes and a dendrimer core-loading therapeutic microRNA can efficiently target neuroblastoma cells in vivo and inhibit tumor growth. Indeed, in this murine model, both cytolytic molecules present in the NK cell exosomes, such as FasL, granzymes, and perforins, together with the microRNA can exert an antitumor effect. Importantly, the targeting of the tumor can be achieved through the expression by NK cell exosomes of the CXCR4 molecule [112]. Altogether, these findings indicate that exosomes from peripheral blood NK cells can exert an antitumor effect in in vitro and in vivo models.

It is well established that peripheral blood NK cells are mainly composed of CD56+CD16+ large granular lymphocytes, which constitutively expressed several inhibiting receptors, besides bearing a plethora of activating receptors such as those mentioned above [113]. These inhibitory receptors are mainly represented by killer-immunoglobulin-like inhibitory receptors (KIRs), C-type lectin inhibitory receptors (CLIRs), and leukocyte immunoglobulin-like receptors (LILRs) [114]. These receptors are involved in the recognition of several different alleles of human leukocyte class I antigens (HLA-I) which are clonally distributed and whose expression is thought to be linked to education processes that occur during NK cell maturation [114]. Indeed, these receptors interacting with appropriate HLA-Class I ligands deliver a strong inhibiting signal that leads to impairment of NK cell-mediated killing and secretion of several antitumor cytokines such as IFN-γ and TNF-α [114]. This is considered the main means by which NK cells do not kill healthy self-cells while they can kill tumor target cells that do not express HLA-Class I molecules such as erythroleukemia K562 [114]. To our knowledge, no reports regarding the expression and function of these receptors on NK cell-derived exosomes have been published. However, it is conceivable that NK exosomes can bear these inhibitory
receptors, because as per definition an exosome looks like the cell from which it derives [107, 108]. Thus, it is still unclear whether the inhibitory receptors expressed on NK-derived exosomes can play a role in delivering signals to tumor cells. Indeed, the presence of a soluble-like form of inhibitory receptors can lead to their interaction with the corresponding ligands on tumor cells [114]. This would inactivate the antitumor function of the NK cell exosomes, but at the same time could augment the cytolytic and cytokine production by the whole NK cell. Indeed, the exosome form of inhibitory receptors would compete with the NK cell surface-bound form, reducing the negative signal mediated of the latter. This would explain the relatively high amount of NK cell exosomes that are needed for delivering a killing signal in tumor targets. A second main NK cell population circulating in the peripheral blood displays a strong expression of CD56 antigen (CD56bright NK cells) but not CD16 [114]. These NK cells are considered as cytokine producers because they can secrete high amounts of IFN-γ, TNF-α, and granulocyte-macrophage colony-stimulating factor (GM-CSF). It is important to note that CD56bright NK cells express CD62L, CCR7, and CXCR3 molecules, which are involved in the localization in secondary lymphoid and nonlymphoid tissues where their corresponding ligands are highly expressed [114]. Furthermore, CD56bright NK cells can show a specific surface phenotype depending on the tissue from which they are isolated [115]. Indeed, based on the expression of CD69, adhesion molecules, and chemokine receptors, the CD56bright tissue-resident NK cells can be distinguished from peripheral blood CD56bright NK cells [114, 115]. To our knowledge, the analysis of exosomes from tissue-resident NK cells is not reported. However, it is conceivable that these exosomes can play a role in the regulation of tissue homeostasis as well as in antitumor or protumor NK cell-mediated effects [114–116]. Thus, it is evident that our knowledge on this specific topic is still limited. It can be suggested that, like the whole NK cell, NK cell exosomes in specific tissues can have a role in regulating angiogenesis and that the tissue microenvironment can shape the function and phenotype of NK cells [17, 117]. Indeed, it has been reported that dNK cells may have a role in placenta formation influencing angiogenesis, trophoblast invasion, and spiral artery remodeling and can express VEGF [16, 17, 118].

Noncoding RNAs

Although proteins are major regulators of cell function, increasing evidences suggest that noncoding RNAs (ncRNAs) are critical regulators of NK cells, thus representing new therapeutic targets to improve the response of immunotherapy and block tumor progression [119–126]. ncRNAs include many different RNAs, such as small nucleolar RNAs (snoRNAs), piwi-interacting RNAs (piRNAs) [119], long ncRNAs (lncRNAs), together with the well-studied microRNAs (miRNAs) and circular RNAs (circRNAs) [125, 126]. In particular, dysregulation of miRNAs and circRNAs has been widely documented to shape the TME and in particular to affect NK cell biology in the context of development, inflammation, and tumor surveillance [124–126].

MicroRNAs

MicroRNAs (miRNAs) are the major studied ncRNAs, transcribed from the DNA in the form of single-stranded RNAs of approximately 22 nucleotides in length, and acting as
negative regulators of gene expression by targeting the messenger RNAs (mRNAs). Numerous studies suggest that miRNAs exert important regulatory functions in innate immunity and cancer immunosurveillance [25, 127–129]. In particular, miRNAs play important roles in NK cell development, maturation, activation, proliferation, cytotoxicity, and cytokine production mainly by targeting receptors or multiple pathways involved in transcriptional regulation [130, 131]. Two studies employing Dicer-deficient NK cells reported a reduced survival/proliferation and maturation in the absence of miRNAs, thus demonstrating the critical role of miRNA in NK cell homeostasis [132, 133]. Transcriptomes analysis identified different miRNA signatures able to discriminate the two main NK cells, CD56bright from CD56dim NK subsets, providing valuable clues of miRNA regulation in human NK maturation [134]. Key miRNAs reported as relevant for the NK cell development and differentiation process are miR-146a-5p, miR-150, miR-181, and miR-583 [131, 135–137].

MiRNAs can affect NK cell cytotoxicity and cytokine production either acting as negative regulators or by enhancing NK cell functions. In this context, miRNAs including miR-27a-5p, miR-378, miR-30e, and miR-150 [138–140] have been reported as negative regulators of human NK cell cytotoxicity by targeting granzyme B and perforin. In contrast, miR-155 overexpression promotes NK-cell effector functions by enhancing IFN-γ release targeting SHIP1, T-bet/Tim-3 [141, 142], or modulating multiple signaling pathways involving PI3K, NF-κB, and calcineurin [143]. Likewise, miR-362–5p overexpression promotes NK-cell effector functions by upregulating IFN-γ, perforin, granzyme-B, and CD107a, by targeting cylindromatosis lysine 63 deubiquitase (CYLD). MiR-181 also promotes IFN-γ production through regulation of the Notch pathway [136].

Within the TME, miRNAs might play a dual role, acting both as tumor suppressor genes or oncogenes. By using genome-wide miRNA expression profiles, a new molecular circuit involved in the pathogenesis of nasal NK cell lymphoma (NNL) was identified. Chen et al. showed that T-bet, PTEN, AKT, and RICTOR are regulated by miR-BART20-5p, miR-494-3p, and miR-142-3p [144]. Similarly, other NKTCL oncomiRNAs, including miR-155 [145], miR-10a, and miR-342-3p, have been suggested to affect NK cell lymphoma survival [146].

Several studies suggest that miRNAs modulate the immune checkpoints/ligands interaction, including the PD-L1 axis or their upstream genes. Pesce et al. found that miR-146a-5p is able to downregulate both KIR2DL1 and KIR2DL2 [134], together with targeting STAT1 and NFκB acting on NK cell maturation [147]. Qing Li et al. found that miR-28 targets TIM-3 that is expressed both in NK and T cells, and is implicated in PD-1 regulation and cytokine secretion [148]. Several other miRNAs such as miR138 [149], miR-4717 [150], miR-195/16 [151], and miR-138 [149] target PD-1 and T lymphocyte antigen-4 (CTLA-4) expression [151].

Notably, in different cancer settings, miRNAs can target the PD-1/PD-L1 axis directly or indirectly by targeting the related signaling pathways. miR-15a, miR-15b, and miR-16 were found to downregulate the PD-L1 expression [152–155]. The ability of miRNAs to hit simultaneously multiple aspects on NK cell functions makes them attractive targets and offers a new therapeutic approach to treat cancer immune escape.

Circular RNAs

Circular RNAs (circRNAs) are small noncoding RNAs, produced during pre-mRNA splicing, and are characterized by a closed ring structure with no free 3' or 5' ends [156]. Although
previously thought to be insignificant by-products of splicing errors, circRNAs are now emerging as new members of the gene regulatory network. circRNAs bind and sequester specific proteins, interact with regulatory RBPs, act as miRNA sponges, affecting the fate of their target mRNAs that might be directly involved in NK cell activities and cancer immune surveillance [157]. Several evidences suggest that tumor-induced circRNAs regulate NK cell activities either by enhancing their cytotoxicity or downregulating their functions via the circRNA-miRNA-mRNA axis. circARSP91 was reported to increase the susceptibility of hepatocellular carcinoma (HCC) cells to NK cell cytotoxicity by upregulating the expression of UL16 binding protein 1 (ULBP1) at the mRNA and protein levels [158], thus representing a potent novel therapeutic strategy [158].

Zhang et al. reported that downregulation of circTRIM33–12 was correlated with reduced number of NKG2D-positive cells in HCC tissues and a poor prognosis for HCC patients. Mechanistically, circTRIM33–12 by sponging miR-191 suppresses HCC immune evasion and progression [159].

The tissue inhibitor of metalloproteinases-3 (TIMP3), a potent inhibitor of the MMP subfamily and some ADAMs, has been associated with MICA and MICB shedding and the enhancement of the lytic activity of NK cells through the immune recognition mediated by the NKG2D receptor [160]. circSMARCA5 promotes the expression of TIMP3 and may facilitate NK cell-mediated immune recognition by the NKG2D receptor by sponging miR-17-3p and miR-181b-5p, making it a potential therapeutic target in HCC [161].

circRNAs act also as immune suppressors. In a recent study, Ou et al. found that circ_0000977 knockdown, sponging miR-153, of which HIF-1α was a downstream target, modulated HIF-1α and enhanced the killing effect of NK cells on pancreatic cancer cells under hypoxia. Thus, the circ-0000977/miR-153/HIF1α axis modulates the HIF-1α-mediated immune escape of pancreatic cancer cells by downregulating the responsiveness to NK cell-mediated lysis [153]. A very recent study revealed new evidences about the cross talk between HCC cells and NK cells linking high levels of plasma exosomal circUHRF with a decreased NK cell tumor infiltration and NK cell-derived IFN-γ and TNF-α secretion. circUHRF1 was able to inhibit NK cell function by upregulating the expression of TIM-3 via degradation of miR-449c-5p, thereby driving resistance to anti-PD-1 immunotherapy in HCC patients [162]. Thus, the above studies indicate that circRNAs can modulate the activity or cytotoxicity of NK cells, thereby mediating tumor immune surveillance. Given their stable structure, compared with miRNA and lncRNAs, circRNAs are emerging as novel targets for immunotherapy and as noninvasive clinical diagnostic and prognostic markers.

Cell-to-cell interactions in the tumor microenvironment

Macrophages

Macrophages are the major myeloid component of mononuclear phagocytes of the innate immune system and endowed with elevated cell plasticity. They possess the ability to adopt varied phenotypes in response to different microenvironment signals [8, 163]. They are divided into two major subtypes: classical macrophage activation (M1), which is involved in promotion to a proinflammatory response, and alternative macrophage activation (M2), which stimulates an antiinflammatory response and exerts protumor activities [164]. M1

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and M2 responses well describe the in vitro opposing features of killing activities and repairing functions exerted by macrophages, respectively; the first cell type in response to IFN-γ and LPS stimulation and the latter under IL-4 and IL-13 stimuli. M1 macrophages are able to produce NOS, ROS, and several types of proinflammatory cytokines, possess high antimicrobial activities, and exert antitumor activity, whereas M2 macrophages are involved in tissue remodeling and repair activities, immune suppressive effects, and proangiogenic functions [9, 163]. Macrophages in the TME are known as tumor-associated macrophages (TAMs) [165] and are preferentially associated with poor prognosis [10, 166, 167].

However, under the multiple stimuli of diverse tumors and their specific TME factors, macrophages can express a wide range of phenotypes endowed with different functional roles that do not fit into the oversimplified M1/M2 classification [8]. Rather, these cells represent a continuum involving heterogeneous effector cells resembling a spectrum of distinct polarization states; currently, researchers refer to M1-like and M2-like to identify the two extremes of these cell subtypes. In the tumor context, TAMs possess mainly M2-like features and protumor functions [9, 163]. They represent the largest population of immune cells infiltrating in all tumors, they are the hallmark of chronic inflammation, and, although they are potentially able to exert important antitumor responses by killing cancer cells during tumor initiation and progression, they preferentially acquire protumor features, being involved in immunosuppression, tumor invasion, angiogenesis and lymphangiogenesis [9].

In the tumor stroma, the action of the inflammatory mediators, such as CCL-2, CCL-5, and CXCL-12, induces the recruitment of monocytes and macrophages and transforms them into TAMs. These chemokines are produced in TME by different types of cells, including NK cells. The activities of NK cells can be inhibited by macrophages with two mechanisms: by the release of soluble factors and cell-to-cell contact. One example of inhibition of cell-to-cell contact is the checkpoint blockade PD-L1 [168]; on the other hand, NK cells could influence the function of macrophages with different inflammatory factors. In the article of Bellora et al., results have been reported showing in vitro cellular interactions between macrophages and NK cells [169]. In particular, they showed that following LPS stimulation, both M0 and M2 macrophages became capable of fully activating NK cells by a cell-to-cell contact-dependent mechanism and through the DNAM-1 and 2B4 interactions [170]. The cross talk between macrophage-NK cell in a tumor setting has been investigated in ascites of ovarian cancer patients [171]. The results showed that while untreated TAMs induced inhibition of NK cell activity, the LPS stimulation of TAMs was capable of restoring NK cell effector functions against the NK-resistant OVACR-3 tumor cell line [171]. Likewise, further experiments on TAMs from ovarian cancer patients have shown that TLR activation can trigger cytolytic activity of NK cells [172].

TAMs can impair NK cell antitumor functions by secreting inhibitory soluble factors such as TGF-β and PGE2. Finally, Molgora M. et al. have pointed out the key role of IL-1R8 on NK cell function during cross talk with macrophages as well as DCs. Indeed, IL-1R8-deficient NK cells were endowed with an antimetastatic effect in two distinct tumor murine models in an IL-18-dependent manner [173].

**Neutrophils**

Neutrophils constitute 50%–70% of circulating leukocytes and are key effector cells of innate immunity involved in the acute phase of inflammation. Their action is fundamental to
fight invading pathogens, particularly bacteria, and in the potentiation of polarized T helper 17 (Th17) immune responses, as well as in the complication of several chronic diseases, including cancer [174–176]. Their trafficking from bone marrow into peripheral blood, as well as their recruitment in the TME, is regulated by expression of CXCR2 and CXCR4. During tumor progression and in the TME, they adapt in response to diverse tumor stimuli, extend their cell survival, and communicate with other immune and stromal cells, being able to orchestrate either protumor inflammation functions such as angiogenesis, tissue remodeling, immunosuppression, and metastasis or antitumor responses [4, 11, 176]. For example, neutrophil killing’s repertory includes expression of NO, TNF-related apoptosis inducing ligand (TRAIL), ROS, and TNF, and it has been shown that expansion of activated antitumor neutrophils (N1 type) in the metastatic niche can inhibit the formation of metastases through the elimination of cancer cells [176].

Tumor-associated neutrophils (TANs or N2) are mainly educated by tumor- or TME-derived TGF-β in their protumor features with the inhibition of N1 phenotypic polarization [177–179]. When activity of TGF-β is blocked, N1 neutrophils become cytotoxic toward tumor cells and activate CD8+ T cells. N2 TAN are long-living, low cytotoxic cells, showing an immature phenotype, and a high proangiogenic, prometastatic, and immunosuppressive activity [177]. Neutrophils play an important role in activating angiogenesis in a previously quiescent tissue during the early stages of carcinogenesis by the release of different proangiogenic factors such as BV8, S100A8, S100A9, MMP9, PDGF, and VEGF [4, 11]. They also are immunosuppressive cells toward NK and T cells by production of ARG1, ROS, NO, and PGE2 [4, 11]. Moreover, TANs can induce the formation of NETs (neutrophil extracellular traps) in the TME, which in turn coat and protect tumor cells from the cytotoxic activity of NK cells and T cells by impairing their contact with tumor cells [180].

Although the existence of an important cross talk between neutrophils and NK cells in the inflammatory context is well established, only limited findings have been reported on the bi-directional interaction between these two innate cells in the TME [181, 182]. In some circumstances, neutrophils and NK cell interactions could result in both activation and suppression [181, 183]. Studies have documented that ROS and arginase I secreted by neutrophils could impair functional activities in NK cells [182]. Moreover, Harlin et al. demonstrated that CD56brightCD16− NK cells were resistant to neutrophil-derived ROS, and that this cell subset was characterized by a higher antioxidative capacity than the CD56dimCD16+ NK cell subtype [184]. This phenomenon could lead to an increased number of CD56brightCD16− NK cells in solid tumors, guided by TANs [184]. Also, neutrophils can secrete several chemokines, such as CCL2–5 promoting NK cell recruitment in the TME and CD56brightCD16− NK cells can secrete CXCL8 inducing neutrophil recruitment [185, 186].

It has been shown that immune checkpoint programmed cell death 1 ligand 1 (PD-L1) could be induced by the hypoxia-inducible factor 1α (HIF1α) pathway in the mouse and in the response to IL-6, IFN-γ, and GM-CSF in humans, and the interaction with PD-1+ NK cells could lead to an inhibitory effect on cytotoxic cells [187], in particular on NK cells with impairment of their cytotoxicity, decreasing the responsiveness of NKp46 and NKG2D and downregulation of CCR1 expression leading to dysregulation of the infiltration capability of NK cells [188].

Recently, in a sarcoma transplantable mouse model, it has been reported that NK cells can impart tumor-promoting angiogenesis features through VEGF-producing neutrophil
interactions via IFN-γ [189]. Interestingly, Spiegel et al. reported that TANs can favor the metastasis process by inhibiting NK-cell cytotoxicity, thus protecting intraluminally trapped tumor cells [190].

Mast cells

Mast cells (MCs) are critical tissue-resident cells with immunoregulatory functions toward inflammation that can trigger not only protective immune responses at interface organs, such as the skin or gut, against bacterial, viral, and parasitic infections [191], but also uncontrolled reactions in allergic diseases. During cancer progression, it has been shown that tumors were infiltrated by a higher number of MCs [192]; in particular, MCs are key cell modulators of tumor angiogenesis in high vascularized tumors (e.g., hemangioma and hemangioblastoma), or several hematological and solid tumors [193]. MCs can also be seen as double-sided cells, because they can exert both detrimental and beneficial effects on tumor progression. As protumor cells, they can release several growth factors that are able to trigger tumor development and angiogenesis including TNF-α, VEGF, PDGF, TGF-β, FGF-2, CXCL-8, osteopontin, and nerve growth factor (NGF) [193]. On the other hand, MCs display antitumor functions such as the ability to produce inflammatory cytokines such as IL-1, IL-2, IL-17, and IFN-γ and they trigger target cell cytotoxicity by releasing TNF-α or ROS. Further MCs enhance NK cell recruitment in an MC-derived CCL3-dependent orthotopic melanoma model and by the release of CXCL8 [194].

Moreover, MCs can also release TGF-β in the TME and this action could be the main drive to switch the CD56dimCD16⁺ NK cell subset to the noncytotoxic CD56brightCD16⁻ type cell.

It has also been reported that MCs can produce adenosine and this metabolite has strong inhibitory effects on NK functions by the decreasing NKG2D and NKp30 expression on NK cells as well as TNF-α and IFN-γ production [103].

The relevance of the cellular dialogue between MCs and NK cells inside the tumor has recently been brought to light, in particular, in the tumor angiogenic process and accumulating data pointed out of that these two cells can interact and synergize with each other in the TME [195].

Tumor-associated fibroblasts

Tumor-associated fibroblasts (TAF) are the fibroblasts present in the tumor tissue (Fig. 1) [196–199]. According to several reports, TAF show unique phenotypic and functional features that are determined by the intense interaction with tumor cells [196–199]. Thus, it is thought that tumor cells can shape the expression in tissue fibroblasts leading to their “transformation” into TAF [196–199]. Also, there is some evidence that TAF can be derived from bone marrow mesenchymal stem cells (BMMSC) and that there are some phenotypic similarities between TAF and BMMSC [196–199]. Indeed, TAF express typical surface molecules shared with all mesenchymal stromal cells such as CD29, CD44, CD73, CD90, CD106, and CD117, besides extracellular matrix proteins, such as collagen, vimentin, α-smooth muscle actin, and nestin.
More importantly, TAF produce VEGF, TGF-β, immunoregulatory cytokines such as IL-10 and IL-4 and display the potential to differentiate into adipocytes, chondrocytes, and osteoblasts [196, 199]. These findings would suggest that TAF conserve some properties of mesenchymal stromal cells (MSC) [197]. Alternatively, some MSC are present among TAF [197]. Whatever be the explanation, it is evident that it is difficult to distinguish among the different mesenchymal stromal cell populations that can be present within the TME [196–199]. Furthermore, a large part of the reported findings is derived from TAF isolated and cultured in vitro. Indeed, a clear characterization of TAF immediately after their isolation from the tissue is hampered by the relative low amount of these cells. Thus, the culture conditions used can favor the overgrowth of some subsets of TAF or MSC or even a unique type of these cells. Moreover, it has been claimed that TAF can express higher amounts of cytokines and growth factors in comparison with other MSC [196–199]. However, a large part of these reports compares cells derived from unrelated donors or established by different research groups [196–199]. This leads to the discussion of unreliable results because there is high variability of the culture conditions and stimulation protocols in the different reports present in the literature [196–199]. Altogether, these findings can give rise to a confusing scenario where some phenotypic and functional features of TAF can be dependent on the culture conditions used to expand and analyze them instead of unique characteristics related to this cell type within TME [196–199]. This is particularly true when the functional behavior of TAF is analyzed in vitro [196–199].

It is well established that TAF can regulate NK cell activation, proliferation, and tumor cell killing through different molecular mechanisms such as indoleamine dioxigenase (IDO), arginase 1 and 2 (Arg1 and Arg2), heme-hoxigenase (HOX), PGE2, TGF-β, IL-10, and adenosine [55, 200–206]. This effect appears to be mainly related to the direct interaction between NK cells and TAF as some observed inhibitory effects are not so evident or can be undetectable when NK cells and TAF are artificially separated by porous membranes [197–199]. It is conceivable that the immunosuppressive effect of TAF on NK cells can be relieved through two possible mechanisms: (a) eliminating TAF and (b) shaping the immunosuppressive properties of TAF with drugs to convert TAF from immunosuppressive to immunostimulant. In this context, it has been reported that NK cells can kill self-TAF [111]. Indeed, LFA-1 expressed on NK cells can interact with ICAM1+ TAF, and eventually the NK cell-mediated cytolysis is triggered through the NKG2D and DNAM1 NK cell receptors. NKG2D recognizes on TAF stressed molecules such as MICA/B and ULBPs while DNAM1 interacts with PVR and nectin-2 expressed on TAF [197–199].

**Mesenchymal stromal cells**

MSC represent a cellular component of the TME involved in several pathophysiological processes that may lead to shape the behavior of tumor cells as well as the immune response and fate of the tumor disease [197–199]. Indeed, MSC can produce several components of the extracellular matrix, factors involved in the angiogenesis, epithelial mesenchymal transition of tumor cells leading to metastasization and regulation of antitumor immune response [197–199]. MSC are cells of mesodermal origin that can show a plastic behavior related to their surrounding microenvironment (Fig. 1) [197–199]. Based on the topological site of...
identification and intensity of expression of a series of nonspecific surface, intracellular markers can be considered as fibroblasts, myofibroblasts (myoF), pericytes (Pc), and TAF, also named as carcinoma-associated fibroblasts, CAF [197–199]. Some of the MSC can be considered as cells with a unique differentiation potential (at least in in vitro models of differentiation) like stem cells, and to reinforce this notion they have been shown to express Sox-2, Nanog, and other stem cell markers. Thus, MSC can differentiate into adipocytes, chondrocytes, and osteocytes under appropriate culture conditions [197–199]. However, a specific cell surface marker of the MSC subset able to differentiate has not been identified yet; and it is not clear whether fully differentiated MSC such as an adipocyte can dedifferentiate to a pluripotent stem cell [197–199]. MSC and their differentiated counterpart can secrete VEGF and several other cytokines involved in the regulation of angiogenesis. Furthermore, Pc show an anatomical localization in strict proximity with endothelial cells (EC), and there are several experimental evidences on the cross talk between Pc and EC both in physiological and pathological processes [207, 208]. Hereafter, we will summarize and discuss the features of the different types of MSC present in the TME and their cross talk with tumor cells and tumor infiltrating NK cells.

Adipocytes

Adipocytes are mesenchymal stromal cells of the adipose tissue involved in several adipose tissue functions such as the storing and metabolism of fat and the interaction with a plethora of immune cells (Fig. 1) [209–212]; thus, these cells together with endothelial cells and adipocyte precursors are in the adipose tissue microenvironment, and they make this tissue an immunological organ linking the organism metabolism and immunity [212, 213]. Also, adipose tissue acts as an endocrine organ that secretes cytokines and is an important reservoir for lymphocytes.

Obesity is associated with dysfunction of the adipose tissue cell components and it is well established that obesity can increase the risk of different cancers [214, 215]. Thus, it is relevant to understand the NK cell interaction with adipocytes because this cross talk can regulate the obesity and consequently the metabolism of the whole organism and finally the fate of cancer cells [214, 215].

Adipocytes are endocrine cells that dynamically produce several cytokines with anti and proinflammatory effects and adipokines that regulate insulin signaling [216]. Indeed, NK cells can respond to stressed cells because of cancer, infection, or inflammation [217]. Obesity can lead to a proinflammatory state that can trigger in the microenvironment the expression of stress molecules such as NKG2D ligands to which NK cells can respond; consequently, NK cells produce proinflammatory cytokines skewing M1 macrophage polarization of macrophage resident into the fat; this promotes macrophage secretion of paracrine (IL6 and TNF-α) and endocrine factors such as leptin and adiponectin, which in turn promote insulin resistance. These findings are mainly found in murine models while the situation in humans is more confusing [218–226]. It is worth noting that human adipose tissue appears to be enriched for a peculiar CD56brightCD16− NK cell subset with low cytolytic activity [227–229]. It is evident that several tumors are associated with obesity and in particular with colon cancer [230, 231]. It has been reported that NK cells preincubated with leptin showed a
decrement of IFN-γ production and cytolytic activity against colon cancer cells besides a decreased expression of NK cell activating receptors. Furthermore, the quantity, size, and weight of the colon tumor induced by azoxymethane was higher in diet-induced obese rats than in normal rats. This was accompanied by lower numbers of NK cells in the spleen and liver with a lower expression of activating receptors [232]. Altogether, these data support the idea that the impairment of NK cell-mediated functions in obese rats may impair the control of colon tumor cell growth [232].

Thus, adipocytes through the adipokine leptin can regulate NK cell function. However, adipocytes can be used to trigger antitumor immune response. Indeed, it has been shown that adipocytes can be considered as a suitable target for a recombinant adeno-associated viral (rAAV) vector to deliver an IL-15/IL-15Rα complex to trigger an antitumor effect in murine models [214]. Indeed, IL-15 is a proinflammatory cytokine playing a key role in the development and maturation of NK cells [214]. IL-15 interacting with its own specific IL-15Rα associated with βγ chains common to IL-2 can trigger proliferation and increase the expression of activating receptors such as NKG2D, NKp30, NKp44, and NKp46. It has been shown that the intraperitoneal injection of this rAAV expressing IL-15/IL-15Rα almost exclusively in adipose tissue led to the expansion in the adipose tissue, spleen, and peripheral blood without evident side effects. These NK cells were preferentially cytotoxic, expressing NK1.1 CD11b^high^CD27^low^ antigens; more importantly, the growth of subcutaneously implanted Lewis lung tumors and B16-F10 melanoma metastases was strongly inhibited [214]. It is worth noting that NK cells were expanded upon the injection of rAAV in mice without implanted tumors too; this indicates that the IL-15/IL15-Rα pathway was activated, but this activation was not accompanied by evident toxic effects. Altogether, these findings suggest that the triggering of the IL-15 signaling pathway in adipose tissue can elicit a well-tolerated NK cell-mediated immune response and that this rAAV.IL-15/IL-15Rα^+^ adipocyte-specific vector can be considered as a new tool for cancer immune gene therapy [214].

Altogether, these data can support the hypothesis that IL-15/IL-15R expression in adipocytes can transform these cells from immunosuppressive to immunostimulant. On the other hand, it has been reported that in obese mice the expression of IL-6 and TNF-α together with NK cell-mediated cytotoxicity in response to influenza virus A/PR8/34 is markedly retarded. This led to an early and strong lung damage, suggesting that obesity can increment morbidity and mortality during viral infections [233, 234].

**Endothelial cells**

ECs are fundamental cells regulating several key processes such as angiogenic programs in both wound healing and tumor switch angiogenesis induction and progression. ECs interact with diverse immune and stromal cells in the TME, and concerning NK cells we have described a peculiar NK cell subset in NSCLC patients, expressing CD56^bright^CD16^−^VEGF^high^PIGF^high^CXCL8^+^IFN-γ^−^ phenotype and able to trigger human umbilical vein endothelial cell (HUVEC) migration and to stimulate capillary-like structure formation [3, 25, 34]. These in vitro proangiogenic abilities exerted by patients’ NK cells were present not only at the tissue level but also in the peripheral blood, indicating that the
angiogenic switch in NSCLC NK cells had occurred also at the systemic level [34]. In an
thrilling way, we observed that patients with squamous cell carcinomas exhibited NK cells
with higher proangiogenic activities as compared with those with adenocarcinomas, being
more efficient in stimulating endothelial cells in vitro, via soluble factors [34].

Cross talks between ECs and NK cells share similarities with the interaction of these two
cell types within the developing decidua during pregnancy. In the decidua, several works
have identified and characterized dNK cells as CD56^{superbright}CD16^- KIR^+ NK cells with
fundamental roles in the establishment of successful placentation, likely by directing the invasion
of trophoblast cells into the decidua basalis and remodeling of the maternal spiral arteries
[17, 117]. dNK cells are Tbet^+ EOMES^+ CD56^{superbright} and represent almost up to 70% of total
tissue leukocytes and 30%-40% of all decidual cells. It has been shown that dNK cells differ
from peripheral CD56^{bright} cells, because the former expresses the tissue residency markers
CD69, CD49a, integrin β7, and CD9, and the majority of them are positive for Nkp46, Nkp80,
NK2D, and CD94/NKG2A receptors [118]. It has been postulated that a combination of
TGF-β, IL-15, and IL-18 could drive different isoform expression inducing regulatory
profiles such as Nkp30c and Nkp44a,c, which are associated with a switch from cyto-
toxic to tolerogenic NK cell features [235–237] with distinction from peripheral blood
CD56^{bright} CD16^-NK cells [238]. Moreover, dNK cells are weakly cytolytic and produce high
amounts of proangiogenic factors, such as VEGF, PIGF, CXCL-8, and IL-10 [17].

We were the first to describe decidual-like proangiogenic polarization of NK cells in dif-
f erent types of tumors, i.e., NSCLC, malignant pleural effusions, CRC, and prostate cancer
[3, 21, 25]. In particular, we showed that TINKs in CRC patients are skewed toward the
CD56^{bright}CD16^-CD9^+CD49a^-subset, and are able to trigger in vitro HUVEC proliferation,
migration, and vessel formation [21]. Interestingly, we demonstrated that CRC TANKs also
express a decidual-like CD56^{bright}CD9^+CD49a^- phenotype, with the ability to produce several
proangiogenic factors such as VEGF, Angiogenin, CXCL-8, MMP1, MMP9, and TIMP-1
and stimulate angiogenesis in vitro [21]. Moreover, CRC TANKs had increased levels of
STAT3 and STAT5 activation, and in vitro blocking of these two molecular pathways through
the use of the antipsychotic agent Pimozide resulted in the reduction of their proangiogenic
(release of VEGF, CXCL-8, Angiogenin) and prometastatic (production of MMP-1 and
MMP-9) activities [21].

Pericytes

Pericytes (Pc), also called mural cells, are located on the abluminal surface of blood cap-
illaries, and interact closely with endothelial cells in the presence of the same basal membrane
(Fig. 1) [239]. Pc is fibroblast-like cells and show quite long cellular processes that adhere to
ECs. This interaction is mediated by both fibronectin and intercellular junctions such as tight-
gap-, and adherent junctions [240, 241]. Pc can influence the venule blood flow and perme-
ability of vascular walls. Pc can be identified in tumor vasculature through their reactivity in
immunohistochemistry and cytofluorimetry with NG2, CD105, CD90, and PDGFβ-R but not
with CD31, LFA-1, and P1H12 [242, 243]. Pc interact with a large part of endothelial sprouts in
tumor murine models and they are recruited, at least in part, through PDGF-mediated signaling
[242, 243]. Pc may be involved in the generation of new, although primitive, vessels [244];
indeed, they produce and secrete VEGF and their expression of matrix metalloproteinases may have a role in driving the degradation of fibronectin and other extracellular matrix proteins of the basal membrane [244, 245]. Thus, the role of Pc in shaping the tumor angiogenesis is evident, although the precise role of Pc in the alterations of tumor neoangiogenesis is still to be defined. On the other hand, Pc are stromal components of the TME, although the degree and the relevance of their interaction with tumor endothelial cells remain an open question [246–248]. Pc can secrete several cytokines and chemokines in response to inflammatory stimuli such as IL-1β, TNF-α, and IFN-γ [249]. CXCL-8, CXCL-1, CXCL-10, CCL-2, CCL-3, CCL-5, and IL-6 are Pc-derived chemokines that, besides stimulating angiogenesis, can recruit into the site of inflammation several types of innate cells such as granulocytes, monocytes, T, and NK lymphocytes. Thus, it is conceivable that within the tumor site, where an inflammatory response is ongoing, the Pc can influence the migration of innate cells to the tumor site. Also, Pc can express toll-like receptors such as TL4 and NOD1, and they can respond to bacterial products secreting chemokines [250]. Several works have pointed out the immunomodulatory role of Pc through several molecular mechanisms shared by MSC [249, 251–258]. Also, it is worth noting that anti-Pc therapy can lead to an increase of CD8+ tumor infiltrating lymphocytes in murine models [249, 251–258]. These findings suggest that the targeting of Pc can improve the antitumor adaptive immune response. About the interaction between NK cells and Pc, the cross talk between NK and Pc of the liver such as stellate cells can be found in the literature [259–261]. Indeed, it has been reported that NK cells can eliminate stellate cells upon activation with IL18 and TLR3 ligand triggering. It is worth noting that this activation is dependent on the signaling through the p38/PI3K/AKT pathway [259]. Also, it has been shown in a murine model of colorectal carcinoma cells growing into the liver that (i) many HSC were localized next to the tumor invasion line, (ii) the HSC showed a strong cross talk with tumor cells [203], and (iii) NK cells can recognize and kill HSC through the triggering via NKp46 and NKG2D activating receptors [262–265]. It has not been demonstrated that NK cells can kill other kinds of Pc in tumor tissues and it is well known that Pc can show a different phenotype depending on the tissue they reside in. However, it is conceivable that the regulatory role of Pc can be counteracted by NK cell-mediated recognition and killing.

Therapeutic TME-targeted therapies

Chimeric antigen receptor (CAR)-NK cells and adoptive NK cell therapy

The clinical success of using chimeric antigen receptor (CAR)-T cell therapy mainly against malignancies of B lymphocyte lineage such as acute lymphoblastic leukemia and non-Hodgkin lymphomas has triggered interest to improve their efficacy in reducing the serious side effects [266]. Among antitumor lymphocytes, besides CD8+ T cells, NK cells can exert HLA-class I independent recognition and killing of several tumor target cells [267]. Thus, ILCs such as NK cells have become a matter of interest for CAR technology (Table 1). Indeed, severe toxicities, manufacturing time, and costs are the main current limitations of CAR-T cell. Usually, 3–4 weeks are needed to derive CAR-T cells from a given patient and the quality of T cells obtained from a chemo-refractory patient in an autologous setting is not always very good (5). Also, the adverse effects of CAR-T cell therapy are quite frequent [268–275]. These side effects including neurotoxicity and cytokine release syndrome can impair the healthy
TABLE 1  Clinical studies using chimeric antigen receptor (CAR)-NK cells for adoptive tumor cell therapy (according to https://clinicaltrials.gov/ct2/results?cond=Tumor&term=CAR+NK&cntry=&state=&city=&dist=).

<table>
<thead>
<tr>
<th>Study number/enrollment/completion/status/results</th>
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<th>Interventions biological</th>
<th>Phase</th>
<th>Sponsor/collaborators</th>
<th>Locations</th>
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<td>NCT03940820 20 May 2020 Recruiting No results</td>
<td>Clinical Research of ROBO1 Specific CAR-NK Cells on Patients with Solid Tumors</td>
<td>ROBO1 CAR-NK cells</td>
<td>Phase 1 Phase 2</td>
<td>Asclepius Technology Company Group (Suzhou) Co., Ltd.</td>
<td>Radiation Therapy Department, Suzhou Cancer Center, Suzhou Hospital Affiliated to Nanjing Medical University, Suzhou, Jiangsu, China</td>
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<td>NCT03415100 30 December 2019 Unknown No results</td>
<td>Pilot Study of NKG2D-Ligand Targeted CAR-NK Cells in Patients with Metastatic Solid Tumors</td>
<td>CAR-NK cells targeting NKG2D ligands</td>
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<td>The Third Affiliated Hospital of Guangzhou Medical University</td>
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<td>NCT03931720 20 May 2022 Recruiting No results</td>
<td>Clinical Research of ROBO1 Specific BiCAR-NK/T Cells on Patients with Malignant Tumor</td>
<td>BiCAR-NK/T cells (ROBO1 CARNK/T cells)</td>
<td>Phase 1 Phase 2</td>
<td>Asclepius Technology Company Group (Suzhou) Co., Ltd.</td>
<td>Department of Oncology, Suzhou Kowloon Hospital, Shanghai Jiaotong University School of Medicine, Suzhou, Jiangsu, China</td>
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<td>Study of Anti-PSMA CAR NK Cell in Castration-Resistant Prostate Cancer</td>
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<td>Early Phase 1</td>
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<td>NCT03692637 30 November 2021 Not yet recruiting No results</td>
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<td>Clinical Research of ROBO1 Specific BiCAR-NK Cells on Patients with Pancreatic Cancer</td>
<td>BiCAR-NK cells (ROBO1 CAR-NK cells)</td>
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<td>Asclepius Technology Company Group (Suzhou) Co., Ltd.</td>
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<td>NCT03056339 36 June 2022 Recruiting No results</td>
<td>Umbilical &amp; Cord Blood (CB) Derived CAR-Engineered NK Cells for B Lymphoid Malignancies</td>
<td>iC9 / CAR.19 / IL15-Transduced CB-NK Cells</td>
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<td>M.D. Anderson Cancer Center</td>
<td>University of Texas MD Anderson Cancer Center, Houston, Texas, United States</td>
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<td>NCT02944162 10 September 2018 Unknown status No results</td>
<td>CAR-pNK Cell Immunotherapy for Relapsed/Refractory CD33+ AML</td>
<td>anti-CD33 CAR-NK cells</td>
<td>Phase 1</td>
<td>PersonGen BioTherapeutics (Suzhou) Co., Ltd.</td>
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<td>NCT03940833 20 May 2022 Recruiting No results</td>
<td>Clinical Research of Adoptive BCMA CAR-NK Cells on Relapse/Refractory MM</td>
<td>BCMA CAR-NK 92 cells</td>
<td>Phase 1</td>
<td>Asclepius Technology Company Group (Suzhou) Co., Ltd.</td>
<td>Department of Hematology, Wuxi People’s Hospital, Nanjing Medical</td>
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<td>Early phase 1</td>
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<td>January 1, 2021</td>
<td>Recruiting</td>
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<td>Study of anti-CD19 CAR-NK Cells in Relapsed and Refractory B-Cell Lymphoma</td>
<td>Phase 1</td>
<td>NCT02892695</td>
<td>September 2019</td>
<td>Recruiting</td>
<td>Beijing Cancer Hospital, China Academy of Medical Sciences, The First People’s Hospital of Hefei, Beijing Cancer Hospital</td>
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<td>CAR-pNK Cell Immunotherapy in MUC1 Positive Relapsed or Refractory Solid Lymphoma</td>
<td>Phase 1</td>
<td>NCT02839954</td>
<td>July 2018</td>
<td>Recruiting</td>
<td>Beijing Cancer Hospital, PersonGen BioTherapeutics (Suzhou) Co., Ltd., Suzhou, Jiangsu, China</td>
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<td>CAR.CD19-CD28-zeta-2A-iCasp9- IL15-Transduced Cord Blood NK Cells, High-Dose Chemotherapy, and Stem Cell Transplant in Treating Participants with B-cell Lymphoma</td>
<td>Phase 1</td>
<td>NCT03579927</td>
<td>October 2019</td>
<td>Withdrawn</td>
<td>MD Anderson Cancer Center, Houston, Texas, United States</td>
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### TABLE 1  Clinical studies using chimeric antigen receptor (CAR)-NK cells for adoptive tumor cell therapy (according to https://clinicaltrials.gov/ct2/results?cond=Tumor&term=CAR+NK&cntry=&state=&city=&dist=)—cont’d

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<th>Phase</th>
<th>Sponsor/collaborators</th>
<th>Locations</th>
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<td>NCT04538599 36 September 1, 2022 recruiting</td>
<td>RD13-01 for Patients with r/r CD7 + T/ NK Cell Hematologic Malignancies</td>
<td>Drug RD13-01 infusion and NK cells</td>
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<td>The First Affiliated Hospital@College of Medicine, Zhejiang University, Hangzhou, Zhejiang, China</td>
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<td>NCT03882840 30 Recruiting January 1, 2022</td>
<td>Induced-T Cell-like NK Cellular Immunotherapy for Cancer Lack of MHC-I</td>
<td>NK cell therapy</td>
<td>Phase 1 Phase 2</td>
<td>Affiliated Hospital of Guangzhou Medical University Hunan Zhaotai Yongren Medical Innovation Co. Ltd.</td>
<td>The Second Affiliated Hospital of Guangzhou Medical University, Guangzhou, Guangdong, China</td>
</tr>
<tr>
<td>NCT0383978 30 Recruiting</td>
<td>Intracranial Injection of NK92/5.28.z Cells in Patients with Recurrent HER2-positive Glioblastoma</td>
<td>NK92/5.28.z</td>
<td>Phase 1</td>
<td>Johann Wolfgang Goethe University Hospital DRK Blutspendedienst Baden-WürttembergHessen gGmbH Georg-SpeyerHaus LOEWE Center Frankfurt Cancer Institute</td>
<td>German Cancer Research Center</td>
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<td>NCT02742727 10 March 2018 Unknown status</td>
<td>CAR-pNK Cell Immunotherapy in CD7 Positive Leukemia and Lymphoma</td>
<td>anti-CD7 CAR-pNK cells</td>
<td>Phase 1 Phase 2</td>
<td>PersonGen BioTherapeutics (Suzhou) Co., Ltd. The First People’s Hospital of Hefei Hefei Binhu Hospital</td>
<td>PersonGen BioTherapeutics (Suzhou) Co., Ltd., Suzhou, Jiangsu, China</td>
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*Interventions: Drug: Fludarabine, Cyclophosphamide, Mesna, Biological: iC9/CAR.19/IL15-Transduced CB-NK Cells, AP1903 (Rimiducid is a lipid-permeable tacrolimus analogue).

+Autologous Hematopoietic Stem Cell Transplantation, Carmustine, Cytarabine, Etoside, Filgrastim, Melphalan.

Note T cells with NK-like activity.
state and be fatal if not treated [275]. NK cells have a clear advantage with respect to CAR-T cells. Indeed, allogeneic haplo-identical NK cells have been used successfully in bone marrow transplantation [276, 277] without generating graft versus host disease; moreover, NK cells are responsible for the graft versus leukemia effect because tumor cells bear stress antigens and ligands for activating receptors such as NKG2D, DNAM1, NKp30, and NKp44, which can trigger NK cell-mediated antitumor cytolysis [278]. Eventually, NK cells can recognize cancer stem cells (CSC) by the same activating receptors, although CSC can release some of their ligands to escape from the immune system mediated control [279]. It is important to note that NK cells can display serial killing ability and are long-lived with a memory-like behavior at least in the case of recognition of cytomegalovirus. Thus, CAR-NK cells can be a valid alternative to CAR-T cells, although the large majority of clinical trials ongoing are using CAR-T cells (Table 1) [266, 280].

CAR-NK cells have been derived from several NK cell populations, including the NK cell line NK92, cord and peripheral blood NK cells, and induced pluripotent stem cells (iPSC)-derived NK cells [281–284]. The main drawbacks in generating CAR-NK cells are their refractoriness to genetic engineering, limited potential of cell expansion, and limited persistence after infusion [278]. These limitations have been partly overcome using new methods to lead genes integration and combinations of cytokines and feeder cell cultures to increase NK cell expansion [282–284]. The hematopoietic cell signal transducer (HCST or DAP10) and the Tyro protein tyrosine kinase-binding protein (TyroBP or DAP12) are key components of CAR-NK cell constructs alongside CD3ζ [281]. The NK92 cell line, besides cord and peripheral blood NK cells, is the more frequent NK cell used in the current recruiting clinical trials [285–287] for treating either solid or hematological tumors. NK92 cells express NKG2D, NKp30, and NKp46 but not CD16. This lack has prompted the engineering of NK92 with a CD16 molecule to trigger the ADCC effect using appropriate humanized monoclonal antibodies [288]. It is important to note that NK92 has been used for generating a large array of NK-specific CAR utilizing four transmembrane domains such as CD16, NKp44, NKp46, and NKG2D and costimulatory domains including 2B4, DAP10, DAP12, and CD137 combined with CD3ζ [281]. This has allowed to define a better combination for the different domains and further use them to generate iPSC engineered CAR-NK cells [281]. Indeed, it has been shown that from iPSC can be generated from NK cells with a strong ability to eliminate mesothelin-expressing tumors in in vitro and in vivo models [281]. Thus, iPSC-derived NK cells expressing these CAR can be a tool ready to treat refractory neoplasia that would substitute the use of other sources of NK cells [281].

### Immunomodulatory drugs

Also, the use of immunomodulatory drugs (IMIDs) such as thalidomide, lenalidomide, and several other derivatives recently identified [289–291] can influence the functional behavior of TAFs or MSCs present in the TME (Fig. 1) [289–291].

Indeed, in multiple myeloma (MM), MSC play a key role in supporting the growth of neoplastic cells and the targeting of bone marrow MSC with IMiDs having a key role in MM therapy as well as in other hematopoietic malignancies such as non-Hodgkin lymphomas, chronic lymphocytic lymphoma, myelodisplastic syndrome, and myeloid leukemias [292–296]. Also,
IMIDs can trigger not only tumor cell apoptosis but also activation and proliferation of antitumor effector cells [297] besides modifying the TME. In this regard, lenalidomide can upregulate the expression of ligands for NKG2D and DNAM1 on MM cells, increasing the ability of NK cells to recognize and kill MM cells. Furthermore, lenalidomide, at variance with thalidomide, downregulates the expression of immune checkpoint inhibitors on stromal cells and MM cells [297]. Altogether, these findings suggest that IMIDs have several effects on the TME [298]. In particular, IMiDs can target cereblon and, consequently, the Ikaros family of transcription factors [299]. In the preclinical murine model, the relevance of NK cells as targets for IMiDs has been pointed out [300]. Also, it has been reported that IMiDs can trigger IFN-γ production by NK cells [301, 302] together with other cytokines, reducing the level of SOCS1 in antigen-presenting cells and T lymphocytes [300]. IMiDs such as lenalidomide and CC-4047 together with either the anti-CD20 antibody Rituximab or docetaxel can increase in vitro the proapoptotic activity against either Raji lymphoma B cells or prostate carcinoma cells of NK cells in a coculture system composed of unfractionated peripheral blood mononuclear cells (PBMCs) and tumor cells [303]. In this context, it has been demonstrated that IMiDs can induce the degradation of Ikaros and Aiolos transcription factors, which downregulate the expression of CD38 at the MM cell surface. Also, these two factors inhibit the expression of interferon stimulated genes through interference with the nucleosome remodeling and deacetylase complex; on the contrary, treatment with IFN-β of MM cells increase the surface expression of CD38 [304]. This upregulation of CD38 renders MM cells prone to NK cell-mediated ADCC induced in the presence of the anti-CD38 antibody daratumumab. These findings indicate that the expression of a therapeutic target molecule such as CD38 on MM depends on the activity of Ikaros and Aiolos factors and IFN-β. Also, NK cell activity is essential to eliminate MM cells. It is worth noting that CD38 can be expressed on several lymphomas such as Hodgkin’s lymphoma, diffuse large B-cell lymphoma, and peripheral T-cell lymphoma [305–307]; this suggests that the molecular mechanisms relevant for increasing the efficacy of anti-CD38 antibodies on MM cells can have a role in several other hematological malignancies that can be a therapeutic target with IMiDs and NK cells [294, 308].

**Conclusion**

It is well established that NK cells can exert a strong antitumor effect by eliminating cancer cells and are influenced by several cytokines and chemokines in the TME. NK cells can directly interact with tumor cells. Further, they secrete nanovesicles expressing activating NK cell receptors, which leads to the release of killing mediators such as perforins. Also, NK cells can influence adaptive antitumor immunity through a cross talk with antigen-presenting cells. This indicates that NK cells are essential in the fight against tumor development and growth.

On the other hand, different cellular components of the TME can downregulate NK cell-mediated tumor cell killing and production of antitumor cytokines, leading to the escape of tumor cells from both innate and adaptive immune system controls. Tumor-infiltrating NK (TINK) cells show a unique phenotype similar to NK cells localized in decidua tissues. These

**V. Targeting NK cells**
NK cells play a key role in the regulation of the angiogenesis of the TME, which may lead to a protumor effect. This facilitates neovascularization of the tumor and eventually promotes tumor metastasization. Both antitumor and protumor effects should be considered if adoptive therapy with NK cells is used to treat tumors. Indeed, CAR-NK cells may have less side effects than CAR-T cells and several trials are ongoing to treat tumors that are resistant to chemotherapeutic agents or relapse. The use of iPSC-derived NK cells as an off-the-shelf source of CAR-NK cells is a very attractive possibility, but the role of these cells in influencing tumor angiogenesis has to be analyzed deeply. Indeed, the features of any NK cell should be considered as a host-related characteristic of cancer that can influence the outcome of both conventional chemotherapy and the targeted therapies.

Acknowledgments

We thank Dr. Matteo Gallazzi, Laboratory of Immunology and General Pathology, Department of Biotechnology and Life Sciences, University of Insubria, Varese, Italy, for support to literature research. We thank Dr. Barbara Bassani, IRCCS National Cancer Institute, Milan, Italy, and Dr. Annalisa Bosi, Immunology and General Pathology Laboratory, Department of Biotechnology and Life Sciences, Università degli Studi dell’Insubria, Varese, Italy, for helpful discussion and revision of the chapter. Dr. Bruno is supported by the Italian Association for Cancer Research (AIRC-MFAG-22818), the Cariplo Foundation (Proj. Id 2019-1609) and the Italian Ministry of Health Ricerca Corrente-IRCCS MultiMedica.

Author contributions

All the authors listed made a substantial, direct, and intellectual contribution to the work and approved it for publication.

References


V. Targeting NK cells
15. The dual role of Natural Killer cells during tumor progression


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References


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Benjamin_Bonavida, 978-0-12-824375-6

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V. Targeting NK cells
Immunometabolic targeting of NK cells to solid tumors

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Abstract

The function of natural killer cells in tumors is often impaired due to metabolic adaptations of cancer cells, which causes reprogramming of natural killer cell effector and metabolic functions in favor of impaired immunity. As a result, targeting natural killer cells to solid tumors grapples with cells that meet a hostile environment unable to support effector functions. This not only results in cancers that are able to evade targeting, but also promotes natural killer cell dysfunction, most commonly manifested through altered phenotypic and functional signatures. In this chapter, we review the key elements driving natural killer cell metabolism, highlight the role of metabolism in supporting their function against solid tumors, and discuss targeting strategies to drive natural killer cells to solid tumors for immunotherapy.

Abbreviations

<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>BIKE</td>
<td>bispecific killer cell engager</td>
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<tr>
<td>CAR</td>
<td>chimeric antigen receptor</td>
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<tr>
<td>DNAM-1</td>
<td>DNAx molecule 1</td>
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<tr>
<td>GBM</td>
<td>glioblastoma</td>
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<tr>
<td>HIF</td>
<td>hypoxia-inducible transcription factor</td>
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<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
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<tr>
<td>IFNγ</td>
<td>interferon γ</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
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<tr>
<td>ITIM</td>
<td>immunoreceptor tyrosine-based inhibitory motif</td>
</tr>
<tr>
<td>ITT</td>
<td>immunoglobulin tail tyrosine</td>
</tr>
<tr>
<td>KIR</td>
<td>killer immunoglobulin receptor</td>
</tr>
<tr>
<td>LDHA</td>
<td>lactate dehydrogenase A</td>
</tr>
<tr>
<td>MDSC</td>
<td>myeloid-derived suppressor cell</td>
</tr>
<tr>
<td>NFAT</td>
<td>nuclear factor of activated T cells</td>
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Although the presence of natural killer (NK) cells in solid tumors has been shown to correlate to improved patient outcomes, their numbers and activity in these tumors are often low. The tumor microenvironment (TME) presents a formidable obstacle to NK cell effector functions, acting, at once, to induce dysfunction, limit persistence, and stunt infiltration of NK cells. Checkpoint molecules, which have resulted in transformative inhibitor-based immunotherapies in a number of cancers, also signal on NK cells and, as such, affect their activities. However, solid tumors are often characterized by severe heterogeneity, which exerts its immunosuppression in a multifaceted fashion and further limits the effectiveness of various immunotherapies [1]. Evidence has indeed shown that NK cells are often dysfunctional in solid tumors, with distinct phenotypic and metabolic profiles. The metabolism of NK cells is emerging as a key player driving the cytotoxic potential of NK cells, their ability to persist inside tumors, and their infiltrating capacity. Metabolic reprogramming is a common hallmark of tumor-infiltrating NK cells in response to altered fuel availability and the rapid glycolytic metabolism of cancer cells [2]. Such reprogramming results in altered glycolysis and oxidative phosphorylation, as well as the production of cytokines by NK cells [3]. Alongside tumor-induced functional inhibition of NK cell responses through metabolic effects, solid tumor heterogeneity also results in downregulation or mutation of target antigens. This includes hypoxia, low pH, and the release of immunosuppressive small molecules, and further promotes immune evasion [4]. Antigen escape on cancer cells results in phenotypic changes that further promote evasion from immune recognition, and a decreased efficacy of cell-based antigen-specific monotherapy, for instance, chimeric antigen receptors (CARs) [5], an effect that has been observed clinically in a number of solid tumors [6–8], triggered by different mechanisms, including differential splicing, missense mutations, or lineage switch. Contributing to evasion mechanisms are the highly selective pressures created by proliferating engineered cells targeting a single antigen. Collectively, the complex reorganization of immune responses induced by the severely immunosuppressive TME is shifting our understanding of NK cell responses in solid tumors toward the need for systems that can help us study responses and develop immunotherapies that act on multiple pathways of immune resistance.

V. Targeting NK Cells
Inhibitory checkpoints

NK cell inhibition and immunosuppression within the TME is often regulated by inhibitory receptors also called “checkpoints,” upregulated within the TME that severely impair or entirely shut down NK cell function and cytolytic activity [9, 10]. NK cell inhibition is controlled primarily by either killer immunoglobulin-like receptors (KIRs) or the CD94/NKG2A complex. The KIR family includes a number of different receptors that bind to HLA-A and HLA-B (KIR2DL1, KIR3DL2, and KIR3DL3), as well as HLA-C (KIR2DL1, KIR2DL2, and KIR2DL3) on target cells [11]. Alternatively, CD94/NKG2A recognizes the nonclassical HLA-E ligand on both mouse and human cells [9, 10]. However, there are many other inhibitory receptors on NK cells including TIGIT, CD96, PD-1, TIM-3, and LAG-3, which modulated NK cell immunosuppression in the TME in an MHC-independent manner [12, 13].

Inhibitory KIRs have been well characterized for their role in NK cell inhibition, through their recognition of MHC class I molecules (HLA-A, B, and C). KIRs, like many other inhibitory receptors, contain an ITIM motif, which, upon activation, recruits SHIP-1/2 and SHIP, inducing an inhibitory intracellular signal [14]. These receptors inhibit NK cell activation against the patient’s own cells, and lack of matching KIRs or HLA will induce NK activation [15]. However, upregulation of KIRs on NK cells induced by cancer cells has been observed within the TME in various cancers [16, 17]. Lirilumab, an anti-KIR blocking antibody targeting KIR2DL1, KIR2DL2, and KIR2DL3, has shown clinical efficacy in treating patients with hematologic malignancies and solid tumors [18, 19]. Further, in vitro studies utilizing cytokines (IL-12, IL-15, and IL-18), aimed to downregulate KIR expression on NK cells, and observed a resultant increase in ADCC-mediated lysis of cancer cells, demonstrating the potential for KIR targeted checkpoint therapies [20]. Unfortunately, lirilumab infusion has also been linked with reduction in KIR2D expression, limiting efficacy of this blockade against multiple myeloma [21].

Recently, NKG2A has also emerged as a promising target for checkpoint inhibition therapy, particularly within the context of solid tumors. NKG2A heterodimerizes with CD94 on NK cells and, upon binding with HLA-E on tumor targets, induces inhibition through ITIM activation and SHP-1 recruitment [22, 23]. NKG2A-positive NK cells as well as cancers expressing upregulated levels of HLA-E have been reported in ovarian, endometrial, CRC, and cervical cancer, with large populations coexpressing both NKG2A and PD-1 [24]. NKG2A blockade with monalizumab has proven effective in enhancing NK effector functions, including degranulation, tumor lysis, and interferon \( \gamma \) (IFN\( \gamma \)) secretion, and has also shown clinical efficacy alone and in combination with PD-1 and EGFR-targeted therapies [24]. Nonetheless, checkpoint inhibition studies targeting both NKG2A and KIR have been relatively limited compared with other checkpoint receptors (PD-1/PD-L1, CTLA-4, etc.) and require further investigation.

Further, TIGIT (T-cell immunoreceptor with Ig and ITIM domains) and CD96, along with activating receptor DNAM-1 (DNAX molecule 1), play an overwhelming role in NK cell functionality within the context of cancer, and specifically with difficult to treat cancers such as glioblastoma (GBM) [25, 26]. The role of the NK cell TIGIT/CD96/DNAM-1 axis with their ligands, particularly CD155, in cancer is complex and nuanced, with multiple players each affecting NK cell responses in different and often divergent ways, and is an example of the sophistication of the interplay between receptors and ligands in the solid tumor TME. TIGIT and CD96 both have

V. Targeting NK Cells
been shown to have inhibitory functions on NK cells in the context of cancer, reducing cytolytic functions [27–30]. TIGIT has an Ig-like extracellular domain along and an intracellular domain with immunoreceptor tyrosine-based inhibitory motif (ITIM) and an immunoglobulin tail tyrosine (ITT) motifs [31–34]. The cytoplasmic ITIM domain is key to TIGIT’s inhibitory function, as it has been well characterized in other inhibitory receptors, such as KIRs and NKG2A [29, 35, 36]. Activation of TIGIT through interaction with CD155 or CD112 effectively diminishes NK cell cytolytic functions by reducing IFNγ secretion and interfering in degranulation [37, 38]. TIGIT has been shown to be upregulated on NK cells within the TME of many different cancers, and TIGIT blockade has proven an efficacious strategy for enhancing NK cell-based therapies, presenting TIGIT as a potential immunotherapy target [39–41]. Interestingly, CD96 contains both inhibitory intracellular ITIM domains, as well as a YXXM motif, which has been characterized on many NK cell-activating receptors, such as NKG2D [42]. This suggests that CD96 may have both immunosuppressive as well as activating functions within the context of NK immunotherapy. However, recent studies have shown that targeting of CD96, via blockade, has proven efficacious in the treatment of glioma, particularly when combined with other checkpoint therapies, such as PD-1 or CTLA-4 blockade [30, 43–46].

In addition, upregulation of other checkpoint molecules, such as PD-1 and CTLA-4, expressed on regulatory lymphocytes, and CD155 or the poliovirus receptor (PVR) expressed on the surface of GBM cells further limits the efficacy of immunotherapies by suppressing the cytolytic activity of adoptively transferred T and NK cells [47–52]. PD-1, expressed primarily on regulatory T cells, has been shown to have immunosuppressive function in cancers, resulting in an exhausted effector cell phenotype and loss of proliferative and cytolytic functions [49]. Further, PD-1 has been extensively studied, and has been shown to be upregulated on NK circulating and tumor-infiltrating NK cells in patients with multiple myeloma, Kaposi sarcoma, head and neck cancer, and ovarian cancer [49, 53–55]. Targeting of PD-1 on NK cells has proven successful, with a phase two clinical trials have shown anti-PD-1 antibody blockade to be efficacious in treating multiple myeloma [54]. Blockade of PD-1 in tandem with blockade of other TME-induced checkpoints on NK cells has also proven efficacious, with PD-1 blockade significantly enhancing the therapeutic effect of blocking many receptors expressed on NK cells and cancer cells within the TME, including CD73, TIGIT, and NKG2A [24, 50, 52]. Therefore, the PD-1/PD-L1 is a very promising target for NK cell-based therapeutics, particularly as a combinatorial therapy.

In addition, CTLA-4 has been linked with immunosuppression of effector T and NK cells through interactions with regulator T cells within the TME of GBM [47]. CTLA-4 has been primarily characterized on T cells, though it is expressed on NK cells as well. It competes with CD28 to prevent NK and T-cell activation, and induce immunosuppression [56]. CTLA-4 has been shown to be expressed on mouse NK cells and may be involved inhibiting NK cell cytokine secretion [57]. However, on human NK cells, CTLA-4 is not well characterized. Anti-CTLA-4 antibody blockade has been shown to enhance NK cell cytolytic functions, likely through targeting of T cells [58]. Blockade of CTLA-4 targets regulatory T cells, minimizing Treg-induced immunosuppression of NK cells and allowing NK cells to lyse tumor cells [58]. Further CTLA-4 blockade has been correlated with increased T-cell activity, specifically IL-2 secretion, which could promote NK cell function [59]. Further, targeting of CTLA-4, like PD-1, has proven successful, when combined with targeting of ectonucleotidase CD73 [50]. Therefore, CTLA-4 checkpoint inhibition may also be a promising strategy in NK cell-based cancer therapies.

V. Targeting NK Cells
Other checkpoint receptors have also been targeted to overcome TME-induced immunosuppression of NK cells, including TIM-3 and LAG-3. TIM-3 has been reported to bind CEACAM-1, Gal-9, HMGB1, and PS on tumor cells, and has been correlated with exhaustion in T cells [60]. However, in NK cells, understanding of TIM-3 has been limited. Increased TIM-3 expression has been shown to correlate with an exhaustion phenotype in NK cells from patients with metastatic melanoma [60]. Further, blockade of TIM-3 has been shown to reverse NK cell exhaustion and restore toxicity against tumor targets [60, 61]. Further, TIM-3 expression is significantly upregulated through cytokine stimulation (IL-2, IL-12, IL-15, IL-18, IFNα), and TIM-3 expression increases correlated with NK functional performance, indicating that TIM-3 may be a maturation marker for NK cells, which limit NK function after prolonged expression to prevent chronic inflammatory responses [60, 62, 63]. Nonetheless, there are currently a number of clinical trials targeting TIM-3, either alone or in combination with other targets, such as PD-1. These studies aim to treat advanced and primary liver cancer, AML, and other advanced malignancies, but have yet to be completed [64]. On T cells, LAG-3 interacts with MHC class II molecules, preventing TCR activation [65]. However, on NK cells, the role of LAG-3 still remains unclear. In mice, deficiency in LAG-3 did not affect lysis of MHC-I-mismatched cells, and blockade of LAG-3 does not directly affect NK cell cytotoxicity [66, 67]. Nonetheless, LAG-3 could be a potential target for checkpoint inhibition therapy, due to its expression on both NK and T cells, but more investigation is needed.

Further, bispecific and trispecific killer engagers (BiKEs and TriKEs) represent a promising alternative immunotherapeutic strategy to overcome TME-induced immunosuppression. Traditionally, NK cell activation is regulated through a large panel of activating and inhibitory receptors [68]. NK cell cytotoxicity occurs through natural cytotoxicity in the case of NCRs (NKp30, NKp44, NKp46), or through antibody-dependent cell-mediated cytotoxicity (ADCC), where CD16 recognizes antibodies bound to a cancer cell surface and elicits a potent antitumor response. BiKEs and TriKEs are multitargeted therapeutics that combine scFv fragments recognizing cancer markers, while also engaging NK cell cytolytic responses through induction of ADCC by direct interaction with CD16 [68]. These engagers have proven efficacious in targeting ligands on B-cell malignancies (CD19 and CD22), AML (CD33), and colorectal cancer (CD133) [68–70]. Further, these engagers can be engineering to release cytokines, such as IL-15, directly into the TME, preventing nontargeted immune activation and, ultimately, enhancing NK cell therapies [71]. In the clinic, CD33-targeted BiKEs and TriKEs have proven efficacious in treating MDS patients, where both degranulation and cytokine production were enhanced in patients receiving the therapeutic [72]. Another study, targeting CD30 showed synergistic activity of a CD30 BiKE when combined with PD-1 blockade, indicating that cotherapies involving BiKEs, TriKEs, and mAB checkpoint therapies may greatly improve clinical outcomes for patients receiving NK-targeted therapies [73].

Immunometabolic reprogramming in solid tumors

Metabolism of NK cells in cancer

Metabolism of NK cells has emerged as a key driver of their effector responses in the context of cancer. The TME induces profound changes of NK cell metabolic responses, rewiring
their effector functions in ways which can further potentiate immune dysfunction in settings of solid tumors. A growing body of work has focused on understanding NK cell metabolic signatures in vivo, particularly with regard to functional markers unique to these cells and their distinct phenotypic and functional subsets.

Most of what is known about metabolism in lymphocytes comes from the studies of CD8+ T cells. While T and NK cells share a number of similarities, their metabolic statuses differ in terms of signaling output and the effector functions they perform in the solid tumor TME.

Glycolytic fueling reduces glucose availability to surrounding immune cells, leading to metabolic reprogramming of the immune system. In NK cells, regulation of metabolic response by upregulation of glucose uptake and glycolysis is mediated by mTORC1 [74]. mTOR is essential for regulating the production of granzyme B and perforin, and can most potently be activated with high concentrations of IL-15 during early infection, though other cytokines (IL-2, IL-12, IL-18) are also implicated [75]. IL-15 activates mTORC1 via PI3K, PDPK1, and AKT. While NK cells do not exhibit increased glycolysis during short-term activation, extended stimulation with high-dose IL-15 over multiple days leads to upregulation of metabolism, enhancing glycolysis. mTORC1 also enhances glycolysis by promoting transcription factor HIFα and mitochondrial biogenesis through PPARγ coactivator 1α (PGC1α) and yin and yang 1 (YY1) [76]. Recently, Srebp, otherwise implicated in de novo lipid synthesis, has been shown to regulate functional responses and NK cell effector function, by supporting glycolysis and oxidative phosphorylation through the citrate-malate shuttle, encoded by its targets Acly and Slc25a1 [77]. mTORC1 activation requires sufficient intracellular nutrients and energy. In response to diminishing glucose supplies, NK cells are thought to undergo metabolic reprogramming by foregoing IL-15 and mTOR dependency, instead becoming driven by activating receptors (e.g., Ly49H in mice and KIR in humans) [78]. However, the metabolic reprogramming of failed NK metabolism in a tumor setting is still the subject of intense study. This reprogramming has been suggested to be activation dependent: cytokine-stimulated NK cells can produce IFNγ independent of glycolysis or mitochondrial oxidative phosphorylation, while activating receptor-stimulated NK cells require oxidative phosphorylation [79].

Evidence is also emerging that metabolic signatures also differ among human NK cell subsets. In addition to greater activation of mTORC1, CD56bright cells are thought to have higher rates of glucose uptake compared with CD56dim cells, associated with their higher expression of IFNγ. While CD56dim cells are more cytotoxic, they also have a lower biosynthetic burden and are likely to have lower metabolic requirements.

Despite these insights, significant gaps in our understanding of NK cell metabolism in pathological settings such as solid tumors still remain. Understanding of the mechanisms by which mTOR regulates the metabolic system and NK cell effector responses in the TME are unknown is still nascent. Little is also known about how these metabolic changes occur in phenotypically distinct NK subsets, particularly licensed cells. For instance, it is not yet known whether licensed NK cells display metabolic responses different to those of unlicensed cells, and whether altered NK phenotypes associated with in vivo NK exhaustion correlate to altered metabolic programs. Moreover, the role of mTOR-mediated regulation of protein translation during NK cell effector responses is entirely unknown. This dearth of knowledge places metabolism as a central question in the development of NK cells as effective immunotherapeutics in the setting of solid tumors.

V. Targeting NK Cells
Metabolic reprogramming of NK cells in solid tumors occurs in large part in response to a number of soluble factors and metabolites.

**Adenosine**

Adenosine in the solid tumor TME is a heavily suppressive metabolite, which accumulates in response to hypoxic fueling of cancer cells. Migrating tumor cells from hypoxic regions lead to severe angiogenesis. As a result, low tumor oxygenation constitutes a major problem for many solid tumor patients [80]. Hypoxia triggers the onset of immunosuppressive adenosine signaling [81]. Elevated concentrations of adenosine in the TME's hypoxic cores lead to immunosuppression via dysregulation of NK cells [82]. Hypoxia is a powerful mediator of the activities of the ecto-nucleoside triphosphate diphosphohydrolase CD39 and ecto-5' nucleotidase CD73 [83–85]. These enzymes constitute a catalytic cascade, which leads to the generation of adenosine [86, 87]. Extracellular ATP is first converted into AMP by CD39, followed by CD73, which dephosphorylates AMP into adenosine [88, 89]. Adenosine signaling plays an important role in tissue adaptation during hypoxia. Adenosinergic immunosuppression of infiltrating NK cells signals through purinergic adenosine receptors, particularly the A2A receptor [90]. Adenosinergic signaling regulates the maturation of NK cells [91], the accumulation of cytotoxic CD56dim cells at tumor sites [91], and NK effector function [92]. Adenosine signaling also results in downregulation of receptor expression on NK cells, specifically NKG2D on cytokine-primed human NK cells [93]. It was also shown that adenosine downregulates the metabolism of NK cells, including glycolysis and oxidative phosphorylation, by downregulating the transcriptional expression of genes including LDHA and GAPDH.

Elevated expression of CD73 [94–96] in GBM tumors was shown to be associated with worse overall survival [97]. As a result, adenosinergic signaling through CD73 is a negative feedback loop that regulates local and systemic antitumor response [98, 99]. CD73 blockade [100] was shown to result in tumor inhibition that relies strongly on recruitment of NK cells, and the presence of NK-produced IFNγ and perforin [101, 102]. In addition, inhibition of CD73 was shown to improve antitumor efficacy of anti-PD-1 and anti-CTLA-4 checkpoint inhibitors in preclinical models of various solid tumors [50]. There are now multiple anti-CD73 antibodies being tested in clinical trials for the treatment of various solid tumors.

Therapeutic relief from adenosine-mediated inhibition of NK cell effector functions has been shown to be induced by blockade of CD73 activity. Data have shown that administration of a CD73 antibody enhanced the effector function of chimeric antigen receptor (CAR)-engineered NK cells both in vitro and in vivo [103]. Similarly, coadministration of adenosine receptor inhibitors with anti-CD73 antibodies is an antitumor strategy that has been pursued in the setting of solid tumors with promising preclinical results.

**Hypoxia**

Oxygen availability is a driver of immune responses. Many solid tumors are characterized by low oxygen levels, which have been shown to correlate to immune cell presence and function. Due to alterations in oxygen levels in the TME of solid tumors to favor hypoxia, low
oxygenation has been recognized as a dynamic regulator of antitumor functions of immune cells.

Though NK cells respond to oxygen levels, the precise role of hypoxia on the function of NK cells in cancer is nuanced. While hypoxia has been shown to impair NK cell cytotoxicity [104–106], alter their metabolism [107], and downregulate the expression of activating receptors NKp46, NKp30, NKp44, and NKG2D as well as perforin and granzyme B [108], these effects are balanced by their responsiveness to specific activation triggers. For instance, activating marker expression on NK cells could be partially restored by treatment with the cytokine IL-2, based on evidence in multiple myeloma [109]. Apart from cytokines, hypoxic heterogeneity—including the extent and duration of hypoxic conditioning—contributes to the divergent responses of NK cells to oxygen levels. For instance, data have shown that priming NK cells with IL-15 under short-term (<48 h) hypoxia can enhance NK cell functions and metabolism, including TCA cycle and oxidative phosphorylation processes [110]. That both stimulation and the hypoxic environment play a significant role in defining NK cell responses to hypoxia was further shown through evidence that while hypoxia is able to downregulate the release of IFN\(\gamma\), TNF\(\alpha\), GM-CSF, CCL3, and CCL5 by NK cells, the same study showed that hypoxia could sustain elevated expression of the chemokine receptors CCR7 and CXCR4, leading to higher NK cell chemotaxis to CCL19, CCL21, or CXCL12 [107].

Mechanistically, the effect of hypoxia on NK cell function is thought to occur via reduced phosphorylation of ERK and STAT3, induced by the activation of protein tyrosine phosphatase SHP-1 (Src homology region 2 domain-containing phosphatase-1) in hypoxia [111].

Immune cells, including NK cells, are able to respond to hypoxia through hypoxia-inducible transcription factors (HIFs)—HIF-1, HIF-2, and HIF-3 [112]. HIFs, in particular HIF-1\(\alpha\) and HIF-2\(\alpha\) in the context of NK cells, are key drivers of immune adaptation to low oxygen. Hypoxia, often encountered by NK cells infiltrating the microenvironment of solid tumors, causes upregulation of HIF-1\(\alpha\) on NK cells. Loss of HIF-1\(\alpha\) on NK cells was shown to result in the inhibition of tumor growth in vivo, alongside an increase in angiogenic cytokine vascular endothelial growth factor (VEGF). This was caused by a lower infiltration of NK cells that express angiostatic soluble VEGFR-1. Similarly, HIF1-\(\alpha\)-deficient NKp46\(^+\) NK cells displayed an enhanced ability to control tumor growth and produce IFN\(\gamma\) [113].

Lactate

The metabolite lactate has been shown to induce dysfunction of tumor-infiltrating NK cells. Lactate dehydrogenase-A (LDHA) is expressed in cancer cells and is responsible for the conversion of excess pyruvate and NADH into lactate and NAD\(^+\). Lactic acid is, in turn, generated in the TME following the accumulation of lactate and protons. LDHA itself has emerged as a negative prognostic marker for many cancer patients. Elevated expression of lactate dehydrogenase A (LDHA) on cancer cells has been correlated with poor prognosis in tumor patients [114]. High lactate concentrations in the TME of solid tumors have been associated with tumor progression, metastasis, and recurrence [115]. Therapeutically, inhibition of LDHA has been explored as a means to restore immune dysfunction on the basis of evidence that blockade of its activity is able to impair tumorigenesis and tumor progression in mouse models of various cancers [116, 117].
Lactate is also not favorable to NK cells, with mounting evidence demonstrating its role in the impairment of NK immunosurveillance. Tumor lactate acidosis has been shown to impair IFN$\gamma$ production by NK cells, which could be reversed pharmacologically [118]. Impaired IFN$\gamma$ production was also reported by NK cells in Ldha$^{\text{low}}$ tumors compared to wild-type tumors, likely by disabling NFAT activation [119]. Similarly, lactate was reported to induce a downregulation in perforin and granzyme B on NK cells, as well as activating receptor NKp30 [120]. In addition to these direct effects on NK cytotoxicity, lactate also impairs NK cell cytotoxicity by increasing numbers of immunosuppressive myeloid-derived suppressor cells (MDSCs). In addition, lactate-induced acidification resulted in the apoptosis of NK cells in colorectal liver metastases, reducing their contribution to antitumor immune responses [121].

**Oxidative stress**

Solid tumors are characterized by high levels of oxidative stress, which impairs immune function through the production of free radicals, including reactive oxygen species (ROS). NK cell functions are impaired in response to the presence of ROS, including their ability to interact with and bind to cancer cells [122]. One pathway involved in NK cell responses to ROS involves the NADase CD38. CD38, expressed intracellularly and on the plasma membrane of NK cells, is an enzyme responsible for degrading nicotinamide adenine dinucleotide (NAD$^+$) into ADP-ribose and nicotinamide. Because NAD$^+$ protects against oxidative stress, NK cells with lower CD38 expression were shown to exhibit higher resistance to cell death due to ROS [123]. Elsewhere, NOX2-derived ROS was shown to promote metastasis of melanoma cells by downregulating NK cell function. Conversely, inhibition of NOX2 was shown to be able to restore IFN$\gamma$ production and tumor control by NK cells [124]. Stimulation of NK cells with IL-15 was able to confer resistance to oxidative stress to NK cells through the thioredoxin system activated by mTOR [125].

**Soluble factors**

NK cell immunosuppression in the TME is further modulated through other soluble factors and cytokines, not directly involved in NK cell metabolism, which includes PGE-2, TGF-$\beta$, and IL-10 [126, 127]. In T and NK cells, TGF-$\beta$ inhibits activation, proliferation, and IL-2 production, suppressing activity of effector cells and promoting regulatory T-cell (Treg) phenotype [128, 129]. TGF-$\beta$ has also been associated with the upregulation of key molecules, such as MMP-2 that promote tumor invasion in GBM [130]. Blockade of the TGF-$\beta$ signaling pathway has proven effective in enhance NK cell cytolytic responses in vitro against breast cancer targets, and represents a promising strategy for overcoming TGF-$\beta$ induced NK cell suppression [131]. Further, TGF-BR1 blockade has also been shown to enhance the efficacy of anti-GD2 blockade, restoring DNAM-1, NKp30, and NKG2D expression of NK cells from neuroblastoma patients, and enhancing degranulation and ADCC cytotoxicity [132]. Studies have also shown that blockade of TGF-$\beta$ with neutralizing mAbs was able to restore NK cell
cytolytic functions, including activating receptor expression, proliferation, and cytokine secretion, demonstrating the efficacy of TGF-β as a potential immunotherapeutic target [133]. IL-10 has been shown to reduce cytokine production in T and NK cells, specifically the production of IFNγ, resulting in a reduction in inflammatory immune responses within the GBM microenvironment [134]. Further, IL-10 reduces expression of target molecules on cancer cells within the TME, and potentially reduces NK cell antitumor effects [135]. Levels of IL-10 have been shown to be elevated in patients with gastric cancer, along with TGF-β, suggesting that gastric cancer cells may secrete IL-10 to reduce NK cytotoxicity [136]. Although IL-10 has not been explored extensively in the context of cancer, it, nonetheless, represents a potential target for NK immunotherapy.

Similarly, PGE-2 has been shown to suppress T and NK cell proliferation, IL-2 receptor expression, and cytotoxicity [137]. PGE-2 induces COX-2 activity in lymphocytes, and its levels in cancer patients are closely correlated with cancer stage and prognosis [138–140]. Further, PGE-2 has been shown to inhibit NK cell activity in thyroid cancer patients, through downregulation of activating receptors and inhibition of differentiation and maturation of NK cells [141]. One study showed that blockade of PGE-2 receptors on monocytes was able to prevent PGE-2-induced immunosuppression of NK cells, and prevent metastasis in breast cancer [142]. Other studies have shown that inhibition of PGE-2 through COX-2 silencing was able to inhibit MDSC-directed immunosuppression of NK cells and restore NK cell activity in melanoma [143]. Therefore, targeting of PGE-2 represents a potential strategy for enhancing NK immunotherapies of solid tumors.

**Trafficking to solid tumors**

The prognostic role of NK cells in tumors suggests that there is benefit to these cells inside tumors, and that the intratumoral presence of NK cells often associates with improved patient outcomes [144]. Reasons for the poor presence of NK cells in many tumors include those already discussed, as well as impaired migration to tumor sites. Infiltration of NK cells to solid tumors is driven by networks of gradients formed between chemokine ligands and their respective receptors on NK cells and cancer cells [145–147]. It is common, however, for solid tumors to alter the expression levels of chemokine ligands or their receptors which, in turn, impairs the ability of NK cells to reach depths of tumors necessary for optimal effector responses. Impaired NK cell migration has resulted in a lower number of NK cells able to infiltrate tumors and carry out effector functions.

Chemokines, together with their receptors, act by directing the migration of NK cells along a chemical gradient of ligands, a process known as chemotaxis. NK cells express several chemokine receptors, including CXCR1, CXCR3, CXCR4, CXCR6, and CX3CR1 [148]. Correspondingly, the ligands of those chemokine receptors can recruit NK cells, including CXCL9, CXCL10, CXCL12, and CXCL16. Although many of these receptors are expressed constitutively, their expression levels can be modulated during NK cell activation. It was demonstrated, for instance, that CXCR3 expression on human NK cells could be decreased following the treatment for 6 h or 24 h with IL-2 and IL-12 either alone or in combination. Downregulation in response to IL-2 stimulation was also observed for CCR7, leading in
all cases, to reduced chemotaxis to their corresponding ligands [149]. Conversely, IL-2 could induce expression of CCR4 and CX3CR1 on NK cells. In response to altered chemokine receptor/ligand expression in solid tumors, strategies aimed at restoring the balance between chemokine ligand/receptor axes in favor of restored NK cell migration is the subject of growing research interest. Genetic engineering of NK cells to express CXCR2 was shown to promote their migration to renal cell carcinoma, which secretes ligands for the receptor, in transwell systems [150]. Similarly, engineering of EGFRviii-CAR-NK cells to express the chemokine receptor CXCR4 promoted their chemotaxis to CXCL12/SDF-1α secreting U87MG glioblastoma cells [151].

Understanding and subsequently modulating chemokine receptor/ligand axes is a promising approach to drive the deeper infiltration of NK cells into solid tumors where they can, in turn, promote more durable antitumor effector responses.

Perspective: Overcoming immune resistance to NK cell targeting in solid tumors

NK cell-based immunotherapy has emerged as a rapidly growing field with huge potential for targeting and treating many difficult-to-cure tumors. While therapies targeting hematologic malignancies, utilizing both T and NK cells, have been relatively successful, the TME of solid tumors represents a significant immunosuppressive burden that needs to be overcome when designing therapies that target solid cancers. Targeted therapies, such as checkpoint blockade, have shown remarkable responses in a number of cancers, including lung cancer and melanoma. However, response rates are still low and many other cancers, such as GBM, are largely unresponsive to checkpoint inhibitors or single-antigen monotherapy. The complex immunometabolic reprogramming that NK cells undergo in the TME alters their glycolytic fueling and dampens their activation and antitumor functions. Soluble metabolic factors such as adenosine and lactate and molecules such as TGF-β must all be considered in the context of enhancing NK cell lysis of solid tumor targets. Multifunctional-targeted therapies that streamline NK cell activation, such as BiKEs and TriKEs, offer a promising approach to overcoming tumor-induced immunosuppression, as do multifunctional CAR-based constructs. The potential conferral of a superior survival advantage to treated subjects by enhancing the duration of tumor suppression and eliminating recognition escape has led to the development of genetic constructs with two antigens expressed on a single immune cell that either share or have completely dissociated cosignaling domains [152]. These strategies—resulting in bispecific, dual, or tandem targeting strategies, can enhance receptor activity, while also selecting for specific signaling components to enhance CAR cell persistence and effector function. In this context, avoidance of activation-induced cell death [153] has been shown to be of particular concern for CAR-T-based therapies [154] and can be addressed by rational design of combinatorially incorporated signaling regions. Multiple CARs with cells engineered to target two antigens [155], such as HER2 and IL13Rα2 [156], have been described preclinically. Multispecific targeting of solid tumors with NK cells is still in its infancy, however, the relatively safer administration of NK cell-based therapies compared to toxicities associated with CAR-T cells makes use of NK cells as platforms for the development of such targeting.

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approaches attractive and the expectation is that these therapies will grow in prominence in the coming years.

Due to the complexity of the TME, including heterogeneity as well as localized low pH and hypoxia, it is likely that no single therapeutic approach will be efficacious in treating these solid tumors, but, rather, a combinatorial therapy targeting multiple immunosuppressive factors in the TME (hypoxia, nutrient depletion, inhibitory checkpoints, trafficking, etc.) will be more successful. The potential in combining the innate ability of NK cells to recognize cancers with the targeting specificity imparted by genetic engineering will play a major role in further expanding the clinical potential of NK cells.

Acknowledgments

The authors acknowledge the V Foundation for Cancer Research and the Walther Cancer Foundation for providing financial support.

References


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Targeting metabolism to potentiate NK cell-based therapies

Payal Dhar and Jennifer D. Wu

Abstract

NK cells are critical components of the immune system that play a major role in immune surveillance against transformed and virally infected cells as well as in immune regulation. Despite their potent effector potential, NK cells face several challenges in pathological conditions, resulting in their impaired functionality and reduced in vivo persistence. These factors limit the overall efficacy of NK cell-based therapies and there is a critical need to understand the mechanisms underlying NK cell dysfunctions. Cell metabolism is now acknowledged to play an important role in determining the functional fate of immune cells. Rapidly emerging evidence suggests that immunometabolism research is opening up new avenues for critical understanding of immune responses and can be targeted to improve NK cell-based therapies. In this review, we discuss the current literature on NK cell metabolism, its regulation in normal and disease conditions, and potential therapeutic approaches targeting NK cell metabolism for improving NK cell-based therapies.

Abbreviations

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<tr>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>ACLY</td>
<td>ATP-citrate lyase</td>
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<tr>
<td>ARID5B</td>
<td>AT-rich interaction domain 5B</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>FADH₂</td>
<td>flavin adenine dinucleotide</td>
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<tr>
<td>FBP1</td>
<td>fructose-1,6-bisphosphatase</td>
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<td>Glut1</td>
<td>glucose transporter 1</td>
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<tr>
<td>IFNγ</td>
<td>interferon gamma</td>
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<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
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<tr>
<td>NADH</td>
<td>nicotinamide adenine dinucleotide</td>
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<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<td>TRAIL</td>
<td>tumor necrosis factor-related apoptosis-inducing ligand</td>
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Competing interest

Authors declare no conflict of interest.

Introduction

NK cells are effector lymphocytes that play a pivotal role in the elimination of tumors and viral infections. NK cell functions are regulated by an array of germline-encoded activating and inhibitory receptors and the balance of these receptors determines their ability to detect and eliminate target cells without prior sensitization [1]. There are two key mechanisms through which NK cells recognize and eliminate target cells. NK cells recognize the transformed or infected cells that present downregulated expression of MHC class I molecules, a mechanism called “missing self.” Alternatively, target cells with upregulated expression of stress-induced ligands of activating receptors are recognized by NK cells through “induced-self” mechanism [1]. NK cells execute direct cytotoxic effects against target cells through the secretion of granzyme B and perforin, engagement of death receptors such as Fas and TRAIL, and also mediate antibody-dependent cellular cytotoxicity (ADCC). NK cells are also potent producers of cytokines such as IFNγ. Apart from being a critical component of innate immunity, NK cells also regulate adaptive immune responses through the secretion of immune-regulatory cytokines and through cross talks with dendritic cells [2, 3]. Their intrinsic ability of spontaneously targeting cancer cells without antigen specificity makes NK cells promising candidates for cancer immunotherapy. NK cell-based therapies are being rapidly developed and tested in clinics [4]. Although NK cell-based therapies have shown remarkable efficacy against hematological malignancies, their efficacy in the treatment of solid tumors still remains poor [1, 5]. Several factors including poor persistence, expansion, and defective functionality in vivo significantly limit their application for immunotherapies, raising a critical need to understand the mechanisms that lead to NK cell dysfunction.

Immunometabolism research in the last decade has clearly established that cell metabolism plays a critical role in regulating immune cell responses [6]. Importantly, in disease conditions such as cancer, viral infections, and obesity, there is a complex interplay between cellular metabolism and immune cell functions because of the high variability in availability of nutrients and metabolites. Rapidly emerging evidence from immunometabolism studies has demonstrated that NK cell functions are highly dependent on metabolic processes. Glucose-fueled oxidative metabolism and glycolysis have been demonstrated to be crucial for NK cell effector functions [7–9]. In addition to the effector functions, various recent studies have described that metabolism plays an important role in NK cell developmental, education process, and adaptive NK cell responses [10–14].

The current major focus in the field is to investigate the key regulatory mechanisms that would facilitate the understanding of how specific metabolic configurations define the function of NK cells in the context of health and disease.

The goal of this review is to summarize the current literature that has examined NK cell metabolism and its impact on NK cell functions, key regulators of NK cell metabolism, and how NK metabolism is impaired in disease conditions, with the prime focus on

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cancer. The understanding of these key aspects provides unique opportunities to target altered NK cell metabolism, thereby modulating NK cell function for effectuating NK cell-based therapies.

**Brief overview of basic cellular metabolic pathways**

The basic immune cellular activities including growth, differentiation, and activation require energy production in the form of ATP synthesis. One of the essential metabolic fuels used by immune cells to generate energy is glucose. Immune cells are known to drive their ATP generation by engaging two major metabolic pathways including glycolysis and oxidative phosphorylation (OXPHOS). Glycolysis occurs in the cytosol and involves the conversion of glucose into pyruvate, other essential biosynthetic by-products, and generating only two ATP molecules for one molecule of glucose. Glycolysis-derived pyruvate can either be converted to lactate and secreted out of the cells to maintain glycolytic flux or it can be converted to acetyl-CoA and enters the TCA cycle (tricarboxylic acid cycle or citric acid cycle) in mitochondria and further metabolized to generate ATP. Reducing equivalents NADH and FADH2 are the two main products of the TCA cycle, which contributes the electrons to be transferred to the electron transport chain (ETC) to fuel the oxidative phosphorylation process that leads to the generation of 34 ATP molecules from one molecule of glucose [6]. Other than glucose, cells also utilize metabolic fuels such as glutamine (glutaminolysis) and fatty acids (fatty acid oxidation) to drive OXPHOS and generate energy by feeding into the TCA cycle. However, the significance of these metabolic pathways in NK cells is not completely understood.

**Basics of the NK cell metabolic profile**

Depending on the activation status of the cells, the basic metabolic phenotype of the cells varies. In resting state, mouse NK cells maintain low basal metabolic activity with a preferential utilization of glucose-fueled oxidative phosphorylation [10, 15]. Upon activation, NK cells undergo distinct stimulation-specific metabolic changes to facilitate functions such as IFNγ production [15]. NK cells mainly use glucose-fueled oxidative phosphorylation to facilitate their functions after short-term activation (4–6 h) with cytokines (IL-12 + IL-15) or activating receptor (NK1.1 or Ly49D)-mediated stimulation, but activation with IL-12 + IL-18 combination is independent of metabolic changes [15]. Pharmacological inhibition of both OXPHOS and glycolysis results in diminished IFNγ production [15]. NK cells upregulate both glycolysis and OXPHOS in response to longer activation period (18 h), preferentially relying more on glycolysis [7, 16]. Along with the increase in these metabolic pathways, there is an upregulation of the glucose transporter Glut1, transferrin receptor CD71, and the amino acid transporter CD98 [7, 10]. In addition, there is a concentration and time-dependent dynamics for IL-15-induced NK cell metabolic reprogramming. At low concentrations and shorter stimulation time points, IL-15 activates IFNγ production through OXPHOS, whereas both glycolysis and OXPHOS are upregulated at higher concentrations and longer stimulation time.
points, however, the metabolic dependence shifts more toward glycolysis, presumably to fulfill biosynthetic requirements [10, 15].

In humans, the two subsets of peripheral blood NK cells are defined on the basis of CD56 expression. CD56\textsuperscript{bright} cells are more potent producers of cytokines including IFN\textgamma, whereas CD56\textsuperscript{dim} cells are more cytotoxic [17]. These two human NK cell subsets have distinct metabolic features. CD56\textsuperscript{bright} cells have been identified to be more robust in terms of modulating their metabolic activities in response to cytokine stimulation to fulfill their biosynthetic demands for IFN\textgamma production compared to CD56\textsuperscript{dim} cells. Similar to mouse NK cells, IFN\textgamma production by human NK cells is also supported by enhanced glycolysis and OXPHOS in CD56\textsuperscript{bright} cells [16]. In response to cytokine stimulation by IL-2 and IL-12 + IL-15, CD56\textsuperscript{bright} cells also display enhanced expression of Glut1, CD71, CD98 as well as increased glucose uptake compared to the CD56\textsuperscript{dim} cell subset [16, 18]. Regulation through nutrient transporter expression is a common feature for both mouse and human NK cell metabolisms. The general metabolic profile of activated NK cells has been depicted in Fig. 1.

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![Basic metabolic profile of activated NK cells. Glucose is the primary metabolic fuel used by NK cells. Glucose uptake occurs primarily through glucose transporter Glut1. Glucose is metabolized into pyruvate via glycolysis in the cytoplasm. The pyruvate is further metabolized to lactate, which is secreted by the cells or converted to acetyl-CoA that feeds into TCA cycle in the mitochondria. TCA cycle and citrate-malate shuttle (CMS) generate reducing equivalents NADH and FADH\textsubscript{2} that provide electrons to be transferred to electron transport chain (ETC) to fuel oxidative phosphorylation for ATP production. Stimulation of NK cells with cytokines IL-2, IL-12, and IL-15 induces metabolic activation of NK cells. Increase in the nutrient transporter expression such as transferrin receptor CD71, amino acid transporters SLC7A5 and CD98 is a common metabolic feature of activated NK cells.](image-url)
Key regulators of NK cell metabolism

Given the critical role of metabolism in NK cell functions, it is very important to identify and have a clear understanding of the factors that regulate specific metabolic activities required for NK cell effector functions. Several signaling proteins and transcription factors have been identified as key regulators of NK cell metabolism. The activity of these regulators is essential in determining the metabolic configurations acquired by the cells and specific immune responses. The following section focuses on the key regulators of NK cell metabolism.

**mTOR**

The mechanistic/mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase that acts as a sensor for essential nutrients such as glucose, amino acids, oxygen, and growth factors and thus regulates several metabolic pathways in various cell types. mTOR exists as two distinct complexes, mTORC1 and mTORC2 [8, 19]. mTORC1 and mTORC2 are defined by their distinct binding partners “raptor” and “rictor,” respectively [20, 21]. Both mTORC1 and mTORC2 play a critical role in NK cell development [11, 22, 23].

Several studies have comprehensively demonstrated the importance of mTOR for NK cell development, metabolism, and functions [7, 10, 24]. Mouse NK cells undergo different maturation stages and are classified into four sequential subsets from immature to mature NK cells based on the expression of CD27 and CD11b: CD11b<sub>low</sub>CD27<sub>low</sub>, CD11b<sub>low</sub>CD27<sup>high</sup>, CD11b<sup>high</sup>CD27<sub>high</sub>, and CD11b<sup>high</sup>CD27<sub>low</sub> [25]. The NK-specific deletion of mTOR using MTOR<sub>lox/lox</sub>NKp46<sub>iCre</sub> mice led to their impaired developmental transition from CD11b<sub>low</sub> to CD11b<sup>high</sup>CD27<sup>high</sup> stage in the bone marrow and defective IL-15 signaling mediated STAT5 activation, suggesting the importance of mTOR in NK cell development process [10]. The mTOR activity was also found to be the highest in immature CD27<sup>high</sup> cells and lowest in mature CD11b<sup>high</sup> cells, and this decreased mTOR activity is associated with reduced expression of nutrient transporters CD71 and CD98. mTOR activity in NK cells has been shown to be induced by IL-15-mediated stimulation [10, 26]. mTORC1-regulated glycolytic reprogramming is critical for NK cell effector functions including IFNγ production and granzyme B expression both in vitro and in vivo [7]. Inhibition of mTORC1 by the specific inhibitor rapamycin significantly diminished the production of IFNγ, granzyme B, and perforin [7, 10]. Yang et al. investigated the individual contributions of mTORC1 and mTORC2 in NK cell development using NK cell-specific conditional deletion of raptor and rictor and found that both mTORC1 and mTORC2 are necessary at distinct NK cell development stages [22]. Deletion of mTORC1 disturbed NK cell homeostasis and early development stages of transition from the CD11b<sub>low</sub>CD27<sub>high</sub> to the CD11b<sup>high</sup>CD27<sup>high</sup> stage, whereas deletion of mTORC2 impaired the terminal maturation stages of NK cell development [22]. Intriguingly, a recent study by Wang et al. identified that mTORC2, as opposed to mTORC1, has an inhibitory effect on NK cell metabolism and functions [23]. They found a unique cross talk between mTORC1 and mTORC2, whereby mTORC1 activity mediated by IL-15 signaling sustained mTORC2 activity, but mTORC2 suppressed mTORC1 through inhibition of both STAT5 signaling and the amino acid transporter SLC7A5 expression. Specific deletion of mTORC2 (by deletion of Rictor) in NK cells led to increased NK cell metabolic...
activity characterized by enhanced nutrient receptor expression, increased mitochondrial activity as well as enhanced NK cell effector functions [23]. Altogether, these findings clearly demonstrate that mTOR activity is critical for NK cell development, metabolic activities, and effector functions.

SREBP

SREBP (sterol regulatory element-binding proteins) are transcription factors that have a canonical role in regulating lipid and cholesterol biosynthetic pathways [27]. A recent study by Assmann et al. demonstrated the previously undiscovered role of SREBP1 in mouse and human NK cell metabolism [8]. SREBP was essential for IL-2- and IL-12-induced metabolic reprogramming of NK cells via activation of the glucose metabolism and facilitated through citrate-malate shuttle (CMS). SREBP upregulates the expression of ACLY and SLC25A1 that are the two key genes essential for driving CMS. By shuttling and exchanging glucose-derived mitochondrial citrate with cytosolic malate, the CMS enables the conversion of NADH generated by glycolysis into mitochondrial NADH that drives OXPHOS and ATP generation in NK cells. NK cells deficient in the Scap gene, which controls the activation of SREBP or direct inhibition of SREBP pharmacologically by oxysterol 25HC or PF429242, resulted in impairment of NK metabolic activities including glycolysis and OXPHOS as well as their effector functions of IFNγ and granzyme B productions. In addition, the cytotoxic potential of NK cells against Yac1 target cells in vitro and antitumor functions in B16F10 melanoma tumor model were severely impaired [8]. These findings clearly highlighted the critical role of SREBP in regulating NK cell metabolic and functional activities. This study also proposed a new concept that instead of the TCA cycle, NK cells might use CMS for driving oxidative phosphorylation.

Myc

Myc is another important transcription factor for NK cell metabolic and functional responses. The role of Myc in the regulation of T cell metabolism is well established, but its role in NK cells has been recently demonstrated by several studies [28, 29]. In T cells, Myc regulates the metabolic reprogramming upon activation by enhancing the expression of the glucose and amino acid transporters as well as enzymes involved in glycolysis and glutaminolysis [30]. A comprehensive study by Loftus et al. showed that cMYC plays a critical role in IL-2/IL-12-induced metabolic reprogramming in mouse NK cells by regulating both glycolysis and OXPHOS. Interestingly, the availability of amino acid glutamine, which although is not a critical metabolic fuel for driving OXPHOS, is the main regulator of cMYC-mediated metabolic responses and antitumor functions of activated NK cells. Glutamine assists in the uptake of amino acids via SLC7A5 (L-type amino acid transporter), which is required for sustaining cMYC protein levels. The absence of glutamine or inhibition of SLC7A5 results in diminished cMYC expression and the associated impairment of NK cell effector functions. By using 13C metabolic tracing experiments, they further confirmed that in response to cytokine stimulation, the OXPHOS in NK cells is mediated by CMS and not by the TCA cycle as previously described [8] or by glutaminolysis [28]. This study also demonstrated that the transcription factor HIF1α,
which is critical for CD8 T cell glycolysis [31], is dispensable for IL-2-/IL-12-mediated metabolic reprogramming and functional responses in NK cells [28].

**PGC1α**

The regulators of NK cell glycolysis are well studied, but the regulators of mitochondrial metabolism in NK cell immune responses remain relatively obscure. One of the key regulators described by several studies is the transcriptional coactivator peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC1α) that is a master regulator of mitochondrial biogenesis. PGC1α has been associated with the regulation of mitochondrial metabolism and function in both CD8 T cells and NK cells [32–36]. In NK cells, PGC1α has been shown to be important for optimum cytotoxic activity and IFNγ production in high-dose IL-2-stimulated lymphokine-activated killer (LAK) cells [34, 35]. These studies provided initial evidence that primary human NK cells stimulated with high doses of IL-2 in vitro display substantially higher mitochondrial mass and mitochondrial membrane potential in a PGC1α-dependent manner and these functional and metabolic features were lost with the NK-specific deletion of PGC1α [34, 35]. Most recently, Gerbec et al. directly investigated the role of PGC1α in NK cell functions and metabolism using in vivo models and found that PGC1α-mediated transcription of mitochondrial activity genes is critical for optimum NK cell functions and metabolism [36]. NK-specific deletion of PGC1α resulted in their diminished cytotoxic potential and cytokine production that correlated with defective OXPHOS. These findings were recapitulated in human NK cells by pharmacological inhibition of PGC1α. Furthermore, PGC1α-deficient NK cells also displayed impaired ability in controlling B16F10 tumors, decreased mitochondrial mass and mitochondrial membrane potential compared to their wild-type counterparts, because of the reduced expression of PGC1α-regulated target genes [36]. Overall, these findings suggested that PGC1α is essential for maintaining NK cell metabolic activity required for facilitating antitumor responses.

**Role of mitochondrial dynamics in NK cell functions**

Mitochondria are highly dynamic organelles in terms of their morphology, which critically regulates their metabolic activity and functions [37]. A number of studies in T cells have established that mitochondrial metabolic activity is associated with their structural integrity and determined by a balance of mitochondrial fission and fusion [38, 39]. A landmark study by Zheng and colleagues demonstrated that aberrant mitochondrial morphology and metabolism are associated with dysfunctionality of tumor-infiltrating NK cells in liver cancer patients [40]. Tumor-infiltrating NK cells from these patients displayed mitochondrial fragmentation and enhanced expression of mitochondrial fission-associated genes. In addition to the morphological defects, tumor-infiltrating NK cells had diminished OXPHOS, mitochondrial mass, mitochondrial membrane potential, and increased mitochondrial ROS (reactive oxygen species). Mitochondrial fragmentation was driven by the hypoxic tumor microenvironment through activation of the mitochondrial fission regulator, dynamin-related protein 1 (Drp1) via mTOR activity. Inhibition of mTOR or Drp1 restored the morphological
defects in NK cell mitochondria and their effector functions [40]. Several other studies have also reported mitochondrial defects in NK cells from cancer patients [41, 42]. In addition, an important role of mitochondria in the formation of NK-tumor cell immune synapse formation has been reported [43]. Mitochondria of human NK cells were found to reorganize toward immune synapse, a process that was accompanied by rapid reduction in mitochondrial membrane potential upon contact with K562 target cells, indicating rapid energy utilization. This study suggests an important role of mitochondrial dynamics for NK cell cytotoxicity, potentially as a source of rapid energy [43]. Together, these studies highlight that targeting mitochondrial defects might be a potential strategy to rescue NK cell functionality in cancer.

Perturbation of NK cell metabolism in disease

There is accumulating evidence from mouse and human studies supporting that impairment of NK cell functions is associated with their altered metabolism in chronic disease conditions such as viral infections, obesity, and cancers. The following section focuses on some of the key studies demonstrating how NK cell metabolism is modulated in disease conditions.

NK cell metabolism and viral infections

Several studies have highlighted the “adaptive” NK cell responses characterized by their ability to form immunological memory and strong recall responses to viral infections [44]. NK cells have been shown to play a critical role in antiviral responses in mice and humans against cytomegalovirus. In humans, NKG2C⁺ CD57⁺ NK cells expand rapidly in response to HCMV infection and controls the infection [45, 46]. In mice, NK cells recognize the MCMV-encoded ligand m157 expressed by the infected cells through Ly49H response, leading to the expansion of Ly49H⁺ NK cells and killing of m157⁺ viral targets [47–49]. Recent studies have implicated the role of metabolism in determining NK cell responses to viral infections. Mah et al. have described the significance of glucose metabolism in regulating NK cell responses in vivo in an MCMV infection model. Blockade of glycolysis using 2-deoxyglucose (2DG) or mTORC1 pathway by rapamycin resulted in enhanced susceptibility of MCMV-resistant C57BL/6 mice to infection and impaired clearance of viral m157⁺ target cells adoptively transferred during the infection [9]. A study by O’Sullivan et al. has reported that in response to MCMV infection, NK cells undergo dynamic changes in mitochondrial activity [13]. In the effector phase, proliferating NK cells displayed mitochondrial depolarization and increased ROS production. But in subsequent response phases, these defective mitochondria and ROS are removed by the process of “mitophagy,” a process essential to promote NK cell survival and NK cell memory [13].

In humans, Cichocki et al. showed that adaptive NK cells from HCMV-seropositive donors had increased OXPHOS, spare respiratory capacity, mitochondrial mass, and membrane potential compared to the canonical NK cells. These enhanced metabolic activities in adaptive NK cells were found to be associated with increased expression of transcription factor ARID5B. Using shRNA-mediated knockdown and lentiviral transduction-mediated overexpression approaches, the authors demonstrated that ARID5B had a significant role in...
in regulating the oxidative metabolism, IFNγ production, and survival of human adaptive NK cells [14]. Recent study by Cubero et al. demonstrated that NK cells experience severe metabolic defects during chronic HIV infection. NK cells displayed defective OXPHOS, fragmented mitochondria, and decreased IFNγ production in response to CD16 receptor-mediated stimulation. Pretreatment with IL-15 rescued the functional defects in NK cells and was found to bypass the OXPHOS requirement for receptor-mediated IFNγ production [50]. Altogether, these studies demonstrate the perturbations in NK cell metabolism in chronic viral infections.

**NK cell metabolism and obesity**

Obesity is linked with the increased risk of predisposing people to cancer and infections [51, 52]. NK cell dysfunctionality including decreased circulating cell numbers, diminished IFNγ production, and cytotoxic potential has been reported in obese patients as compared to healthy controls [53–56]. Recent studies have associated aberrant metabolism with NK cell dysfunction in obesity in mice and humans (adults and children) [54, 56]. Michelet et al. demonstrated that obesity induces metabolic defects in mouse and human NK cells as a result of peroxisome proliferator-activated receptor (PPAR)-mediated lipid accumulation. Stimulation of NK cells with agonists of PPARα/δ that are transcription factors regulating lipid metabolism resulted in enhanced lipid uptake by NK cells that correlated with diminished IFNγ production and granzyme B expression. Upon cytokine stimulation, NK cells from obese humans or mice displayed severely impaired metabolic rates, mTOR activity, and reduced potential to kill tumor cells as compared to healthy controls [54]. Overall, these results clearly suggested that obesity induces severe metabolic defects and impaired antitumor responses in NK cells.

**NK cell metabolism and cancer**

NK cell functions are known to be severely impaired in various cancers [42, 57, 58]. Recent studies have identified defective cellular metabolism as one of the key mechanisms underlying NK cell functional impairment. A recent study by Cong et al. demonstrated inhibition of glycolysis as the key mechanism of NK cell dysfunctionality during tumor progression in the KRAS-driven lung cancer [59]. The inhibition of glycolysis was mediated by fructose-1,6-bisphosphatase (FBP1), which is a rate-limiting enzyme in gluconeogenesis. Using a KRAS-driven spontaneous lung tumor model, the authors found that FBP1 expression was highly upregulated in tumor-infiltrating NK cells as tumors progressed to advanced stages in response to elevated levels of tumor-driven TGFβ. Remarkably, pharmacological inhibition of FBP1 partially rescued NK cytotoxicity and cytokine production via restoration of glycolysis [59]. Another recent study by Kobayashi et al. reported that NK cells from aggressive B cell lymphoma patients and Eμ-myc lymphoma-bearing mice had dampened IFNγ production, mTORC1 activity, and mitochondrial metabolism. Considering the lipid-rich lymphoma environment, these functional and metabolic defects were demonstrated to be associated with excessive lipid accumulation characterized by elevated plasma fatty acid levels and upregulation of lipid transporter CD36 [60]. In addition to the preclinical evidence, several
studies in cancer patients have also attributed NK cell dysfunction to their metabolic defects. Slattery et al. have reported that NK cells from metastatic breast cancer patients fail to engage in normal OXPHOS and glycolytic activities and have impaired ability to produce IFNγ as compared to the NK cells from healthy controls [61]. NK cells from these patients further displayed dysregulated mitochondrial function as characterized by very high mitochondrial membrane potential, mitochondrial ROS levels, and fragmented morphology [61]. Overall, these findings demonstrate that NK cells have severely impaired metabolism and functions in cancer and highlight the potential of rescuing NK cell functions in cancer patients by restoring their metabolism.

The following section focuses on the details of how NK cell metabolism and functions are hampered in the immune suppressive tumor microenvironment.

**Impaired NK cell metabolism and functions in the TME**

NK cells play a major role in tumor immune surveillance. But there is accumulating evidence showing that NK cells inevitably become dysfunctional in the tumor microenvironment (TME). The TME is frequently characterized by scarcity of oxygen and essential nutrients for immune cells. NK cell metabolism has been shown to be impaired in the TME owing to the presence of highly immune-suppressive factors that contribute to the pathogenesis of solid tumors. The presence of such immunosuppressive factors in excess in the TME incapacitates effector cells to kill malignant cells. The following section focuses on some of the major suppressive factors of the TME and their impact on NK cell metabolism and effector functions.

**Lactate**

Excessive levels of lactate accumulation in the TME occur as a result of high rates of glucose metabolism in tumor cells via glycolysis [62]. The enzyme lactate dehydrogenase (LDHA) converts glycolysis-derived pyruvate into lactate and is found to be upregulated in various solid cancers [62]. Several studies have reported negative influences of high lactate levels on NK cell functions. High concentration of tumor-derived lactate has been shown to correlate with high incidence of metastasis in human cervical and head and neck cancers [63–65]. Walenta and colleagues demonstrated that primary human cervical tumors with metastatic spread had significantly higher lactate levels than the tumors without metastasis. Moreover, the overall survival of patients with low tumor lactate levels was significantly higher than the patients with high tumor lactate levels [65]. A direct correlation of reduced number of liver-resident NK cells with high levels of lactate in the TME was reported in patients with colorectal liver metastasis (CRLM). CRLM tumors secreted excessive lactate, resulting in reduced pH in liver NK cells, reduction in their mitochondrial mass, enhanced accumulation of ROS, and NK cell apoptosis [41]. Husain et al. examined the impact of tumor-derived lactate on NK cell activity in vivo using a mouse pancreatic tumor model and found that LDHA-depleted tumors were significantly smaller than LDHA+ tumors. The cytotoxic potential of splenic NK cells from LDHA+ tumors against YAC target cells was significantly reduced compared to
their LDHA⁻ counterparts. Mechanistically, the suppressive role of lactate was demonstrated in vitro where lactate pretreated NK cells displayed significant downregulation of the activating receptor NKp46 and reduction in granzyme B and perforin expressions [66]. Following this, Brand et al. also reported significant impairment of tumor-infiltrating NK and T cells by tumor-derived lactic acid in a melanoma tumor model [67]. They found that tumors with low lactate acid production (LDHA\textsuperscript{low}) had reduced growth rate and increased infiltration with functionally competent NK and T cells compared to the LDHA\textsuperscript{high} counterparts. High concentrations of lactic acid induced reduction in ATP generation and downregulation of NFAT-mediated IFN\(\gamma\) production in T cells and NK cells [67]. Overall, these findings clearly indicate the suppressive effect of tumor-derived lactate on NK cell metabolic and functional activities and that targeting excessive lactate production in the tumor microenvironment has the potential to rescue NK cell antitumor functions.

### Adenosine

One of the characteristics of the TME is the accumulation of tumor-derived metabolites that obstruct immune cell functions. Adenosine is a highly immunosuppressive metabolite that is present in the tumors at very high concentrations and is induced by hypoxia [68, 69]. Adenosine is generated from extracellular ATP by the activity of tumor-associated ectonucleotidases CD73 and CD39, which are prevalently expressed by various solid tumors. Adenosine binds and signals via four G-protein-coupled adenosine receptors A\(_1\), A\(_{2A}\), A\(_{2B}\), and A\(_3\), among which the A\(_{2A}\) receptor (A\(_{2A}\)R) is most predominantly expressed on immune cells including NK cells [70]. Raskovalova and colleagues investigated the impact of adenosine and its analogs on the cytolytic activity of IL-2-activated NK cells against Lewis lung carcinoma cell line 3LL using NK cells. Through genetic and pharmacological blockade of adenosine receptors, their findings revealed significant impairment of cytotoxicity of NK cells against tumor cells and this inhibitory role of adenosine was mediated by A\(_{2A}\)R [71]. Excessive accumulation of extracellular adenosine in tumors has been shown to be one of the immune evasion mechanisms adopted by the tumors [72]. Studies from different groups have demonstrated that targeting adenosine leads to enhanced antitumor immune responses [72–74]. Mice deficient in A\(_{2A}\)R or treated with A\(_{2A}\)R inhibitors display superior tumor control compared to their wild-type counterparts in different mouse tumor models [73, 74]. Interestingly, A\(_{2A}\)R-mediated adenosine signaling was identified as a negative regulator of NK cell maturation and deficiency of A\(_{2A}\)R resulted in accumulation of highly proliferative and mature, cytotoxic CD56\textsuperscript{dim} NK cell population [74]. Recently, Chambers et al. demonstrated that adenosine induced functional and metabolic impairments in IL-2/IL-15-activated NK cells [75]. In the presence of adenosine, NK cells displayed diminished activating receptor NKG2D expression, IFN\(\gamma\) production, and cytotoxicity against target tumor cells. In addition, adenosine also inhibited the glycolytic capacity, OXPHOS, and downregulated other NK metabolism-associated genes [75].

Overall, targeting adenosine represents a promising therapeutic strategy to enhance the efficacy of NK cell-based therapies. Indeed, several A\(_{2A}\)R antagonists and anti-CD73 antibodies are currently being evaluated in clinical trials [70].

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Transforming growth factor beta

The negative influences of immunosuppressive cytokine TGFβ (transforming growth factor beta) on NK cell development and functions have been demonstrated by multiple studies [76–80]. One of the mechanisms by which TGFβ secreted by tumors inhibits NK cell cytotoxicity functions in cancer patients is by downregulating the NK cell-activating receptor NKG2D [58]. Several recent studies have reported TGFβ-mediated alterations of NK cell metabolism as a potential mechanism of NK cell dysfunctionality in the solid TME. TGFβ was also found to be the key suppressive mechanism underlying NK cell mitochondrial metabolic defects and impaired IFNγ production in metastatic breast cancer patients [61]. Viel et al. reported an interesting mechanism, whereby TGFβ inhibits NK cell functions by repressing their mTOR activity in response to IL-15 stimulation [81]. Mouse and human NK cells treated with TGFβ in vitro displayed significantly reduced glycolysis and oxidative phosphorylation, CD71 and CD98 expressions, and reduced activity of mTORC1 as measured by the phosphorylation of mTOR substrates S6, 4EBP1, and Akt. In addition, the mTORC1 inhibitor rapamycin and TGFβ displayed similar effects in inhibiting NK cell metabolic activity and cytotoxic functions. Specific deletion of TGFβR2 in NK cells (Ncr1CreTGFβR2fl/fl mice) resulted in reduction of metastasis in B16F10 melanoma and RM1 prostate adenocarcinoma mouse tumor models [81]. Following this, another study by Bittencourt et al. reported similar findings on the role of TGFβ in significant inhibition of NK cell bioenergetic metabolism and effector functions through the canonical TGFβ signaling pathway rather than the noncanonical pathway that attenuates mTORC1. In the presence of TGFβ, IL-2-stimulated human NK cells displayed reduced glycolytic capacity, OXPHOS, and maximal respiration. Additionally, these cells displayed decreased levels of IFNγ, granzyme B, CD69, and CD71 expressions. Interestingly, these metabolic and functional defects were reversed with the TGFβR1 inhibitor [82]. Taken together, these studies clearly highlight the negative impacts of TGFβ on NK cell metabolism and functions.

Therapeutic opportunities for improving NK cell therapies

Currently, various NK cell-based therapies are being developed and tested in clinics [4]. Adoptive transfer of NK cells expanded and activated ex vivo with cytokines and chimeric antigen receptor-engineered CAR-NK cells are the two major NK cell-based therapies that are being actively harnessed for treatment of cancer. Although, these therapies are safe to use, their therapeutic efficacy remains limited [1, 4]. Given that NK cell metabolism and effector functions are intricately linked, and NK cell metabolism is altered in pathological conditions, modulating the metabolism represents a promising strategy to restore and further potentiate their effector functions for therapies. Immune-stimulatory cytokines IL-2 and IL-15 are used to sustain and enhance the NK cell survival and functions during adoptive cell therapy [83, 84]. The impact of these cytokines on NK cell metabolism has been demonstrated by various studies, but the complete understanding of their impact in clinical application is still lacking. A preclinical study by Felices et al. has reported the effect of continuous vs intermittent stimulation of NK cells with 10 ng/mL of IL-15 [85]. NK cells receiving continuous IL-15 stimulation displayed defective OXPHOS and decreased cytotoxic potential both in vitro and

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in vivo when compared to intermittent IL-15 stimulation. Reducing the strength of IL-15 signaling by using rapamycin, which inhibits mTOR activity downstream of IL-15, restored the metabolic activities, and cytotoxic functions of NK cells continuously stimulated with IL-15, suggest that continuous IL-15-stimulation of NK cells could have a negative impact on overall NK cell cytotoxic functions [85]. These findings highlight that it is very critical to evaluate the impact of cytokines on NK cell metabolism for selecting appropriate dosing strategies for clinical use and to develop more efficient immunotherapies.

Additionally, a large number of CAR-NK cell-based strategies are currently being evaluated in preclinical and clinical studies [86, 87]. Studies in T cells have identified potential impact of distinct CARs on T cell metabolic reprogramming that supports their antitumor functions and persistence. Recent studies investigating CAR-NK cell metabolism are also emerging [88]. Interestingly, several groups have reported the impact of a cytokine-inducible SH2-containing protein (CIS), on NK cell functions and metabolism [88, 89]. This protein is encoded by the CISH gene and has been identified as a negative regulator of IL-15 [90]. CISH-deficient iPSC-derived NK cells displayed enhanced persistence both in vitro and in vivo and antitumor functions. The mechanisms underlying the improved functionality of these cells were identified to be their enhanced metabolic activity including both glycolysis and OXPHOS [89]. Additionally, the combined therapeutic strategy of engineering cord blood-derived NK cells with CAR-IL-15 constructs and depletion of CISH, resulted in enhanced NK antitumor functions via mTOR and cMYC-mediated increase in glycolysis [88]. These findings provide proof-of-concept evidences that the persistence and function of adoptively transferred engineered NK cells could be improved by modulating their metabolism.

**TABLE 1** Important pharmacological inhibitors for modulating NK cell metabolism.

<table>
<thead>
<tr>
<th>Targets</th>
<th>Drug name</th>
<th>Mechanism of action</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexokinase</td>
<td>2-DG</td>
<td>Glycolysis inhibition</td>
<td>[15, 59]</td>
</tr>
<tr>
<td>ATP synthase</td>
<td>Oligomycin</td>
<td>Inhibition of OXPHOS</td>
<td>[15]</td>
</tr>
<tr>
<td>mTOR</td>
<td>Rapamycin and Torin 1</td>
<td>Inhibition of mTOR pathway</td>
<td>[7, 24, 26, 81]</td>
</tr>
<tr>
<td>CPT1a</td>
<td>Etomoxir</td>
<td>Blockade of fatty acid oxidation</td>
<td>[15, 54]</td>
</tr>
<tr>
<td>TGF-βRI</td>
<td>SB-431542</td>
<td>Inhibition of kinase activity of TGF-βRI</td>
<td>[81, 82]</td>
</tr>
<tr>
<td>FBP1</td>
<td>MB05032</td>
<td>Inhibition of FBP1 enzymatic activity and gluconeogenesis, restoration of glycolysis</td>
<td>[59]</td>
</tr>
<tr>
<td>LDHA</td>
<td>Sodium oxamate</td>
<td>Pyruvate analog, inhibits enzymatic activity of LDHA</td>
<td>[91]</td>
</tr>
<tr>
<td>CD73, CD39</td>
<td>Anti-CD73, anti-CD39 blocking antibodies</td>
<td>Blocking of ectonucleotidase activity</td>
<td>[70, 75]</td>
</tr>
<tr>
<td>A2AR</td>
<td>SCH58261</td>
<td>Adenosine receptor A2A inhibition</td>
<td>[70, 75]</td>
</tr>
<tr>
<td>Drp1</td>
<td>Mdivi-1</td>
<td>Prevents mitochondrial fission</td>
<td>[40]</td>
</tr>
<tr>
<td>ROS</td>
<td>N-Acetyl-l-cysteine</td>
<td>ROS scavenger</td>
<td>[13]</td>
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</table>

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Moreover, targeting the immunosuppressive cytokines and metabolites in the TME that limit NK cell functions and metabolic activities will also generate an environment conducive for NK cell antitumor activity. Further advances in the understanding of NK cell metabolism would be critical to design novel strategies for improving NK cell-based therapies. Some potential metabolic inhibitors discussed in this chapter are presented in Table 1.

**Concluding remarks**

Given the critical role of NK cells in immunosurveillance against tumors and viral infections, substantial efforts are currently being made to harness NK cell effector functions for therapeutic purposes. Immunometabolism has become an important component of NK cell research in the recent years and the field is rapidly evolving. Cellular metabolism has been demonstrated to be critical for NK cell development, activation, and antitumor and antiviral responses. In pathological conditions, NK cells exhibit significant metabolic defects. In cancer, various immunosuppressive factors present in the TME significantly impair NK cell antitumor responses by modulating their metabolic activities (Fig. 2).
Although several regulators of NK cell metabolism have been identified, the understanding of regulatory mechanisms of NK cell metabolism in pathological conditions is still limited. Improvement in the understanding of NK cell metabolism regulation will definitely help in the development of novel therapeutic strategies to reprogram NK cell metabolism and restore NK cell functions for effective therapies.

References

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New therapeutic modalities in breast cancer by targeting NK cell inhibitory and activating receptors

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Abstract
Since the discovery of natural killer cells in 1975, immunotherapies targeting the potent cytolytic activity and robust surveillance of the innate immune system have increased significantly. Many studies have explored the crucial role of NK cells in various cancers; however, the literature exploring the role of NK cells in breast cancer is limited. Despite the efficacy of conventional therapies in breast cancer, innate and induced resistances persist. Treatment strategies and resistance to such therapies vary across breast cancer subtypes, including the Luminal A, Luminal B, HER2-enriched, and triple-negative/basal-like subtypes. Here, we delineate the various potential signaling cross-talk pathways between NK cells and breast cancer. These pathways include, but are not limited to, the following: (i) NK cell activating receptors (e.g., NKG2D, DNAM1, and CD16), (ii) NK cell inhibitory receptors (e.g., NKG2A), and (iii) checkpoint inhibitors (e.g., PD-1). The stimulation of activating receptors or the disruption of inhibiting signaling with various postulated combination therapies may be key to overcoming resistance and unlocking the power of NK cell immunotherapy against breast tumors. This review provides significant support for targeting the NK cell activating or inhibitory receptors to activate the antitumor activities of NK cell-based therapies in breast cancer.

Abbreviations
ADCC antibody-dependent cellular cytotoxicity
DNAM1 DNAX accessory molecule-1

* Contributed equally.
The authors declare no conflict of interest.

**Introduction**

Current breast cancer therapies continue to contend with the issue of resistance; thus innovative new targets for immunotherapies are necessary to overcome this obstacle. The following section delves into the diversity of breast cancer subtypes, and subsequent sections delve into the mechanism of action of NK cell cytotoxic functions and immune surveillance.

**Breast cancer subtypes**

The highly heterogeneous nature of breast cancer characterized by its various molecular subtypes poses therapeutic challenges and, thus, it is important to distinguish among the various molecular and prognostic characteristics of these various subtypes. The presence, or lack thereof, of the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor 2 (HER2) receptor proteins are the primary molecular markers that help distinguish the various breast cancer subtypes.

The Luminal A subtype of breast cancer is characterized by the presence of the ER and/or PR hormone receptor and the absence of the HER2 receptor (HR+/HER2-). Luminal A breast cancers use estrogen and/or progesterone receptors to take up hormones that signal to the cell to grow and replicate, whereas the HER2 receptor is absent. Breast cancers that are ER and PR positive grow in response to the estrogen and progesterone hormones, respectively. A cancer cell positive for the ER and/or PR is termed hormone-receptor positive (HR+). Identifying the hormone receptor status, often using immunohistochemistry, of a breast cancer sample is crucial in treating it. For instance, an ER+ breast cancer patient may be administered hormone therapy to combat the levels of estrogen and its activity on the breast cancer cells. One key characteristic of the Luminal A breast cancer subtypes is the relatively low levels of the Ki-67 protein, a prognostic and predictive indicator of tumor cell proliferation and growth [1]. Luminal A breast cancers are often slow growing and are not typically treated with chemotherapy as they respond well to endocrine therapy alone [2].

The Luminal B subtype of breast cancer is characterized by the presence of the ER and/or PR hormone receptor as well as the HER2 receptor (HR+/HER2+). Those with the Luminal B subtype are triple positive for all three receptor targets, which makes targeted therapeutic strategies more feasible and, in turn, increases survival rates [3]. The Luminal B subtype, therefore, is the best prognostic rate among the other subtypes. Recent evidence suggests...
the presence of potential cross talks between ER and HER2 receptors when both are present in breast cancer cells [4]. The Luminal B subtype of breast cancer does not respond as well to hormone therapy as the Luminal A subtype; however, these breast cancer cells are sensitive to dual therapies targeting both receptors [5]. In contrast to the Luminal A subtype, the Luminal B subtype is more fast growing and expresses relatively higher levels of the proliferative Ki-67 protein [1]. Current therapies used for the treatment of Luminal B breast cancers include chemotherapy, hormone therapy, and anti-HER2 targeted therapy.

The HER2-enriched subtype of breast cancer is characterized by the absence of the ER and PR hormone receptors and the presence of the HER2 receptor (HR+/HER2+). HER2 (ERBB2), an oncogenic receptor tyrosine kinase, is found to be overexpressed in 20%–25% of breast cancers [6]. As a common oncogene in breast cancer, HER2 plays a crucial role in the development and progression of breast cancer and, therefore, poses a large demand for effective anti-HER2 therapies. Current anti-HER2 therapies include monoclonal antibodies, antibody-drug conjugates, and kinase inhibitors. Anti-HER2 monoclonal antibodies (trastuzumab, pertuzumab) are engineered antibodies that specifically bind the HER2 protein on target cancer cells to prevent growth and proliferation. Antibody-drug conjugates (TDMI, Enhertu) are monoclonal antibodies that are linked to a chemotherapeutic drug to help signal the chemo directly to the HER2 protein. Lastly, kinase inhibitors (lapatinib, neratinib, tucatinib) block the HER2 kinase to turn off signals that normally instruct these cells to grow. Among breast cancer patients positive for the HER2 receptor target, stage IV patients are thought to respond to HER2-targeted therapies more successfully than stages I–III. This may be due to the fact that when given these therapies early in the disease process, many patients develop resistance and, thus, do not respond when given these treatments at the time of distant metastatic recurrence.

Lastly, termed rightfully so, the triple-negative or basal-like subtype of breast cancer is characterized by the absence of ER, PR, and HER2 receptors (HR-/HER2-). TNBC is often the hardest to treat and suffers the worsts prognosis among breast cancer patients given that those with TNBC lack any of the receptor targets (ER, PR, HER2) [3]. TNBC is currently treated with conventional therapies such as chemotherapy, radiation therapy as well as other targeted therapies; however, much like other cancers treated with conventional therapies, cancer cells of this subtype are quick in developing resistance [7].

**Natural killer cells**

Natural killer (NK) cells are bone marrow-derived innate effector lymphocytes that are found in most organs, with the largest concentrations of NK cells found in the blood. NK cells are large granular lymphocytes that orchestrate the immune response by releasing chemokines and cytokines that mediate cross talks with signaling pathways that regulate cells of both the innate and adaptive immunities [8]. Low levels of infiltrating NK cells have been associated with negative responses to antitumor treatment and poor prognosis, particularly in breast cancer. For that reason, NK cells play a pivotal role in the recognition and orchestrated attack of abnormal cells without prior sensitization [9, 10]. Currently, the majority of existing immunotherapies utilize T lymphocytes, however NK cells, which lack T-cell receptors (TCRs), hold the potential to be a universal cell therapy product with the capacity to treat...
a multitude of cancer patients [8, 11]. Thus, engineering NK cells and harnessing this innate defense have incredible potential to overcome resistance in anticancer therapeutics.

Recent studies have found diversity in the function and receptor expression among various subpopulations of NK cells, suggesting that NK cells are not a homogenous population but rather have unique subsets [12]. This diversity is noted in the differential expression of the neural cell adhesion molecule (NCAM), also known as CD56. Human NK cells can be classified as CD56bright CD16dim NK cells, also known as immature NK cells, and CD56dimCD16bright NK cells, also known as mature NK cells. Immature NK cells are instrumental in the immunomodulation and production of cytokines, particularly interferon gamma (IFNγ), whereas mature NK cells, which make up 90% of NK cells in the peripheral blood (PB) mediate the cytotoxic immune function of NK cells [13, 14].

NK cells distinguish cancer cells and virally infected cells from their healthy counterparts through an array of germline-encoded activating and inhibitory receptors [15]. Inhibitory receptors include the recognition of MHC class I molecules, such as the killer-cell immunoglobulin-like receptors (KIRs), the heterodimer CD94-natural killer group 2A (NKG2A), and other immune checkpoints such as PD1. Activating receptors include the natural killer group 2D (NKG2D), DNAX accessory molecule-1 (DNAM1), and the natural cytotoxicity receptors NKP46, NKP44, and NKP30 [15] (Fig. 1). These activating receptors recognize ligands that are commonly upregulated on infected, tumorigenic, or stressed cells [16].

Without prior sensitization, these large NK granular lymphocytes are able to detect and kill tumorigenic cells, eliminate virally infected cells, and secrete cytokines that mediate various cross talks and regulate other immune cells, all while tolerating healthy self-tissue [10]. NK cell discrimination of self is based on the expression of MHC class I proteins as well as non-MHC-1 molecules, including glycans and collagen [17]. Upon cell-to-cell encounter, NK cells determine cell action and transmit activating or inhibitory signals, depending on the engagement of cognate ligands [10, 18].

Inhibitory and activating signaling occur simultaneously upon each NK cell-to-cell encounter. NK cells are filled with potent lytic granules that are ready to be released and kill target cells within 30 min of engagement, albeit if the receiving cell has been deemed non-self. Inhibitory receptors otherwise dull NK cell activation and prevent “self” lytic attack [10]. For this reason, NK cells can be considered to be in a constant state of inhibition as they more often than not encounter healthy self-cells. In virally infected or malignantly transformed cells, the surface expression of these inhibitory proteins is often inactivated, thereby designating as nonself, activating NK cells, and killing the target cells [19]. Upon detection of activating ligands, NK cells kill infected or tumorigenic cells by several mechanisms of action, including exocytosis of lytic granules loaded with proteases and pore-forming proteins such as granzymes and perforin, release of cytokines such as tumor necrosis factor alpha (TNFα) and interferon gamma (IFN-γ), upregulation of FasL and TNF-related apoptosis-inducing ligand (TRAIL), and by antibody-dependent cellular cytotoxicity (ADCC) [10, 20].

Resistance to NK cell therapy is a frequent occurrence and is caused by various factors including the genetic profile of the individual, resistance of cancer cells to apoptosis, and the immunoeediting of cancer cells [16]. Resistance to antitumor therapies can be divided into two categories—intrinsic resistance, a lack of response to therapy, and acquired resistance, disease progression after an initial clinical response. The inability of NK cells to infiltrate solid tumors limits the application of NK cell therapies and contributes to the issue of intrinsic resistance [14]. Likewise, the genetic architecture that dictates NK cell life and function may be a
significant indicator for the degree of NK cell therapy effectiveness and the potential resistance to antitumor treatment [16]. In acquired resistance, tumor cells evade NK cell recognition by upregulating inhibitory ligands, such as MHC class I molecules, and inhibiting NK cell cytotoxic functions by downregulating activating ligands, such as NKG2D [10, 12]. Within the tumor microenvironment, the tumor also secretes inhibitory cytokines such as IL-10 and TGF-β in order to inhibit NK cell antitumor functions [12].

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Recent studies have found NK cell exhaustion to be a key determinant in the issue of resistance, as continuous exposure to particular target antigens results in the exhaustion of NK cells [21]. Despite an initial strong antitumor function and cytokine production early after adoptive transfer therapy, after 5 days posttransfer, NK cells often exhibited weak IFN-γ production and cytotoxicity. Repeated adoptive transfer, additional immunotherapy, or checkpoint inhibitors may remedy this issue and may rescue NK cells from exhaustion to unleash their powerful attack [12].

A new direction for NK cell therapy stems from the success of CAR-T-cell therapy against hematological malignancies and recently solid malignancies. Clinical trial NCT02839954 was the first to provide strong support for the effectiveness of CAR-NK cells in patients with solid tumors, such as in the case of breast cancer. NK-92 cells expressing a HER2-specific CAR proved promising in the targeting of cancer cells in mouse breast cancer cell lines [22]. Similarly, CAR-engineered NK cells targeting EGFR have been effectively used in the treatment of TNBC [23]. This potential for an off-the-shelf product with minimal off-target side effects holds promise for the treatment of breast cancer.

In this review, the focus will be on the targeting either the activating or inhibitory NK receptors to render them functional and treat resistant breast cancers.

Immunotherapy by NK cells in breast cancer

Activation of NK cells in vivo

NK cells have great potential as new immunotherapeutic targets. Current therapies promoting NK cells include monoclonal antibodies that promote NK cell antibody-dependent cell-mediated cytotoxicity (ADCC), hematopoietic stem cell transplantation (HSCT), adoptive transfer of NK cells, redirection of NK cells using chimeric antigen receptor (CAR)-NK cells, and the use of cytokines and immunostimulatory drugs to boost the antitumor activity of NK cells [16]. These therapies are currently in use and harness the power of NK cells, however they range in effectiveness. To increase the antitumor functionality of NK cells, administration of stimulatory and activating cytokines such as IL-2 and IL-15 are being explored. Despite initial NK expansion after IL-2 injection in patients, tumor relapse rates and overall survival of patients were not significantly changed. However, to reduce the toxicity side effects of IL-2, IL-15 is a potential alternative [14].

Targeting NK cell activating receptors in breast cancer

While the expression of NK cell activating receptors decreased during breast cancer progression, the expression of NK cell inhibitory receptors increased [24]. Here, we focus on the downregulation of various NK cell activating receptors and, in turn, its effect on NK cell activity in breast cancer.
**Natural killer group 2D**

The natural-killer receptor group 2, member D (NKG2D), a lectin-like type II transmembrane receptor, has been previously identified as an NK cell activating receptor via binding to several ligands poorly expressed on normal cells to prevent an autoimmune tissue damage; however, these ligands have been found to be upregulated during stressing conditions in cancer cells [25]. The increased expression of NK2GD ligands acts as a signal for NK cell-mediated cytotoxicity of target cells [26]. Upon binding of the NKG2D receptor to its cognate ligand, the receptor becomes internalized from the plasma membrane and degraded, which supports evidence demonstrating decreased NKG2D surface expression following prolonged exposure to cells expressing its cognate ligands [27]. The NKG2D receptor is found to bind a number of self-proteins enhanced by stressing stimuli in cancer cells, such as MHC class I-related proteins MICA/MICB as well as the UL16-binding protein family [27]. Overexpression of such ligands and subsequent shedding have led to the controversial problem of whether the expression of NKG2D ligands results in the activation of an effective immune response or leads to the downregulation of NKG2D on immune cells and, in turn, the immune response. Tumor cells shed the overexpressed NKG2D ligand to produce an immune soluble form in order to evade the immune response of activated NK cells and, thus, increased circulation of the soluble NKG2D ligand is associated with poor clinical prognosis and metastasis in various cancer types, including breast cancer [28]. de Kruijf and colleagues found various NKG2D ligands to be overexpressed and, in some cases, found coexpression of these ligands in a cohort of 677 breast cancer patients [29]. MICA/B proteins were found to be frequently expressed in 50% of the breast cancer samples as well as over 90% of these cases expressing ULBP-1,2,3,5 [29]. Coexpression of NKG2D ligands resulted in significant improvements in relapse free period in these patients, particularly the coexpression of MICA/B and ULBP-2 [29]. Although the presence of such ligands may invoke an immune response leading to longer relapse free periods, the overexpression of the NKG2D ligands may result in the degradation of cell surface NKG2D expression and, thus, decreased NK cell-mediated cell lysis (Fig. 2A). Nevertheless, the presence of various NKG2D ligands on breast cancer subsets makes these subtypes of cancer an attractive target for NKG2D-based therapy treatments.

**DNAX accessory molecule-1**

The DNAX accessory molecule-1 (DNAM1/CD226), a costimulatory adhesion receptor, is responsible for the identification of tumor cells and the cytolytic response of NK cells to such tumors. Once DNAM1 interacts with its cognate ligands on target cells, actin polymerization and activation of other surface receptors allow for the stable interaction of NK cells and target cells [30]. The DNAM1 receptor, expressed on both cytotoxic T lymphocytes (CTLs) and NK cells, binds the poliovirus receptor (CD155) that is upregulated in tumor cells to mediate the cytotoxic activity of NK cells against tumors [31]. Thus, DNAM1 plays a pivotal role in the antitumor response by both recognizing target tumor cells and the mobilization of NK cells to such target cells [32]. In addition to the role of DNAM1 in promoting adhesion of NK cells to target cancer cells, the combined role of DNAM1 and NKG2D have been identified as being one of the most significant activating receptors of the cytotoxic function of NK cells [33].
FIG. 2  (A) Role of NKG2D in NK cells and breast cancer. Under normal conditions, the NKG2D transmembrane receptor present on NK cells binds its respective ligand on target cells to initiate NK cell-mediated cytotoxicity. In breast cancer, the NKG2D receptor is internalized and cancer cells begin to shed the NKG2D ligand and soluble forms of the NKG2D ligand are produced. Thus, disruption of NKG2D receptor-ligand binding aids in the immune escape of breast cancer cells from NK cell-mediated cytotoxicity. (B) Role of DNAM1 in NK cells and breast cancer. Binding of the DNAM1 activating receptor found on NK cells to the CD155 ligand on target cells is crucial in the identification of target cancer cells to initiate the cytolytic response of NK cells. Conversely, in breast cancer, expression of the DNAM1 receptor is found to be decreased. Moreover, serum levels of a soluble form of CD155 are increased in breast cancer patients and are correlated with disease progression in breast cancer patients. sCD155, which lacks the transmembrane region, is not able to successfully bind the target cell to the DNAM1 ligand on the NK cell and, thus, is unable to effectively activate an NK cell-mediated immune response. (C) Role of CD16 in NK cells and breast cancer. The CD16 transmembrane receptor is responsible for binding antibodies presented on the surface of target cancer cells to activate antibody-dependent cellular cytotoxicity (ADCC). ADAM17, a membrane-bound protease, acts as a scissor to shed the extracellular domain of CD16 to prevent recognition and binding of NK cells to target tumor cells. The overexpression of ADAM17 in various cancers, including breast cancer, is correlated with disease progression likely due to decreased ADCC activity by NK cells. Thus, the use of ADAM17 inhibitors may decrease disease progression in breast cancer patients by allowing binding of the CD16 receptor to antibodies expressed on target cancer cells. Created with BioRender.com.
A decrease in the expression of the DNAM1 activating receptor by NK cells was found to be associated with tumor progression in a subset of breast cancer patients [34]. Moreover, a meta-analysis of primary breast tumors showed variable expression of various NK cell receptors; however, the ligands for both NKG2D and DNAM1 were found to be widely expressed among various breast cancer subtypes [34]. One study using gene expression profiling, RT-PCR and IHC analyses of primary breast cancer samples identified that the expression of DNAM1 was increased in patients with more favorable prognosis [33]. One recent study reported increased serum levels of a soluble form of CD155 (sCD155), a splicing isoform of CD155, that lacks the transmembrane region, in breast cancer patients and identified a positive correlation between increased sCD155 levels and patients age, disease stage, and invasive tumor size [31]. sCD155 levels were found to be increased in ER-negative breast cancer patients when compared to ER-positive tumors. Moreover, serum levels of sCD155 were reportedly higher in Ki67-high breast cancer samples rather than in Ki-67-low samples [31]. Thus, given that increased serum levels of sCD155 correlated with disease progression in breast cancer patients, one could identify the important role of DNAM1 in the NK-mediated cytotoxic response against breast tumors expressing CD155 (Fig. 2B).

**CD16**

CD16 (FcγRIIIa) is a type I transmembrane Fc receptor that plays an important role in the antibody-dependent cellular cytotoxicity (ADCC) by NK cells. Following the recognition of antibody-expressing cells, CD16 directly signals NK cells to target and destroy such cells. ADAM17, a member of the disintegrin and metalloproteinase (ADAM) family, is a membrane-bound protease that is known to shed the extracellular domain of numerous receptors or its cognate ligands, one of which includes CD16. The overexpression of ADAM17 in various malignant tumors is well known and, in particular, is involved in the progression and tumorigenesis of breast cancer [35]. The activation of NK cells was found to result in the downregulation of CD16 due to the function of ADAM17; therefore, the selective inhibition of ADAM17 was found to prevent shedding of CD16 to enhance CD16-mediated ADCC by NK cells [36]. The role of CD16 in enhancing the antitumor properties of herceptin antibodies used in the treatment of breast cancer has been reported [37]; however, CD16 expression is decreased on NK cells in breast cancer. The ADAM17 inhibitor, INCB7839, was previously used in combination with trastuzumab to treat HER2-positive breast cancer patients with limited success [38]. However, a more recent study found that inhibiting ADAM17 significantly enhanced the ADCC activity of NK cells against various breast cancer cell lines [39] (Fig. 2C).
maintain peripheral tolerance and prevent autoimmunity; however, this tolerance is often
exploited in the breast tumor microenvironment to evade NK cell attack [40].

Conventional chemotherapy is highly effective in the prognosis of most breast cancers;
however, immunotherapy has shown great promise in targeting the primary tumor,
preventing metastases, and deterring recurrence [41]. In particular, immunotherapy has be-
come a front-runner in the treatment of TNBC, considering the lack of either hormone recep-
tor or HER-2 [42]. Potential immunotherapeutic targets for checkpoint blockade in NK cells
include PD-1, TIGIT, CD96, and TIM-3 among others [40]. By activating the innate immune
response by inhibiting these inhibitory checkpoints, we enable the cross talk between the in-
nate and adaptive immune system to unleash the full potential of NK cells and also stimulate
T cells through the secretion of IFN-γ by NK cells [43]. Despite an explosion in the research of
NK therapies, only a small subset of patients, approximately 20%, experiences long-term ben-
efits from these immune checkpoint therapies [43]. Thus, further research into various com-
bination immunotherapies and immune targets, such as checkpoint inhibitors and inhibitory
receptors on NK cells, is necessary to overcome mechanisms of resistance to unleash the
power of the innate immune system.

PD-1/PD-L1 axis

The PD-1/PD-L1 axis is important in inhibiting the response of NK cells, yet blocking this
axis is also a potential target for breast cancer immunotherapies to reduce the inhibition of NK
cells. PD-1 is used in both T cells and NK cells to regulate activation, yet this checkpoint is
often exploited by PD-L1 and PD-L2 expressing tumor cells as a mechanism for tumor im-
mune escape [9, 15]. PD-1 is often hijacked by tumors to suppress immune control such that
PD-L1 was found to be significantly upregulated in TNBC cell surfaces [43, 44] (Fig. 3). Stud-
ies have also found that PD-1/PD-L1 interactions can strongly suppress NK antitumor activ-
ity and PD-1+ NK cells at the tumor site exhibit an exhausted phenotype in murine tumor
models [15].

Anti-PD-L1 mAbs are the subject of numerous ongoing clinical trials, particularly in TNBC
[9, 43, 45]. Currently, six PD-1/PD-L1 inhibitors have been approved by the Food and Drug
Administration (FDA): three against PD-1 (pembrolizumab, nivolumab, and cemiplimab)
and three targeting PD-L1 (atezolizumab, durvalumab, and avelumab) [15]. Despite the pro-
gress in the development of PD-1/PD-L1 inhibitors, predicting tumor response continues to
be an issue as a significant subset of tumors do not respond to therapy [15].

Correct identification of PD-L1 expression may be an advantageous first step in predicting
the efficacy of using mAb combination therapies to disrupt PD-1/PD-L1 interactions [15].
However, there is debate as to if PD-L1 expression, when measured on tumor cells, is a valid
prognostic biomarker of checkpoint inhibitor blockade efficacy in breast cancer. Rather, clin-
ical predictors of therapy efficacy may be more insightful in breast cancer, such as the number
of past lines of treatment and the number of metastatic sites [45].

Although previously deemed as being weakly immunogenic, breast cancer has been found
to be responsive to anti-PD-1/PD-L1 blockade, particularly in TNBC [45]. In PD-L1+ tumors,
MHC class I is often lost, yet in this absence, NK cell therapy in combination with anti-PD-L1
mAbs to induce ADCC is a potential solution to overcoming resistance [46]. The majority of

V. Targeting NK Cells
anti-PD-1/PD-L1 mAbs contain an altered Fc receptor (FcR) binding site in order to prevent ADCC against PD-L1-expressing immune cells. Yet, promoting ADCC antitumor activity by NK cells can launch a potent attack on tumor cells. Therefore, the use of a fully human IgG1 anti-PD-L1 mAb containing wild-type FcR, such as avelumab, can bring about ADCC. This potential therapeutic is being investigated in various clinical trials with breast cancer patients [46]. In an in vitro study in HNSCC and NSCLC cell lines with high PD-L1 expression, it was confirmed that NK cell cytotoxic functions could be augmented by ADCC using anti-PD-L1 mAbs in PD-L1 positive cancer cell lines [46].

Avelumab is a human anti-PD-L1 IgG1 mAb that inhibits the interaction between PD-1 and PD-L1, but not PD-1/PD-L2 and also exhibits ADCC [45]. Avelumab-mediated ADCC through NK cells was drastically improved in TNBC cells in vitro [42]. Juliá et al. found that avelumab in vitro significantly improved NK-cell mediated cytotoxic functions against TNBC cells and PD-L1 expressing tumors were more affected by avelumab-mediated ADCC. Further, IL-2 and IL-15 further enhanced the NK cell avelumab-triggered cytokine production, degranulation, and lytic activity against TNBC cells [42]. Further, Dirix et al. found in a cohort of 168 metastatic breast cancer patients, including 58 TNBC, whose tumors with an available expression of PD-L1 tumor-associated immune cells, and treatment with avelumab resulted in an ORR of 16.7% overall and 22.2% in TNBC patients.

V. Targeting NK Cells
This immune checkpoint blockade shows great promise in increasing the efficacy of treatment of the most difficult subset of breast cancer patients by harnessing the power of the innate immune system.

**NKG2A/HLA-E axis**

Another potential immunotherapeutic target that is involved in the activation of NK and T cells is the inhibiting receptor, NKG2A. NKG2A is a type II transmembrane receptor that binds to HLA-E, a nonclassical HLA molecule [15]. HLA-E is an important signal of “self” cells and is found in most tissues that also express classical HLA class I molecules [48]. Yet, in breast cancer, tumor cells are shaped through their interactions with the immune system and are often optimized to evade detection and to produce highly tumorigenic clones. Upon downregulation of classical HLA class I receptors to escape T-cell immune surveillance, malignant cells often upregulate HLA-E to further escape immune recognition by NK cells [48]. Likewise, NK cells are modulated by the tumor microenvironment such that NKG2A expression is upregulated, thus decreasing NK cell cytotoxic function [7]. In breast cancer patients, expression of HLA-E has been found to be an independent prognostic marker of worse patient outcomes and targeting NKG2A alone or in combination with other therapeutic mAbs disrupting PD-1/PD-L1 holds promising results in breast cancer patients [15, 48].

In a study of 677 early breast cancer patients primarily treated with surgery, HLA-E expression was classified in 50% of patients. In the case of the loss of HLA class I tumor expression, the expression of HLA-E resulted in a worse relapse-free period. Thus, HLA-E is also critical in predicting outcomes of breast cancer patients [48]. de Kruijff et al. suggest that in breast cancer tumors lacking in classical HLA class I expression, the upregulation of HLA-E expression may counteract the otherwise activating signaling and contribute to immune escape [48]. Masking the HLA-cl I-specific NKG2A receptor may prompt NK cell-mediated activity against HLA-cl I+ target tumor cells [15]. A recent study found that monalizumab, an anti-NKG2A antibody, increased NK cell cytolytic activity against tumor cells and recovered CD8+ T-cell function alone or in combination with the PD-1/PD-L1 axis blockade [49]. The use of monalizumab in breast cancer is promising in stimulating NK cell activity and clinical trials are underway exploring the combination of monalizumab and trastuzumab in HER-2+ breast cancer (Clinical Trial: NCT04307329).

Further combinations of NKG2A targeting and anti-PD-1 mAbs have shown promising results in restoring NK cell antitumor activity, particularly as the majority of patients do not respond to anti-PD-1 mAbs alone [49]. In addition to PD-L1, HLA-E is also overexpressed in many aggressive tumors, including a subset of breast cancers [15]. Recent in vitro studies have found the combination of monalizumab, an anti-NKG2A mAb, with durvalumab, an anti-PD-L1 mAb, restored IFN-γ production and cytolytic activity of NK cells and T cells restored in HLA-E+ tumors coexpressing PD-L1 [15]. Inhibiting NKG2A is expected to uncheck not only NK cells but also T lymphocytes with potential antitumor activity and is a potential mechanism to prevent immune escape in combination treatments.

**V. Targeting NK Cells**
This review focused on the various potential crosstalk pathways by which NK cells play a pivotal role in breast cancer. Herein, we explored three pathways by which NK cells may communicate with breast cancer cells to elicit a cytotoxic response, including the targeting of (i) NK cell-activating receptors (NKG2D, DNAM1, and CD16), (ii) NK cell inhibitory receptors (NKG2A), and (iii) checkpoint inhibitors (PD-1).

The role of the NKG2D, DNAM1, and CD16 receptors is to activate NK cells via binding their respective ligands presented on target tumor cells. Under normal conditions, these activating receptors are expressed on NK cells and successfully bind to target cells to initiate the NK cell immune response. In breast cancer, however, activating receptors and/or ligands are aberrantly expressed or structurally altered to prevent binding of NK cells to target tumor cells; thus, allowing tumor cells to evade NK cell-mediated cytotoxicity.

The role of the PD-1 and NKG2A receptors is to inhibit the attack of NK cells to prevent self-reactivity against healthy “self” cells; yet, this mechanism is exploited by the tumor microenvironment and the corresponding ligands are often upregulated in breast cancer. This is evidenced by the poor prognosis of PD-L1+ and HLA-E+ breast tumors. By enabling the activation of NK cells via the promotion of activation signaling, or interrupting the inhibitory receptor-ligand interactions, the antitumor potential of NK cells can be fully harnessed.

While other subtypes of breast cancer are responsive to conventional therapies, a subset remains resistant, as is the case, particularly for TNBC. Thus, the use of immunotherapies and combination treatments require further research as NK cells are also potential targets for a standardized cell therapy product with the capability to treat a vast subset of patients, unlike other T-cell therapies. The potential of drugs that either enhance NK cell-activating receptors and/or downregulate such inhibitory receptors/checkpoint inhibitors in combination with other well-established chemo and immunotherapeutic therapies targeting breast cancer. The combination treatment of atezolizumab (Tecentriq), a PD-L1 inhibitor, and the chemotherapeutic drug nab paclitaxel (Abraxane) was the first immunotherapeutic treatment be approved by the FDA for its promising effects in TNBC [50]. Therefore, exploring the various combination treatments involving the targeting of NK cell activating and inhibitory receptors may be a novel therapeutic approach in breast cancer.

Acknowledgments
We acknowledge the Department of Microbiology, Immunology, and Molecular Genetics and the David Geffen School of Medicine for their continued supports.

References
18. NK-mediated therapy of breast cancer


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V. Targeting NK Cells
PART VI

NK immunotherapy in various cancers
Adoptive NK cell therapies in children with cancer: Clinical challenges and future possibilities

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Abstract

Natural killer (NK) cells can kill cells without prior sensitization. They eliminate tumors via antibody-dependent cell-mediated cytotoxicity (ADCC) or through NK cell receptor-ligand interactions that trigger intrinsic NK cytotoxicity. When the human leukocyte antigen (HLA)-killer immunoglobulin receptor (KIR) mismatch was discovered as one of the mechanisms of intrinsic NK cytotoxicity, adoptive NK cell transfers moved into the spotlight of cellular therapies. Emerging technology to perform KIR and HLA genotyping of donors and recipients allowed the selection of suitable donors for this purpose. The adoptive transfer of NK cells has since evolved into an investigational therapy for hematologic and solid malignancies of childhood. Although early studies have demonstrated that HLA-KIR-mismatched donor transplants extend survival in patients with acute myeloid leukemia, the indication and activity of adoptive HLA-KIR-mismatched NK cells remain under active investigation. Current challenges include short persistence and limited efficacy of adoptive NK cells \textit{in vivo}, features that may be attributed to suboptimal activation and expansion \textit{ex vivo}. Therefore, research efforts are geared toward refining the procedures to manipulate donor NK cells in culture and identifying combinations to further enhance their efficacy. This chapter reviews the current state of adoptive NK cell therapies in children with cancer and highlights challenges and ongoing attempts to improve these strategies.

Abbreviations

- ADCC: antibody-dependent cell-mediated cytotoxicity
- AML: acute myeloid leukemia
- BLT: bone marrow, liver, thymus
- CAR: chimeric antigen receptor
NK cells and their biological role in tumor surveillance and elimination

Principles of “missing self” and ADCC

Natural killer (NK) cells are large granular lymphocytes and play a critical role in viral defense and tumor surveillance. Despite being part of the group of lymphocytes, they belong to the innate arm of the immune system because they do not require priming like T and B cells to execute their immunologic function [1]. T and B cells undergo homologous recombination and enter a process of positive and negative selection in the thymus and bone marrow, respectively. This process selects for the clones that lack autoreactivity and are diverse enough to confer broad adaptive immunity [2]. NK cells cannot undergo somatic rearrangement, but pass through a comparable selection process termed as licensing. During licensing, the maturing NK cells interact with major histocompatibility complex (MHC) class I molecules via their cell surface receptors. If they recognize MHC class I, indicative of self, they are deemed tolerant of the body’s antigens and advance developmentally into mature NK cells. In contrast, NK cells that lack self-recognition are eliminated to prevent autoimmunity [3].

Two major subgroups of NK cells exist in the peripheral blood [4, 5]. They are divided based on the density of expression of CD56 on the cell surface: CD56bright or CD56dim NK cells.
CD56\textsuperscript{dim} NK cells account for about 90% of all NK cells and are considered the progeny of CD56\textsuperscript{bright} NK cells. They possess potent effector function, whereas CD56\textsuperscript{bright} NK cells secrete cytokines primarily. Both attributes are essential to contain viral infections and prevent the development of cancer [6].

**FIG. 1** NK cell cytotoxicity through HLA–KIR mismatch or ADCC. (A) Under conditions where NK cells engage their cognate ligand, tumor cells are not eliminated (top). However, if KIRs are unable to engage their ligand, for example, when tumor cells downregulate HLA, NK cells get activated (color change to red) and tumor cells are killed (bottom). (B) NK cells can induce antitumor cytotoxicity by binding of NK-expressed CD16 (FcγRIIIA) to the Fc portion of the antibody (top), thereby, triggering the elimination of antibody-coated tumor cells (bottom).

NK cells eliminate infected, stressed, or transformed cells through complex interactions between NK cell surface receptors and associated ligands that are expressed on target cells (Fig. 1). Their effector function is determined by the cumulative balance of activating or inhibitory signals that they receive from NK cell receptors. For example, NK cells can be activated by a loss of human leukocyte antigen (HLA) class I expression on target cells, which are referred to as “missing self” [1, 7, 8]. The lack of HLAs removes the cognate ligands for inhibitory killer immunoglobulin-like receptors (KIRs) that then fail to send an inhibitory signal to the NK cell. In the presence of HLA class I, NK cells can also be stimulated when activating NK cell receptors, such as NKG2D, or CD16 are engaged.

Humans have 17 KIRs, nine of which are inhibitory receptors, six that are activating receptors, and the remaining two are encoded by pseudogenes with unknown functions (Table 1) [9]. The KIR genes are densely packed in a gene region on chromosome 19 that gained its diversity through the variability in gene content and allelic polymorphisms [10]. The KIR genotypes comprise various combinations of KIRs and pseudogenes and are classified as

**TABLE 1** Overview of human KIRs.

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<thead>
<tr>
<th>Inhibitory KIRs</th>
<th>Activating KIRs</th>
<th>Pseudo-KIRs</th>
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<tbody>
<tr>
<td>KIR2DL1, -2, -3, -4, -5A, -5B</td>
<td>KIR2DP1-2, -3/2D55C, -3/2D55T, -4</td>
<td>KIR2DP1</td>
</tr>
<tr>
<td>KIR3DL1, -2, -3</td>
<td>KIR3DS1</td>
<td>KIR3DP1</td>
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*Haplo-type A-related KIRs are shown in red, haplo-type B-related KIRs in blue*

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Benjamin_Bonavida, 978-0-12-824375-6
haplotype A or B. Haplotype A donors express six inhibitory KIRs and the activating KIR2DS4. Haplotype B donors have a variable gene content (Table 1). HLA and KIR genes are inherited independently, and the expression of KIRs occurs randomly, leading to an extensive KIR repertoire [10]. Therefore, in a donor search for an allogeneic bone marrow transplantation, only a few HLA-matched donors will also be an HLA–KIR match. In that event, engrafted stem cells will give rise to NK cells that, in theory, exert potent antitumor effects because the cognate HLA class I ligands are absent in the recipient [11].

NK cells are effective in eliminating antibody-coated tumor by engaging the Fc portion of the antibody with the NK cell surface receptor CD16 (FcγRIIIa; Fig. 1) [12]. FcγRIIIa is a low-affinity receptor for Fc that is exclusively expressed on human NK cells and monocytes/macrophages [12]. The effectiveness of ADCC depends on the FcγRIIIa ligation on the NK cell [13]. Previous studies identified single-nucleotide polymorphisms (SNPs) that may alter the affinity of FcγRIIIa for the Fc region [14, 15]. Specific SNPs have shown to correlate with an altered response to antibody therapy in some adult immunotherapy trials [16–19]. Studies are currently underway to investigate such associations in the pediatric population.

Antibody-dependent cell-mediated cytotoxicity (ADCC) is a powerful mechanism by which NK cells can be therapeutically harnessed. Cytolysis through this pathway can still occur despite the presence of HLA–KIR interactions or lack of other activating signals. For example, patients with neuroblastoma had improved outcomes with monoclonal antibody therapy when their genotype encompassed all KIR ligands and the inhibitory KIR2DL2 and KIR3DL1 [20]. Other corroborating studies, also in neuroblastoma, showed that patients with KIR3DL1 and HLA-B expression were predicted to have weak receptor-ligand interaction. This subgroup was found to have superior outcomes compared with patients that were predicted to have strong HLA–KIR interactions [21]. These data indicate that ADCC surmounts specific HLA–KIR interactions, suggesting that a subset of KIRs has more critical functions in NK cell activity and disease control than others. The functional and evolutionary significance of these qualitative rather than quantitative variations among NK cell receptors is not well understood and requires mechanistic and correlative studies in the future.

**Life cycle of an NK cell (persistence, proliferation, and equilibrium)**

Most NK cells have a finite life span of 2 weeks [22]. After being generated in the bone marrow or secondary lymphoid tissues, NK cells start circulating in the body and scan the environment for abnormal cells. For a long time, they have not been associated with immunologic memory, which is the ability of the immune system to protect the human body from pathogens that have been previously encountered. Immunologic memory classically forms in B and T cells of the adaptive immune system. For example, naïve T cells expand into effector T cells after their first encounter with a pathogen. The cells mount an immunologic response during which they fight off the infection and subsequently contract into a small pool of self-renewable memory T cells [23]. The same process occurs in B cells that contract to memory B cells and plasma cells after the disease is overcome [24]. Upon reactivation with the antigen, the memory cells rapidly expand and provide potent immunity. Although most NK cells die after 2 weeks, infection models with mouse cytomegalovirus (MCMV) demonstrated that virus-specific Ly49H+ NK cells could form memory and provoke a robust immune response.
after adoptive transfer into naïve animals [25]. Although the properties of the memory NK cells were like that of CD8+ T cells (i.e., antigen specificity, expansion, contraction, and recall response), the magnitude and kinetics of the cellular expansion were more modest in NK cells compared to CD8+ T cells [25]. Since this discovery in mice, many studies have proven the existence of memory NK cells in humans. Analogous to the MCMV model, human CD94-NKG2C-expressing NK cells undergo clonal expansion in cytomegalovirus (CMV)-seropositive patients during CMV reactivation [26]. Separately, memory B and T cells can secrete antibodies or cytokines [e.g., interferon (IFN)-γ] without encountering their specific antigen. This immunologic involvement is termed as bystander response and triggered by pattern recognition receptors or cytokines [e.g., interleukin (IL)-12] [27, 28]. Although memory NK cells can elicit a robust recall response, they engage far less in the bystander response than do memory B and T cells [29]. Nevertheless, the discovery of memory NK cells and their properties may have essential implications in the future clinical trials. An important challenge of adoptive NK cell therapies is the limited capacity to expand NK cells in culture. In the clinic, bulk NK cell products are used for infusion and selected based on CD3 depletion and CD56 enrichment. A more refined selection of donor NK cells based on their NKG2C expression could yield a product with more expandable NK cells and mediators of ADCC [30].

Mature nonmemory NK cells have a minimal ability to expand and contract [22]. For example, patients with acute mononucleosis have overall markedly elevated NK cell numbers, but the NK cell kinetics remain unchanged. Consistent with that, most studies that measure donor cell chimerism in patients who had received adoptive unmanipulated NK cells report decreasing donor chimerism levels after 2 weeks from the time of NK cell infusion [31–33]. These reports suggest that even adoptive NK cells follow the same kinetic trend as primary NK cells. It has been shown that therapeutic injection with IL-15 and, to some degree, IL-2 induces a robust in vivo expansion of endogenous NK cells [34–36]. IL-15 has not been tested with adoptive NK cells in children yet but holds promise in the future to extend the longevity of the transferred cells and potentially improve cytotoxicity in vivo.

Characteristics of NK cell-tumor cell interactions

Several studies indicate that the infiltration of activated NK cells into solid tumors is associated with better prognoses (see below for more detail) [37–41]. The interaction between NK cells and the tumor determines the composition and quality of the tumor cells (Fig. 2) [42]. Tumor cells that survive NK cell surveillance mechanisms are those that are insensitive to NK cell cytolysis and that have rearranged their surface receptor repertoire to adapt to this selection pressure. Remodeling the tumor microenvironment (TME) can also contribute to escape mechanisms, for example, through the presence of myeloid-derived suppressor cells.

NK cells orchestrate the inflammatory response by secreting specific chemokines that attract other immune cells and reshape the TME [43]. CCL-5-directed homing of conventional type 1 dendritic cells (cDC1s) and CD8+ T cells play an integral part in the tumor inflammation. These cells then engage in antigen presentation and cytotoxic activity or cytokine release (e.g., IFNγ) to further propagate the immune response. The roles of the immune cells in the TME can be complementary to each other. For instance, tumor cells evade CD8+ T-cell cytotoxicity by downregulation of MHC class I [1]. However, by doing so, they become more
susceptible to NK cell-mediated cytolysis. Efforts to improve adoptive NK cell therapy include directly injecting NK cells into tumors (NCT04254419) or combining NK cells with tumor-targeting monoclonal antibodies (NCT02030561, NCT03242603) [44], immune checkpoint blockade (ICB) therapy (NCT03958097), immunomodulatory drugs (NCT00720785), or recombinant and engineered cytokines such as an IL-15 superagonist (NCT02890758).

Associations between NK cells and survival in patients with cancer

Multiple biologic studies have substantiated the correlation between tumor-infiltrating NK cells and survival in adults with cancer [11]. For example, in acute myeloid leukemia (AML), the expression of NKp46, an activating NK cell receptor, is correlated with superior clinical responses after allogeneic stem cell transplantation [45]. The presence of mature NK cells in diffuse large B cell lymphoma (DLBCL) confers improved remission rates after treatment with rituximab and CHOP chemotherapy (i.e., cyclophosphamide, doxorubicin, vincristine, and prednisone) [46]. Other examples that corroborate these observations were reported in patients with melanoma and HER2-positive breast cancer [47]. Interestingly, a tumor gene signature marked by transforming growth factor-β (TGF-β)-induced epithelial-to-mesenchymal transition was found in NK cell-deplete tumors and negatively affected the survival [41]. Altogether, these reports indicate that NK cells have a direct role in antitumor immunity and provide a rationale for the use of adoptive NK cells in cancer therapy.

VI. NK immunotherapy in various cancers
We will discuss adoptive NK cell therapies in two exemplary pediatric cancers, AML and neuroblastoma. The rationale for using NK cells is very different when comparing these two diseases. Arguably, in AML, NK cells are used for immune surveillance [11, 48–50] in the setting of minimal residual disease. In contrast, in neuroblastoma, they have been employed to enhance ADCC induced by a monoclonal antibody against a tumor antigen (GD2) that is now part of the standard of care in patients with high-risk disease [51–54].

**Pediatric AML**

Acute leukemias are the most common cancer in the pediatric age group, of which AML accounts for approximately 15% [55]. The all-comers’ survival for patients with AML has improved from 20% in the 1970s to almost 70% with contemporary therapy regimens [56]. Patients with AML are assigned to risk categories based on cytogenetic and molecular features. While 90% of patients with favorable-risk AML (e.g., core-binding factor AML, PML-RARA translocation) achieve a cure with chemotherapy alone, children with intermediate and particularly unfavorable risk (e.g., monosomy 5 and 7, KMT2A rearrangement) make up the one-third of patients with disease relapse despite risk-adapted intensive multiagent chemotherapy with or without hematopoietic stem cell transplantation [57]. Therefore, more effective consolidation therapies for the latter group of patients are urgently needed to increase survival rates, while limiting therapy-related toxicities.

The first *bona fide* proof for the therapeutic effectiveness of NK cells in cancer was demonstrated in patients with AML. In a study of 57 adults, all 20 individuals who had received a haploidentical stem cell transplantation from a donor with HLA–KIR mismatch remained relapse free [11]. This remarkable observation has since been reproduced in the laboratory and confirmed in some studies [48, 49, 58], but not in others [59–61]. As a result, two therapeutic approaches evolved to harness the antileukemic effect of adoptive alloreactive NK cells. When patients receive myeloablative chemotherapy followed by an allogeneic hematopoietic stem cell transplantation, they reconstitute with stem cells from a donor with KIR–HLA-mismatched NK cells. Alternatively, when patients receive lymphodepleting chemotherapy followed by the adoptive transfer of HLA–KIR-mismatched NK cells, they experience transient engraftment of donor NK cells and, in theory, a temporary expansion of the transferred NK cells due to homeostatic lymphocyte proliferation.

In hematopoietic stem cell transplantation with an HLA–KIR-mismatched donor, the engrafted stem cells are a renewable source of alloreactive NK cells. They therefore provide a lifelong pool of endogenous alloreactive NK cells. The drawback of this therapy is the limited HLA-matched donor pool. On the other hand, finding an HLA–KIR-mismatched NK cell donor is more likely due to the mechanisms of inheritance of the HLA and KIR genes, and the likelihood is highest in haploidentical relatives [62]. This is a logistical advantage in pediatrics because most children are accompanied by their biological parents, who are willing to undergo testing and donate NK cells if found to be a suitable donor. The clear advantages of adoptive NK cell infusions are the less-invasive nature of the intervention compared to a hematopoietic stem cell transplantation and the feasibility of the procedure. However, because
NK cells have a short life span, adoptive NK cell infusions last only transiently. Although hematopoietic stem cell transplantsations with an HLA–KIR-mismatched donor have proven to be efficacious in AML [63], there is an ongoing research using adoptive NK cells in other hematologic and solid malignancies.

Several studies demonstrated the safety and feasibility of adoptive NK cell therapy in adult AML [31, 64, 65]. The first pediatric Phase I trial with adoptive NK cells for the treatment of AML enrolled 10 children with favorable- (N = 4) and intermediate-risk AML (N = 6; NKAML, NCT00187096) at St Jude Children’s Research Hospital [66]. Participants had completed chemotherapy and were in complete remission at the time of the NK cell infusion. They received a conditioning regimen comprised of cyclophosphamide and fludarabine. On alternate days, low-dose IL-2 (1 million U/m^2) was administered subcutaneously for six doses starting a day before NK cell infusion to expand circulating donor NK cells. Eligible parents underwent leukapheresis, and the cell product was purified by depleting CD3+ T cells and enriching for CD56+ NK cells using the CliniMACS system. The donor NK cells were immediately infused after NK cell enrichment without ex vivo expansion or activation. Peripheral blood from the patients was obtained for NK cell chimerism studies, phenotyping, and functional assays on days 2, 7, 14, 21, and 28 after NK cell infusion. Bone marrow evaluation for minimal residual disease (MRD) was performed by flow cytometry prior and 1, 2, and 4 months after the NK cell infusion.

In this study, T-cell contamination was detected in only one patient and measured 1 × 10^3/kg, which is 10-fold lower than the level commonly associated with GVHD. Patients received a median of 29 × 10^6/kg NK cells (range 5–81 × 10^6/kg), and all participants tolerated the adoptive NK cell transfer. Of the 10 patients, 9 expanded KIR–HLA-mismatched donor NK cells in their blood within the first 14 days. The intrinsic cytotoxicity of peripheral blood NK cells was measured against the erythroid leukemia cell line K562 in vitro. Using a 2:1 effector-to-tumor cell ratio, the average lysis of K562 cells by resting NK cells was between 10% and 30%, which lies within the expected range of activity. All patients remained in complete remission with a median follow-up time of 964 days (range 569–1162 days).

After safety and feasibility were confirmed in the Phase I trial, the next step was to determine whether adoptive haploidentical NK cell therapy prolongs the event-free survival (EFS) in children with AML in a Phase II study (AML08, NCT00703820) [32]. This St Jude trial enrolled children with intermediate-risk AML who were in first complete remission after completion of four or five courses of chemotherapy as part of a randomized controlled Phase III clinical trial. Patient and donor eligibility criteria were adopted from the Phase I trial mentioned above, and participants with recovering bone marrow received the identical conditioning regimen and IL-2 treatment as in the Phase I trial. Patients were monitored for 45 days after NK cell infusion for hematologic toxicities and GVHD. The survival of the patients was compared to children with intermediate-risk AML who did not receive NK therapy but had completed at least four courses of chemotherapy as part of the same Phase III clinical trial [32]. NK cell studies were conducted weekly for 4 weeks postinfusion.

In all, 21 patients participated in the trial and received a median NK cell dose of 12.5 × 10^6/kg (range 3.6–62.2 × 10^6 cells/kg). This cell dose was much lower compared to the number of infused cells in the Phase I study (12.5 vs 29 × 10^6/kg) but still higher than the median doses in adult studies [64, 65]. All but one patient developed grade 3 neutropenia and five (24%) developed grade ≥3 thrombocytopenia. GVHD did not occur in any of the patients. While the
white blood cell counts continuously rose after NK cell infusion, the NK cell concentration remained stable in the peripheral blood consistent with the natural kinetics of NK cells. The weekly chimerism studies revealed that donor NK cells initially increased but steadily decreased after 2 weeks from the time of NK cell infusion, leaving only 11 of 18 (61%) patients with detectable donor cells above the threshold of 1% at 4 weeks after adoptive transfer. This trend resembles the life span and persistence of primary NK cells in the peripheral blood. The NK cell dose was not correlated with the level of alloreactive NK cells in the peripheral blood or donor NK cell chimerism.

Eight patients (38%) relapsed between 186 and 629 days after NK cell infusion. Adoptive NK cells did not improve EFS (60.7 ±10.9% vs 69.1 ±6.8%; P = 0.553) or overall survival (84.2 ±8.5% vs 79.1 ±6.6%; P = 0.663) in these children compared to chemotherapy alone since a statistical difference would have required for all patients with NK cell infusion to remain relapse free (100% vs 69.1%). Interestingly, six of eight patients with disease relapse received less than the median NK cell dose. EFS was associated with specific NK cell surface markers (e.g., KIR2DL1, KIR2DL2/3, and NKG2D) and a higher NK cell count on day 7 but not with number of alloreactive NK cells. Hence, the failure of adoptive NK cells to decrease relapse or increase survival compared to chemotherapy may have been due to the limited persistence of donor NK cells and low cellular infusion dose in this trial.

Thus, the future of adoptive NK cell therapies in pediatric AML may require the use of ex vivo manipulations to enhance persistence and increase the expansion capacity of these cells (discussed further below). Alternatively, a refined selection of donor T cells (e.g., NKG2C-expression memory NK cells) could yield a more reactive cell product with enhanced function. Whether NKG2C NK cells engraft long term and retain the same safety profile as bulk NK cells remains to be shown in the future studies.

Neuroblastoma

Neuroblastoma, a developmental cancer of childhood, belongs to the group of neuroblastic tumors that arise from primitive sympathetic ganglion cells. With about 650 new cases in North America each year, it is the most common extracranial solid tumor of childhood [55]. It accounts for almost 15% of all cancer-related deaths in the pediatric population. Neuroblastoma mainly affects toddlers; however, it is the most common cancer in infants and can present in adolescents, or even rarely, in adults [67]. Because these neuroectodermal tumors arise from the sympathetic ganglia, patients typically develop tumors in the adrenal glands, abdomen, or along the cervical and thoracic paraspinal area. Presenting signs typically depend on the location of the primary tumor [68].

The natural history of neuroblastoma spans a remarkably broad clinical spectrum. For example, infants with stage 4S neuroblastoma have disseminated disease confined to skin, liver, bone marrow, and many 4S patients will eventually experience spontaneous regression over time [69, 70]. In contrast, older patients with stage 4 disease have a poor prognosis despite intensive multimodal therapy. Patients with worse survival tend to be older than 18 months and have a higher tumor stage, unfavorable histology (e.g., undifferentiated histology with high mitosis-karyorrhexis index), and specific genetic (e.g., MYCN amplification) and chromosomal abnormalities (e.g., 1p and 11q deletion or 17q gain) at diagnosis [71–73]. Based on
such predictive factors, children with neuroblastoma are assigned to risk groups that dictate the intensity of their therapy. About 50% of all newly diagnosed patients have high-risk disease, which has been historically associated with dismal survival. The treatment of these patients has evolved and now includes multiagent induction chemotherapy, surgery, and consolidation therapy with radiation, autologous tandem bone marrow transplantation, differentiation therapy with cis-retinoic acid, and immunotherapy [52].

Immunotherapy has, until recently, included an anti-GD2 monoclonal antibody, IL-2, and GM-CSF. After patients have attained a state of MRD, they received immunotherapy together with 13-cis-retinoic acid for five cycles, followed by an additional cycle of 13-cis-retinoic acid alone [52]. The addition of immunotherapy has significantly lengthened the 2-year EFS of patients with high-risk neuroblastoma compared to monotherapy with cis-retinoic acid (66% vs 44%) [52] and has marked one of the most significant therapeutic breakthroughs in the recent pediatric solid tumor therapy. Recent data from Europe have cast significant doubt on the added value of subcutaneous IL-2 both in terms of efficacy and the added toxicity [74–76]. For these reasons, IL-2 has been eliminated in the ongoing Children’s Oncology Group (COG) trials for newly diagnosed children with high-risk disease. Nevertheless, indications and benefits of intravenous recombinant IL-2 and engineered IL-2 muteins remain to be determined.

One-third of patients that achieve the state of MRD experience therapy failure within the first 2 years despite receiving immunotherapy, of which less than half can be cured. More effective therapies are still needed to improve the survival rates of patients with high-risk neuroblastoma.

As mentioned beforehand, NK cells are one of the primary effector cells of ADCC. To enhance immunotherapy for patients with neuroblastoma, NK cells have been adoptively transferred into patients who receive monoclonal antibody and cytokine infusions. This was attempted in a Phase I clinical trial by the Memorial Sloan Kettering group for the first time (NCT00877110) [53]. In this trial, the investigators treated patients with relapsed/refractory high-risk neuroblastoma with a lymphodepleting conditioning regimen [i.e., cyclophosphamide, vincristine, and topotecan [77]], haploidentical CD3-CD56+ NK cells, and m3F8, a monoclonal anti-GD2 antibody. The harvested NK cells were activated ex vivo overnight with 500–1000 U/mL of IL-2 at a concentration of 2 × 10^6 cells/mL. After 24 h, the NK cells were thoroughly washed before infusion into the patients. The release criteria for the final product required

\[ <2 \times 10^5 /kg \text{CD3}^+ \text{cells}, \geq 90\% \text{CD3}^{-}\text{CD56}^+ \text{NK cell purity by flow cytometry, and } \geq 70\% \text{viability.} \]

Although the study was planned to enroll patients according to a 3 + 3 NK cell dose-escalation schema, more patients ended up being treated at lower NK cell dose levels due to variations in the yield of donor NK cells. Specifically, isolation of NK-cell numbers \(>5 \times 10^6 /kg\) was feasible in only two-thirds of donors. The ex vivo procedure of IL-2 activation led to high cell loss (possibly overactivation) and required the IL-2 dose to be lowered from 1000 to 500 U/mL during the study. Due to this procedural limitation, the maximum tolerated dose was not reached. Among the 35 patients that participated in the trial, 14/35 (40%) were infused on dose level 3 (10–30 × 10^9 NK cells/kg). Dose-limiting toxicities were observed in two patients at dose level 1 (grade 3 hypertension and ventricular dysfunction).

VI. NK immunotherapy in various cancers
In all, 10 of 35 (29%) patients achieved a complete (N=5) or partial response (N=5), 17 (47%) had no response, and eight (23%) had progressive disease. Interestingly though, the authors noted that NK cell doses ≥10 × 10⁶/kg were associated with objective MIBG responses and improved progression-free survival, which suggests that the transferred cells contributed to the antitumor effect.

Phenotypic analysis revealed that more than 85% of the peripheral blood NK cells in the patients before and after NK cell infusion expressed NKG2A. NKG2A is an inhibitory KIR that corresponds to HLA-E on target cells and prevents alloreactivity. In comparison, NKG2A expression in healthy adult controls was approximately 55%, which presumably represents the levels in the parentally derived NK cells. Thus, the infusion of haploidentical and HLA–KIR-mismatched NK cells likely increased the pool of alloreactive NK cells representing one of the mechanisms of antitumor activity. Future investigations are necessary to confirm this hypothesis.

Another lesson learned from this trial pertains to the choice of conditioning regimen. All patients experienced lymphopenia (grade 4), thrombocytopenia (grade 4), and neutropenia (grades 3 and 4) [53]. Therefore, to reduce the incidence of severe myelosuppression, the Phase II trial by this group will employ single-agent cyclophosphamide 50 mg/kg/day IV for 2 days (NCT02650648).

Other correlative studies assessed HLA–KIR mismatch and FcγRIII receptor polymorphisms and revealed that these parameters did not correlate with the patient outcome. Therefore, the role of HLA–KIR mismatch and FcγRIII receptor polymorphisms as donor selection criteria remain elusive.

Despite administering a lymphodepleting conditioning regimen, most of the NK cells were undetectable at 7 days postinfusion. A possible explanation for such rapid clearance could be cytokine withdrawal, which can lead to quick NK cell death, especially when these cells were prestimulated with high doses of IL-2. Therefore, the follow-up study by this group incorporates in vivo administration of IL-2 (NCT02650648).

In another Phase I clinical trial (NCT01576692), haploidentical NK cells were isolated from a biologic parent via leukapheresis and infused into patients receiving chemoimmunotherapy [54]. When both parents were eligible for NK cell donation, the parent with the highest degree of HLA–KIR mismatch was chosen. Mononuclear donor cells were isolated using the ClinicMACS system by first depleting CD3+ T cells and then purifying CD56+ NK cells. Patients on this trial received six courses of chemotherapy with concurrent anti-GD2 antibody (hu14.18K322A) and cytokine infusion (GM-CSF and IL-2). As part of the courses 2, 4, and 6, patients were eligible to receive a haploidentical NK cell infusions, which were timed to be administered after chemotherapy and antibody infusions had been completed before or shortly after GM-CSF and IL-2 were started. The parentally derived NK cells were infused without ex vivo manipulation (e.g., no activation or expansion). Patients underwent chimerism studies by detection of variable number tandem repeats to monitor the persistence of the adoptively transferred NK cells 14 and 21 days after infusion. The cell surface expression of three KIRs (i.e., KIR2DL1, KIR2DL2/3, and KIR3DL1) was measured in the donors, and this information was used jointly with the HLA genotype of the patient to determine the HLA–KIR mismatch status between patient and parent.

In this Phase I trial, 11 patients received 29 haploidentical NK cell infusions with a median NK cell dose of 15.5 × 10⁶/kg (range 4.7–59.5 × 10⁶/kg). The infusions were tolerated without
reported side effects. Interestingly, the persistence of adoptive NK cells on day 14 after infusion varied between patients. Eight of the 11 (73%) children yielded similar individual chimerism results with repeated NK cell infusions, suggesting that the survival of the adoptive NK cells is dependent on intrinsic donor and recipient factors that remain to be determined. However, the persistence was not correlated with any of the measured clinical features in this trial (i.e., HLA–KIR mismatch status, donor age, patient and donor gender mismatch, and NK cell infusion dose). Consistent with the average life span of human NK cells, most patients (8/11; 73%) had undetectable donor NK cell levels by day 21 postinfusion. Of note, the median NK cell concentration in the peripheral blood on day 14 appeared to be higher with courses 2, 4, and 6, courses given with parental NK cell infusions, than courses 1, 3, and 5, courses without parental NK cell infusions [54]. However, due to the small size of this Phase I study, the significance of these elevated NK levels and the clinical impact of adoptive allogeneic NK cells were not able to be determined. Altogether, this study showed that adoptive allogeneic NK cells are a feasible therapeutic option in patients with neuroblastoma receiving chemo-immunotherapy. Moreover, the trial highlighted that our understanding remains limited regarding the in vivo behavior and persistence of adoptive NK cells in the therapeutic setting.

Based on the results of the Phase I trial, the combination of chemo-immunotherapy, cytokines, and adoptive NK cell therapy was further evaluated in a Phase II trial for patients with newly diagnosed high-risk neuroblastoma (NCT01857934) [44]. This prospective nonrandomized, single-arm study used the induction chemotherapy backbone of COG ANBL0532 and added an anti-GD2 monoclonal antibody (dinutuximab), GM-CSF, and IL-2 to each of the six induction courses. During consolidation, unmanipulated haploidentical NK cells were administered when available, 4–5 days after autologous stem cell infusion, followed by four doses of subcutaneous IL-2 (1 × 10^6 units/m²; every other day) and daily intravenous GM-CSF (250 μg/m²) [78]. The donor selection criteria were identical to the ones applied in the Phase I trial, and NK cells were purified using the same techniques as described above.

However, unlike the preceding trial, more detailed longitudinal NK cell studies were conducted to understand the impact of chemo-immunotherapy and adoptive NK cell infusion on NK cell concentrations in the peripheral blood [33].

Moreover, detailed phenotyping with multicolor flow cytometry, molecular studies of NK cells using single-cell RNA sequencing, and functional analyses to delineate intrinsic NK cell functions were performed. A total of 63 patients each provided 11 peripheral blood samples for research purposes. The time points were distributed throughout therapy and chosen to reflect best the fluctuations in cell numbers and possible changes in NK cell function, phenotype, and transcriptome.

This chemo-immunotherapy induced profound NK cytopenia, but all children reliably recovered their cell counts at the end of each induction course. The intrinsic NK cell function was measured as cytotoxicity against the cell line K562 in vitro. Surprisingly, the natural NK cytotoxicity improved during induction therapy compared with that of baseline measurements (at diagnosis), and the cells demonstrated a strong activation response to IL-2 and IL-15 stimulation in vitro. Altogether, this suggests that NK cell function is enhanced possibly due to the generation of a new NK cell pool and/or activation with cytokine infusions in vivo.

In this study, 31 patients received haploidentical NK cell therapy without remarkable toxicities. This study was not designed to discern the impact of adoptive NK cell transfer on EFS.
In concordance with previous studies, haploidentical NK cell infusion transiently increased the NK cell concentration in the peripheral blood, an effect that was noted 7 but not 21 days after NK cell infusion. Donor NK cells were undetectable in patients when they received an NK cell dose below \(25 \times 10^6\)/kg.

Alloreactive donor NK cells express HLA-mismatched KIRs but lack NKG2A and KIRs corresponding with patient HLAs. A total of 17 patients received alloreactive NK cells from their donor, and all evaluable children demonstrated an expansion of this subpopulation from day 7 to 21 posttransplant. The role that NK cell dose and alloreactivity play in persistence of NK cells needs further study.

The comprehensive phenotypic and functional information from these patients were integrated with primary outcome measures and examined for their relationship. CD56\(^{\text{bright}}\) NK cell count and degree of tumor response segregated the study population by their tumor volume at diagnosis and therapy-induced tumor regression after two courses. This suggests that NK cell properties and tumor responses are correlated. Future broad, unbiased biologic studies of NK cells in this patient population may identify factors to stratify patients by their therapy response and individualize chemo-immunotherapy in future studies.

Five patients from this trial underwent single-cell RNA sequencing of their peripheral blood NK cells at diagnosis and after two courses of induction chemo-immunotherapy [79]. It is known that NK cells divide into two groups by their CD56 expression status. These two subgroups were also transcriptionally defined, and the ratio of CD56\(^{\text{bright}}\) and CD56\(^{\text{dim}}\) NK cells at diagnosis remained stable in these cell clusters after two courses of therapy. However, analyses that compared the transcriptional signature of NK cells derived from these patients with healthy controls revealed a deficiency in a subgroup of active NK cells. These active NK cells arise after stimulation with, for example, cytokines [80]. The lack of this cluster was not only found in children with neuroblastoma but also in NK cells from a humanized mouse model with a patient-derived xenograft, when compared to healthy controls. Our observation suggests that tumor-related factors may lead to a depletion of active NK cells in neuroblastoma. It is unknown if cytokine therapy can replete this subpopulation. Whether the lack of this population is important for tumor growth and progression needs further study.

In summary, this trial demonstrated that adoptive NK cells can be administered safely during immune reconstitution after consolidation and stem cell rescue, bridging the profound NK cytopenia after myeloablation [44, 78]. The correlative biology studies provided detailed information on NK cell kinetics, phenotype, and function in patients with neuroblastoma during multimodal therapy [33]. However, because this study was not designed to evaluate the effects of adoptive NK cell therapy on the overall outcome, future trials are required to assess the efficacy of haploidentical NK cells in the setting of antibody administration in patients with neuroblastoma.

Adoptive CAR cell therapy

Engineered NK cells

T cells underwent a revolutionary transformation and repurposing for therapeutic use when T cells derived from patients were reengineered in vitro to express artificial chimeric
antigen receptors (CARs), targeting specific cancer antigens. This has been very successful in treating some hematologic malignancies. T cells expressing a CD19-specific CAR induce a sustained complete remission in patients with pre-B cell leukemia and are now approved by the FDA as a therapy for this group of diseases [81]. However, the current challenges of this therapy include logistics to produce these complex cell products, the lead time from collection to infusion, and the associated financial burden for manufacturing autologous CAR T-cell products. On the clinical side, toxicities with CAR T-cell therapy, such as off-target effects, cytokine release syndrome (CRS), or immune effector cell-associated neurotoxicity syndrome (ICANS) remain a challenge and limit clinical applications [82].

NK cells share many features with CD8+ T cells. Both populations express some identical germline-encoded receptors, such as KIRs, NKG2D, TIGIT, and KLRG1. They both express receptors for the γ-chain family of cytokines (e.g., IL-2 and IL-15) and require the same transcription factors for their development and for the regulation of cytotoxic function (e.g., EOMES and TBX21) [83]. Importantly, the way both cell types eliminate their targets overlaps in terms of effector molecules (i.e., granzyme subtypes and perforin) and cytokine profile (e.g., IFN-γ). Nevertheless, one significant difference between the two populations is that NK cells kill in an MHC I-unrestricted manner and, therefore, are also effective against cancers with low mutational burden as in pediatrics. They execute their function rapidly due to constitutively high levels of mRNA transcripts of effector molecules that can be translated and released in a more timely manner than in T cells [41].

Although the transduction efficiency of NK cells is lower and NK cell expansion less efficient than in T cells, CAR NK cells have, in many regards, theoretical advantages when compared to CAR T cells. Adoptive NK cells have a limited life span compared to T cells, which are advantageous from a toxicity standpoint because they last only transiently. CAR T cells cause CRS by secreting large amounts of IFN-γ, tumor necrosis factor-α, IL-1, and IL-6 [84]. NK cells, on the other hand, produce IFN-γ, IL-3, and granulocyte-macrophage colony-stimulating factor (GM-CSF) and are not usually associated with the development of CRS [85]. NK cells also do not cause graft-versus-host disease (GVHD) or ICANS by themselves and, therefore, may serve as an off-the-shelf cell product, alleviating the logistic and financial burden associated with the production of the CAR T cell product (Fig. 3).

Conceivably, the challenges with CAR NK cells relate to the rejection of these allogeneic cells after adoptive transfer. CAR T cells that are produced from autologous T cells do not face this issue. In addition, CAR NK cells do not undergo clonal expansion like T cells after encountering their antigen. Therefore, the lymphodepleting conditioning regimen becomes a critical component when designing therapeutic strategies with CAR NK cells [86]. Investigators have also found other ways to genetically engineer CAR NK cells to produce transgenic cytokines. These cytokines stimulate the cells in an autocrine and paracrine manner and boost the expansion capacity of CAR NK cells in vivo [87]. Alloimmunization to one donor product may occur with repeated infusions. Given the diversity of NK cells receptors, as discussed exemplarily for KIRs, patients with alloimmunization may tolerate adoptive NK cells from donors that lack the offending antigen.

The first human Phase I study of HLA-mismatched CAR NK cells was launched by the MD Anderson group and utilized the CD19–CD28–CD3ζ CAR construct to transduce allogeneic NK cells [87]. A transgenic IL-15 gene for activation and inducible caspase 9 suicide gene were integrated into the construct for efficacy and safety, respectively. Seven of the 11 treated...
patients with mature CD19-expressing B cell malignancy or chronic lymphocytic leukemia achieved a complete response after lymphodepleting chemotherapy and CAR NK cell infusion. The completed Phase I study demonstrated excellent tolerability of allogeneic CAR NK cells. None of the participants developed CRS or ICANS. Therefore, this trial marked a milestone in adoptive NK cell therapy because it demonstrated that allogeneic HLA-mismatched CAR NK cells are a safe cellular therapy.

Ongoing adult Phase I and II trials utilize HLA-mismatched CAR NK cells to target CD19 (expansion of the trial mentioned above; NCT03056339), ROBO1 (NCT03940820), NKG2D (NCT03415100), and BCMA (NCT03940833). Studies that will soon accrue patients will test CD22- (NCT03692767), PSMA- (NCT03692663), and mesothelin-targeting CAR NK cells (NCT03692637). The results of these studies will give us more insight into the efficacy and cellular kinetics of CAR NK cells in vivo.

**Engineered NKT cells**

NKT cells are a small population of lymphocytes that constitute <1% of the peripheral blood T cells. They possess the cytotoxic properties of NK cells but express the αβ T-cell receptor and recognize foreign and endogenous lipid antigens in a restricted manner like T cells when presented by CD1d [88]. Vα24-invariant NKT cells were found to naturally home to the TME in patients with neuroblastoma where they eliminate protumor macrophages and the presence of these NKT cells in tumors was associated with a better prognosis [88–90]. In the preclinical studies by this group, GD2-CAR-IL15 NKT cells were found to express less exhaustion markers, have enhanced in vitro cytotoxicity in tumor rechallenge experiments,
and show improved tumor control compared to GD2.CAR NK Ts in vivo, supporting the use of this construct in their clinical trial [88].

A Phase I trial by the group at Texas Children’s Hospital uses invariant NKT cells and transduces them with the abovementioned disialoganglioside (GD2)–CD28 CAR construct that contains a transgenic IL-15 gene (NCT03294954). After transduction and expansion, the GD2-CAR-IL15 NKT cells are infused in escalating doses, into patients with refractory/ recurrent neuroblastoma. The study is ongoing and preliminary results are expected soon.

Interventions to enhance adoptive NK cell function and persistence

The role of cytokines and conditioning regimens in NK cell therapies

We have learned that the efficacy of autologous NK cells is significantly compromised by the chronic immunosuppression induced by cancer [91]. The first-in-human studies that harnessed the antitumor effect of endogenous NK cells attempted to overcome these negative effects by activating NK cells with IL-2 in vivo [92]. Although IL-2 has been one of the oldest approved immunotherapies in patients, it has significant toxicities, especially at the higher doses that are needed to activate cells in vivo. Lower doses of IL-2 stimulate T regulatory cells that have an immunosuppressive effect [93, 94]. These caveats remain the main limitations of this agent [95, 96]. In an attempt to circumvent these issues, the subsequent studies obtained autologous [97] or allogeneic [86] NK cells via apheresis and performed the IL-2 stimulation ex vivo.

The disappointing clinical response of autologous NK cells is possibly explained by a KIR ligand match (i.e., expression of “self” HLA alleles by cancer cells), which results in the inhibition of the antitumor activity of autologous NK cells. To overcome this problem, the NK cell field moved toward using allogeneic HLA–KIR-mismatched NK cells from healthy related donors. This has several advantages. In contrast to patient NK cells, the allogeneic cells were also fully functional and spared from the immunosuppressive effects of the cancer. From the first-in-human trial with allogeneic NK cells, we learned that the conditioning regimen is a critical factor for successful adoptive allogeneic NK cell therapy because it makes space for the adoptive cells and suppresses the endogenous immune system, thereby, preventing rejection [86].

This trial was conducted with 43 adult patients with metastatic melanoma, metastatic renal cell carcinoma, and high-risk AML who received adoptive NK cells that were collected from the peripheral blood of haploidentical donors (Fig. 4). The NK cells were enriched and incubated in high-dose IL-2 (1000 IU/mL) overnight before the infusion into the patient. The patients received either a high-dose lymphodepleting regimen, a reduced-intensity regimen, or fludarabine alone, and all participants were supplemented with IL-2 daily for 14 days.

Interestingly, NK cell persistence was only observed in patients with AML who had received the intensive lymphodepleting conditioning regimen. Among this group, 5/19 high-risk AML patients achieved a complete remission. This correlated with the persistence of donor NK cells 7 and 14 days after infusion. This study has sparked the interest of multiple groups to refine the conditioning regimen and determine ex vivo manipulations to achieve optimal in vivo persistence and expansion of allogeneic NK cells.

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As mentioned above, initially, IL-2 was the main cytokine in use to achieve expansion and activation of NK cells ex vivo. However, over time, soluble or membrane-bound cytokines have been developed to further improve this process. For example, K562 cells were transduced to express membrane-bound 4-1BB/IL15Rα [98, 99] or IL-21 [100] (Fig. 4). These cells served as feeder cells for NK cell expansion in coculture. A 10^2- to 10^3-fold increase in NK cell numbers can be achieved that way, and the procedure induces an effector-like phenotype with potent antitumor activity in vitro. Investigators at the NCI tested this expansion strategy in a Phase I study, using allogeneic NK cells that were grown in coculture with IL-15/4-1BBL-expressing K562 cells for 9–11 days [98]. The cells were infused into the patient following an HLA-matched, T-cell depleted, nonmyeloablative peripheral blood stem cell transplantation. The first eight patients received an NK cell dose of 1 × 10^5/kg and one patient received 1 × 10^6/kg.

Unexpectedly, five of the nine participants developed acute GVHD, which was classified as grade 4 GVHD in three subjects. These patients were also found to have higher donor CD3+ T-cell chimerism. Given that the T-cell dose in the stem cell infusion product was below the threshold that is required for the development of GVHD, it was concluded that the administration of adoptive IL-15/4-1BBL-expanded NK cells contributed to the development of GVHD.
Another Phase I study (NCT01787474) at the MD Anderson Cancer Center utilized haploidentical NK cells, which were expanded from CD3-depleted peripheral blood mononuclear cells \textit{ex vivo} by coculture with irradiated membrane-bound IL-21 K562 feeder cells [101]. Expanded cells were infused following an allogeneic bone marrow transplantation. In all, 11 of 13 patients tolerated three NK cell infusions, of which 10 remained relapse free with a median follow-up period of 14.7 months. Seven patients (54%) developed grade 1–2 acute GVHD, but curiously, none had grade 3 or 4 GVHD or chronic GVHD.

In another trial by the group at the MD Anderson Cancer Center, haploidentical NK cells were activated with 1000 IU/mL of IL-2 for about 16 h overnight and administered to patients with myeloid malignancies undergoing allogeneic bone marrow transplantation [64]. NK cells were infused between the completion of the busulfan-/fludarabine-containing conditioning regimen and the antithymocyte globulin and stem cell infusion. A subset of patients also received in vivo IL-2 treatments following NK cell transfer. Only two (10%) patients developed ≥grade 3 GVHD. Despite the small sample size of 21 patients, survival was highly associated with the NK cell dose and the development of ≥grade 3 GVHD. However, the concentration of purified NK cells was only about $0.02 - 8.32 \times 10^6$/kg [64]. This group is planning a follow-up trial focusing on expansion methods \textit{ex vivo} to increase the NK cell numbers available for administration, and other methods designed to increase NK cell persistence.

In a head-to-head preclinical comparison, freshly obtained and activated NK cells were compared to cryopreserved and expanded NK cells [102]. Activation was induced through overnight culture with 1000 IU/mL of IL-2 for about 16 h overnight and administrated to patients with myeloid malignancies undergoing allogeneic bone marrow transplantation [64]. NK cells were infused between the completion of the busulfan-/fludarabine-containing conditioning regimen and the antithymocyte globulin and stem cell infusion. A subset of patients also received in vivo IL-2 treatments following NK cell transfer. Only two (10%) patients developed ≥grade 3 GVHD. Despite the small sample size of 21 patients, survival was highly associated with the NK cell dose and the development of ≥grade 3 GVHD. However, the concentration of purified NK cells was only about $0.02 - 8.32 \times 10^6$/kg [64]. This group is planning a follow-up trial focusing on expansion methods \textit{ex vivo} to increase the NK cell numbers available for administration, and other methods designed to increase NK cell persistence.

\textbf{Cytokine muteins as adjunct to NK cell therapy}

The emerging field of cytokine engineering has led to the development of muteins, a protein containing changes in amino acids, where these genetically mutated cytokines have an enhanced affinity or specificity [105]. These changes result in an improved biologic effect or reduced toxicity. Some strategies directly engineer the receptor-binding site or diversify the protein. The latter can be achieved by randomly introducing mutations into the respective gene or by site-directed mutagenesis that targets amino acid residues, which are part of the receptor-binding site. In phage, yeast, or mammalian cell display, the gene encoding the mutein is inserted into the respective cell, leading to the display of the mutein on the outside. These systems serve as screening platform for protein-protein interactions and in the cases of mutein engineering, help identify variants based on their affinity to the target receptor.

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Several IL-2 muteins have emerged with diverging affinities to the IL-2 receptor subunits. These subunits are differentially expressed on the various immune cell lineages. For example, IL-2 antagonists with enhanced affinity for IL2-Rα and reduced affinity for IL2-Rβ and IL-2Rγ bind more robustly to, and inhibit regulatory T cells, thereby shifting the effect toward an overall immunostimulatory response [106]. The “super-2” molecule has a 200-fold enhanced affinity for IL-2Rβ and potent preclinical antitumor activity with reduced pulmonary edema. In another study, “super-2” was modified to reverse its binding capacity to IL-2Rγ and showed effects in a model of GVHD [107].

While the IL-2 muteins are still in preclinical development, bempegaldesleukin has completed preclinical studies and is currently being tested in patients [108]. Bempegaldesleukin, also known as NKTR-214, is IL-2 conjugated to six releasable polyethylene glycol (PEG) chains and serves as an IL-2Rβ-preferential IL-2 agonist [109]. The PEGylation of IL-2 prevents the molecule from binding to the IL-2Rα subunit of the heterotrimERIC IL-2Rαβγ complex, which is expressed primarily on regulatory T cells. This altered binding profile skews the activity of bempegaldesleukin toward CD8+ T and NK cells. Upon infusion, the fully PEGylated bempegaldesleukin is a prodrug and only gains biologic activity after the PEG chains slowly dissociate from the molecule over time, improving the toxicity profile. In a Phase 1 study, single-agent bempegaldesleukin was administered to 28 patients with locally recurrent or metastatic solid tumors, including melanoma, renal cell carcinoma, bladder cancer, and colorectal cancer [108] (NCT02869295). Based on pharmacodynamic markers and safety assessments, a dose of 0.006 mg/kg, given once every 3 weeks, was recommended for further efficacy evaluations. Nine of 26 (35%) evaluable patients had a reduction in tumor size ranging from 2% to 30%. A Phase III study is ongoing and combines bempegaldesleukin with nivolumab compared with nivolumab alone in adult and pediatric patients with untreated inoperable or metastatic melanoma (NCT03635983). Expansion of the use of this agent in pediatric cancers with adoptive NK cells should be considered, given the favorable toxicity profile.

Another promising new cytokine is the IL-15N72D:IL-15RαSu/IgG1 Fc “superagonist” complex (N-803, formerly known as ALT-803) that contains two molecules of an amino acid-substituted (N72D) IL-15 and two molecules of the IL-15α receptor “sushi” domain fused to a dimeric human IgG1 Fc that confers stability and prolongs the half-life up to 70 h in animal studies [110]. Preclinical studies with this complex demonstrated immunomodulatory effects in mice with various tumors, most effectively against melanoma, myeloma, and urothelial cancers. Because the immune-modulatory effects lasted up to 7 days in the preclinical studies [110, 111], the Phase I trial in humans used a weekly intravenous schedule for administration [112]. Additional animal studies later indicated that the maximum concentration with subcutaneous dosing was eightfold lower than with intravenous infusion [111] and may have a better tolerability and toxicity profile. Thus, the final regimen for the Phase I studies in solid tumors [112] and hematologic malignancies [103] was changed to an outpatient regimen with subcutaneous injections. Subcutaneous N-803 was tolerated without significant side effects and led to a substantial NK cell expansion. A combination trial with N-803 and nivolumab was safely administered to 21 adults with nonsmall cell lung cancer in an outpatient setting [104].

Preclinical studies in neuroblastoma with precomplexed IL-15 and IL-15Rα have demonstrated antitumor efficacy in an integrated chemo-immunotherapy regimen. Mice with...
patient-derived neuroblastoma xenografts were given irinotecan/temozolomide alone, or with GM-CSF, with GM-CSF/IL-2, or with IL-15/IL-15Rα and an anti-GD2 monoclonal antibody [51]. Substitution of IL-15/IL-15Rα for IL-2 resulted in greater tumor regression. These preclinical data along with the convenient dosing schedule of N-803 and the more favorable toxicity profile as compared to IL-2 support further testing of N-803 in children with neuroblastoma.

Altogether, the use of these cytokines or engineered muteins enhances the quality and quantity of the immune response, leading to an improved efficiency of cell killing by NK cells and an increase in circulating NK cell numbers. They need to be evaluated in combination with adoptive NK cells in the future studies.

**Immune check point blockade to enhance NK cell function**

Immune checkpoints are inhibitory pathways that counterbalance the activation process in immune cells [113, 114]. These pathways prevent a sustained immune response and potentially dangerous consequences such as immune-mediated tissue damages. However, in immune evasion, cancer cells can hijack these pathways by expressing immune checkpoint ligands that engage the cognate receptors and inhibit immune function [115–117]. The most prominent examples are the PD-1/PD-L1 and CTLA-4/CD80/86 axis in T cells. The development of PD-1 and CTLA-4 blocking antibodies, also referred to as immune checkpoint blockade (ICB) therapy, has led to unprecedented objective responses in multiple patients with a variety of advanced stage cancers (e.g., melanoma, nonsmall cell lung cancer, etc.); a discovery that was honored with the Nobel Prize in Physiology or Medicine in 2018 [118–120]. NK cells also possess immune checkpoints, which include KIRs, NKG2A, CTLA-4, and PD-1, and other emerging molecules, such as B7-H3, TIGIT, LAG-3, TIM-3, Siglec-7/9, CD200, and CD47 [113]. The receptors are divided into those that contain a cytoplasmic immunoreceptor tyrosine-based inhibition motif (ITIM) domain (e.g., KIR2DL2/3, NKG2A, TIGIT/CD96, PD-1, and Siglec-7/9) and those that lack such a signaling domain (e.g., LAG-3, TIM-3, CD200, CD47, and CTLA-4). The inhibitory regulation of NK cells through these receptors is termed as dominant inhibition, whereby NK cell activation is prevented by abrogating the activating signal [3, 121]. Dominant inhibition sets NK cells apart from classically exhausted T cells because they lack anergy or apoptosis [121].

Given the success of anti-PD-1 and anti-CTLA-4 blocking antibodies in cancer therapy, other antibodies that target inhibitory NK cell receptors are currently an active focus of clinical development (Table 2).

Current clinical trials administer NK cell-specific ICB therapy to overcome the inhibition of endogenous NK cells in patients with cancer. However, it is conceivable that such interventions may enhance the biologic activity of adoptive NK cells, too, when administered consecutively. Possible regimens may include one or even a combination of different checkpoint inhibitors since dual targeting has demonstrated synergy in preclinical models [122] and some clinical trials [123]. Particularly, the blockade of TIGIT appears to prevent the functional exhaustion of NK cells and promote NK cell-dependent tumor immunity [124]. Laboratory-based studies are needed to better understand the contribution of the individual inhibitory receptors as they pertain to the function of adoptive NK cells. Based on such studies, meaningful combinations of NK cell-specific ICB therapy can then be evaluated in the clinic.

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### TABLE 2
Overview of therapeutic antibodies targeting NK cell checkpoints (excluded from the list are anti-CTLA-4 and anti-PD-1 antibody trials).

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Antibody</th>
<th>Active clinical trials</th>
<th>Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>KIR2DL2/3</td>
<td>Lirilumab</td>
<td>HNSCC (NCT03341936)</td>
<td>II</td>
</tr>
<tr>
<td>NKG2A/CD94</td>
<td>Monalizumab</td>
<td>HNSCC (NCT02643550)</td>
<td>I/II</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hematologic malignancies (NCT02921685)</td>
<td>I</td>
</tr>
<tr>
<td>TIGIT/CD96</td>
<td>BMS-986207</td>
<td>Multiple myeloma (NCT04150965)</td>
<td>I/II</td>
</tr>
<tr>
<td></td>
<td>MK-7684</td>
<td>Melanoma (NCT04303169, NCT04305054, NCT04305041)</td>
<td>I/II</td>
</tr>
<tr>
<td></td>
<td>BGB-A1217</td>
<td>Metastatic solid tumors (NCT04047862)</td>
<td>I</td>
</tr>
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<td></td>
<td>COM902</td>
<td>Advanced solid tumors (NCT04354246)</td>
<td>I</td>
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<tr>
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VI. NK immunotherapy in various cancers
Challenges of development and future directions of adoptive NK cell therapy

Reasons for NK cell therapy failure (microenvironment, persistence, exhaustion)

With infections, NK cell activation is a highly controlled process resulting in containment of inflammation and pathogen clearance. In the solid TME, NK cells face several challenges [91]. Solid tumors are heterogeneous and contain spatially divergent gene expression and mutations that influence the potential of cells to metastasize, proliferate, and evade the immune system.

Structurally, NK cells encounter environmental conditions such as hypoxia, lactic acidosis, and exposure to TGF-β, which inhibit NK cells and compromise their ability to kill tumor cells.
Abnormal angiogenesis gives rise to a defective vasculature that adds to hypoxia and lactic acidosis and prevents cellular extravasation, suppressing immune function [125–127]. Factors that are the most influential at inhibiting NK cell function in the TME are still incompletely understood.

NK cells scan their environment for abnormal cells. In this process, they influence the expression of ligands, referred to as immunoediting, and enable tumor cells to evade NK cell detection by downregulating activating and upregulating inhibitory NK cell ligands [128]. In a murine fibrosarcoma model, tumors are more immunogenic in NK cell-deficient mice than in those with regular NK cell numbers [42]. Tumors, however, also possess other powerful ways of decreasing NK cell activity. For example, they can secrete soluble factors that negatively regulate NK cell function. TGF-β changes the expression of the transcription factor Eomesodermin (EOMES) and thereby reduces degranulation and cytokine release in NK cells [129].

NK cells themselves shape the immune landscape of tumors through the secretion of chemokines and cytokines. Among these molecules are GM-CSF, CCL5 (RANTES), XCL1 (lymphotactin), and FLT3L [43, 130]. For example, conventional type 1 dendritic cells (cDC1) migrate into the tumor in response to XCL1 release by NK cells. In conjunction with FLT3L and GM-CSF, these cDC1 proliferate, differentiate, and assume their function as antigen-presenting cells in the TME. CCL5 recruits CD8+ T cells into the tumor, where they encounter their neoantigen and undergo clonal expansion. This recruitment has proven to be critical in inducing tumor control in vivo [130, 131]. A better understanding of these complex interactions is essential to improve responses to adoptive NK cell therapy.

Immune-cold tumors are also referred to as immune deserts. They lack the infiltration of immune cells and signs of inflammation. For NK cells to be effective against tumor cells, they are required to continuously migrate into the TME and convert the immune-cold into an immune hot tumor [128, 132]. A number of anticancer therapies have been evaluated for their ability to transform a “cold” tumor to a “hot” tumor including chemotherapy [133], radiation therapy [133], small molecule inhibitors [134] as well as various cytokines/muteins (NCT02350673, NCT01579318, NCT00323206, NCT01502293, and NCT02345330) [135,136]. The effect of chemotherapy on tumor cells and their expression of NK cell ligands and reactivity is incompletely understood. For some compounds, these effects have been studied and reported. Standard therapies, including cisplatin, 5-fluorouracil, radiation therapy, and TNF-α supplementation, upregulate the expression of B7-H6, the NKp30 ligand, in tumor cells and enhance their sensitivity to NK cell-mediated cytolysis [133]. Interventions that interfere with DNA-damage repair mechanisms such as PARP inhibitors were demonstrated to upregulate NKG2D ligands in AML stem cells, making them susceptible to NK cell clearance [137]. But beyond this, PARP inhibitors also act through the mechanisms described above, where they activate DC and attract CD8+ T cells through an increase in CCL5 expression via the STING pathway [138]. Elegant studies in KRAS and TP53-mutant lung cancer cell lines showed that the combination of a MEK and CDK4/6 inhibitor increased the susceptibility of these cells to NK cell-mediated lysis in vivo [134]. This process occurs by upregulating NKG2D ligands and ICAM-1 expression in the tumor cells and increasing the secretion of IL-15, CCL-5, and TNF-α to attract NK cells to the TME. The antitumor effect was reversed in vivo when animals were depleted of NK cells or treated with antibodies targeting ICAM-1 or TNF-α. In another study, the use of a BET protein inhibitor also induced the
expression of the NKG2D ligand MICA in multiple myeloma cells effectively by downregulating IRF4, rendering them susceptible to NK cell degranulation [139].

As reviewed above, cancers, particularly solid tumors, employ a variety of mechanisms to hide from immune surveillance, resulting in decreased NK cell trafficking, targeting, and killing of tumor cells. To make the most of adoptive NK cells in the future, it seems clear that an integrative therapy approach will be necessary, employing many of the strategies we have discussed including: engineering of NK cells, monoclonal antibodies against tumor cells or NK cell checkpoints or inhibitory receptors, cytokines/muteins as well as chemotherapy, and/or radiation therapy. All these interventions have a direct impact on cancer cells and NK cells and the challenge for the future is to find the most synergistic way to employ these strategies to overcome the immunotherapy resistance of immune-cold tumors.

Preclinical models to test NK cell therapies

Mouse models have contributed significantly to our understanding of cancer biology but have significant limitations, especially when evaluating immunotherapeutic agents [140, 141]. Nowadays, immunocompetent and humanized animals are used complimentarily to help us better understand the immune response in cancer and test therapeutic combinations with translational relevance [142]. The main drawback of syngeneic tumor models is the intrinsic difference between mice and humans, which have made direct clinical translation challenging. For example, when planning preclinical antibody studies, it is essential to acknowledge that most human Fc receptors have an equivalent orthologous nomenclature in mice, but that both systems differ in their affinity, expression patterns, and cross-reactivity [12]. As such, all human activating Fc receptors can bind IgG1, the main IgG subclass [143]. In mice, only FcγRIII binds mouse IgG1 [144]. The inhibitory Fc receptors in humans have the lowest affinity for IgG among all Fc receptors [143]. In contrast, the affinity in the murine counterpart is higher when it comes to binding IgG1 and IgG2b [145].

Aside from these intrinsic differences, there is also arbitrary cross-reactivity between the species, which engages different subsets of effector cells due to divergent expression. While murine Fc receptors generally cross-react with all human Fc domains, human Fc receptors have a more selected binding ability to mouse IgG subclasses [146]. The half-life of human antibodies is usually shorter in mice than vice versa because the murine neonatal Fc receptor (FcRn), which is responsible for lysosomal antibody degradation, can recognize human antibody. Human FcRn, on the other hand, does not bind mouse IgG1 [147]. Lastly, the expression of human FcγRIIIa, but not the orthologous mouse FcγRIII, is restricted to NK cells and monocytes/macrophages [148].

Humanized mouse models can overcome some of these inherent differences (Fig. 5). Immune-deficient mice allow the engraftment of human tumors, such as patient-derived xenografts (PDXs). PDXs recapitulate more faithfully human histology, the molecular landscape, growth behavior, and resistance to therapy than immortalized cell lines or syngeneic tumors [149]. For immunotherapy studies, these mice can be reconstituted with human immune cells.

The humanization of effector cells can be achieved with an adoptive transfer of mature effector cells (e.g., PBMC, purified NK cells) or CD34+ hematopoietic progenitor cells that later reconstitute the mouse with a human immune system [150, 151]. The development and
education of innate human immune cells can be achieved in conventional NSG and related strains (e.g., NOG, NRG, BRGS). MISTRG mice that have five human transgenes have a superior development of innate immunity, including NK cells, then the previous models. It is also possible to use endogenous NK cells in CD-1-deficient nude mice for antibody testing [51].

Nude mice lack a thymus and functional T cells but can establish PDX growth. The levels of NK cells in these animals are lower than in immunocompetent mice, but they are functional and can elicit ADCC. Most importantly, they allow testing of immune-modulatory interventions. This type of testing is not usually possible in adoptive NK cell regimens. Adoptive NK cells are stimulated in IL-2 ex vivo resulting in an activated phenotype and enhanced cytotoxicity [150] before they are injected. These manipulations decrease the chances of detecting a stimulatory effect of in vivo cytokine therapy. In addition, the cell doses that mice receive as adoptive cell transfer are significantly higher, when extrapolated to human equivalent dosing.

Regardless, the main limitation of all humanized animal models pertains to the absence of adaptive immunity. Although low levels of human T cells are present in the mice, they are educated based on murine MHC in the mouse thymus. HLA-restricted T cells can develop in these models through the implantation of fetal human thymus and liver tissue and

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simultaneous injection of autologous fetal liver hematopoietic progenitor cells ("BLT" mouse; bone marrow, liver, thymus) [152]. However, with federal restrictions on the use of fetal tissue, these models are now being validated with embryonic tissue. Although these new models (i.e., MISTRG and BLT mice) hold promise in the future, factors such as availability of the animals and cell sources, expertise, and costs have limited their current applicability in preclinical testing.

Conclusion

Adoptive NK cell therapies in children are an area of continued interest and investigation. We have reviewed basic NK cell mechanisms of tumor control and current approaches to exploit these mechanisms for antitumor therapy. Recent landmark trials have paved the way for adoptive NK cell therapies in children but also highlight the current challenges in preparing and using these cell products. The areas that require further investigation pertain to improved methods of NK cell activation, expansion, and refinement of cell isolation. With the emergence of new cytokines and molecular techniques to manipulate and study the NK cell subpopulations, we anticipate new methods for the production of cell products and new combinations, which have the promise to overcome the current limitations of adoptive NK cell therapy in children with cancer.

References

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anti-GD2 antibody dinutuximab beta (DB) long-term infusion with and without subcutaneous interleukin-2 (scIL-2) in high-risk neuroblastoma patients with relapsed and refractory disease: results from the SIOPEN LTTrial. J Clin Oncol 2019;37 [suppl; abstr 10014].


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References


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CHAPTER 20

NK cells in prostate cancer

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Abstract

Advanced modalities in the treatment of prostate cancer, an important health problem among men, draw attention to the field of cancer immunotherapy. Although the immune system holds promise for prostate cancer treatment, the biological processes associated with prostate cancer cells and immune cells have not yet been fully elucidated. The prostate cancer microenvironment is a complex system containing a large number of immune cells. Among multiple immune cells, natural killer (NK) cells have enormous potential in targeting various cancer cells. NK cells, which are an important part of the innate immune system, play a very important role as a first-line defense against prostate cancer cells. Various strategies have been adopted by researchers to increase the efficiency of NK cells. However, there are many difficulties to increase the therapeutic relevance of NK cells. This review will provide a perspective on the phenotypic and functional properties of NK cells, molecular links between NK cells and prostate cancer cells, and the therapeutic and prognostic significances of NK cells in both advanced hormone naïve prostate cancer and castration-resistant prostate cancer.

Abbreviations

- AP-1: activator protein-1
- APM: antigen-processing machinery
- bi-Tg: bi-transgenic
- CSCs: cancer stem cells
- C2GnT: core2 β1,6-N-acetylglucosaminyltransferase
- CLEC2D: C-type lectin-like domain family 2
- CIK: cytokine-induced killer cell
Introduction

Prostate cancer is still one of the leading causes of cancer-related deaths in men. In 2020, new prostate cancer cases and deaths from prostate cancer were estimated to be 191,930 and 33,330, respectively [1]. Adenocarcinomas comprise a large extent of all prostate cancers with a rate of 98% [2]. Early diagnosis and pathological classification take an important place in the survival of patients. Prostate-specific antigen (PSA), also known as hK3, has been defined in the 1970s. In 1986, the FDA has approved the PSA as a marker for the management of patients who have already been diagnosed with prostate cancer. In 1994, PSA has been accepted as a marker for diagnosis. There are still some concerns about the use of PSA as a diagnostic marker in prostate cancer [3]. The Gleason grading system is still used to help evaluate the prognosis of men with prostate cancer using prostate biopsy samples. This histological categorization is the most commonly used grading system and is still one of the most useful predictors for the prognosis of the patients’ classification.

The clinical profile shows quite a wide range because of the stage of the disease. Besides incidentally diagnosed patients with no symptoms, there are also patients with distant
metastasis in whom castration treatment resistance can also be observed. Radical prostatectomy for local tumors, adjuvant chemotherapy, and medical-chemical castration treatments can be used to improve survival rates. Although most of the prostate cancer patients typically respond to initial medical-chemical castration treatment, later on these group of patients returns to the androgen independent-castration-resistant prostate cancer (CRPC) form. Second-generation antiandrogens (apalutamide, abiraterone, enzalutamide), cytotoxic agents (docetaxel or cabazitaxel), radium-223, and autologous active cellular immunotherapy have been developed for CRPC patients but even though these treatments improved the quality of life and survival, recurrence occurs most of the time [4,5]. Therefore, there is still a need for new agents with alternative pathways.

Nature killer (NK) cells have an important role in cancer immunity. Monoclonal antibodies (mAbs) such as cetuximab and trastuzumab are used for the treatment of metastatic solid tumors via antibody-dependent cellular toxicity. Preclinical studies also show the effect of NK-mediated antitumor immunity via the NK group 2D receptor (NKG2D) activation [6,7]. Various studies have indicated that microenvironment regulation is almost the first step of tumor development in solid tumors and plays a crucial role in immunoregulation [8]. Various tumor antigens have been identified for prostate cancer but due to its immunosuppressive microenvironment, prostate cancer is eventually poorly immunogenic [9,10]. NK cells have a major impact on remodeling the tumor microenvironment and NK cell infiltration in solid tumors has been associated with improved clinical outcome [11,12]. Pasero and colleagues also stated that cytotoxic and antitumoral effects of NK cells have been diminished by the prostate cancer tumor microenvironment thus recovery of NK cell functions in the prostate cancer microenvironment should be considered in terms of immunotherapy [13]. In 2010, the FDA approved the first autologous cancer vaccine for men with CRPC, asymptomatic or minimally symptomatic and metastatic prostate cancer. However, there has been growing evidence and attention on the effect of immunotherapy on cancer treatment. Immune checkpoint inhibitors (ICPI) inhibit checkpoint proteins that are responsible for blocking the immune response against cancer cells. In recent years, ICPIs have been highly researched in the treatment of advanced tumors. Unfortunately, their success on CRPC has not been satisfactory in phase 3 studies. Clinically, ICPIs can be examined in two classes as cytotoxic T lymphocyte antigen-4 (CTLA-4) inhibitors and the programmed death receptor-1 (PD1)/programmed death-ligand 1 (PDL1) inhibitors. Both groups of immunotherapies have clinical trials in CRPC. While nivolumab, durvalumab, avelumab, and atezolizumab target PD-1 receptors, ipilimumab and tremelimumab target the CTLA-4 receptor. Early results of clinical studies did not reveal great success suggesting that further studies on the prostate cancer microenvironment should be necessary to discover advanced agents for treatment.

Understanding the general biology of NK cells: An overview

NK cells are a type of cytotoxic lymphocytes critical to the innate immune system. They are related to group 1 innate lymphoid cells (ILCs) since they can produce interferon-gamma (IFN-γ) and tumor necrosis factor (TNF)-α upon stimulation [14]. However, NK cells differ from Group 1 ILCs as they also have cytolytic functions similar to those of CD8+ cytotoxic
T lymphocytes [14]. The cytolytic function of NK cells can initiate through a variety of processes such as degranulation and death receptor ligation [15]. This process is crucial for the clearance of diseased and dysfunctional cells without prior sensitization by utilizing the unique set of receptors expressed on their surface via interaction with ligands on infected and transformed cells [16–19]. On the other hand, NK cells do not express clonotypic T-cell receptor (TCR) of T and NK T cells and its associated signal-transducing adaptor, CD3ε. However, they can mediate antitumor cytotoxicity and produce significant amounts of proinflammatory cytokines [20]. The cytolytic function of human NK cells resides primarily in the CD56dim population [21]. The subsets of NK cells express the activating Fc receptor, CD16 and most express the CD56 [neural cell adhesion molecule (NCAM) or Leu-19] in humans [22]. However, evidence suggests that their capabilities regarding antitumor cytotoxicity and inflammatory cytokine production may not be acquired equally [23]. The current knowledge of NK cells’ biology and the challenges facing NK cell antitumor strategies in solid tumors were recently reviewed [24].

NK cells are involved in unique signaling pathways. Unfortunately, the ligands that NK cells interact with are not characterized completely. NK cell receptors are categorized as activating, inhibitory, and mixed functions. NK cells integrate signals from a variety of activating and inhibitory receptors in a hierarchical manner to establish an activation state [20] (Fig. 1A). The natural cytotoxicity receptors, comprised of Nkp30, 44, and 46 and Fcγ-RIII

![Diagram](image-url)

**FIG. 1** Strategies aimed at improving the reactivity of natural killer cells against prostate cancer cells. (A) The interaction of NK cell with prostate cancer cell by ligation of NK cell-activating receptors and inhibitory receptors with corresponding ligands on the targets. Cytokines can increase NK cell antitumor immunity by increasing the production and release of cytolytic granules (perforin, granzymes). (B) Soluble factors and some cytokines released from cells may affect NK cell and the prostate cancer cell interaction through signaling pathways. (C) Cross talk between prostate cancer cells and macrophages can modulate the anticancer properties of NK cells.

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A (CD16) that trigger antibody-dependent cellular cytotoxicity are included as activating receptors [25]. On the other hand, CD161, KLRG1, PD1, TIM3, LAG3, CD96, and TIGIT are listed as inhibitory receptors [16]. In addition to these activating and inhibitory receptors, there are NK cell receptors that have a mixed function such as the killer cell Ig-like receptors (KIR) and the NK group 2 (NKG2) receptors. For instance, KIR2DS, KIR2B4, and KIR3DS are shown to activate NK cell cytolytic activity in contrast to other KIR with inhibitory functions. Another example can be given for the NKG2A receptor which inhibits NK cell activity. However, NKG2D, which is highly conserved from mice to humans, is involved in the initiation of cytotoxic activity upon binding to specific stress-induced ligands selectively expressed on tumor cells such as the MHC class I chain-related molecules (MIC), MICA and MICB, and the viral UL16-binding proteins [16,26,27]. The NK cell receptors that interact with MHC-I belong primarily to the KIR family in humans and the lectin-like homodimeric Ly49 receptor family in mice. The receptor-proximal signaling molecules activate the CBM signalosome containing Carma1, Bcl10, and Malt1, as well as Akt and the MAPKs, Erk1/2, Jnk1/2, and p38 [28–30]. NKG2D-related NK cell activation leads to the mobilization of lytic granules as well as cytokine production via activation of transcription factors such as activator protein-1 (AP-1) and NF-κB [28,29].

The cytokine receptors including IL-2 receptors, IL-4, 10, 12, 15, 18, and 21 receptors, as well as TGF-β receptors (TGF-β-Rs) are also expressed by NK cells [16,26]. Proinflammatory cytokines such as TNF-α induce tumor cell necrosis through the interaction of NK cell FASL and tumor cell FAS complex, thereafter triggering apoptosis process in the target tumor cell. On the other hand, the release of some cytokines such as CCL5, XCL1, and XCL2 involves the recruitment of dendritic cells into solid tumors through NK cells association [16,31]. NK cells can also promote the release of IFN-γ, which induces a proinflammatory tumor microenvironment by recruiting additional immune effector cells such as macrophages and dendritic cells, ultimately driving the TH1 immune response [32]. Another mechanism that NK cells are actively involved is that they can also attack immature dendritic cells and sparing mature ones [33]. However, the cascade of the cross talk between NK cells and lymphocytes, myeloid cells, or nonimmune cells such as epithelial or endothelial cells in a successful immune response is also far from fully being understood. Furthermore, NK cells play an important role in a variety of autoimmune disorders acting as drivers of pathologic inflammation [34], and also they can regulate the tissue repair process through antiinflammatory programs [35].

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The prostate is the largest accessory sex gland in the male reproductive system. It employs an exocrine function by secreting proteolytic enzymes, prostatic acid phosphatase, fibrinolysin, zinc, prostate-specific antigen, etc. [36] and also displays endocrine functions involved in the production of thyrotropin-releasing hormone, inhibin, etc. [37]. The prostate gland is of interest since it is considered an immunocompetent organ in contrast to the immune privilege of the testis by the presence of the blood-testis barrier. The studies have indicated that there were several immune cells, such as mast cells, lymphocytes, NK cells, and macrophages in the stroma and interstitium of prostate tissue [38]. These immune cells seem to be involved in
both male infertility [39] and the prevention of autoimmune reactions toward intraprostatic and sperm antigens [40]. The analysis of intraprostatic lymphocyte profiles in Sprague-Dawley rat prostate revealed unique characteristics of lymphocytes compared with those in other tissues, consisting of three major subsets of cells as $\alpha\beta$ TCR $^+ \text{T cells}$, CD161a $^+$ $\alpha\beta$ TCR/CD8 $^-$ NK cells, and CD161a $^-$ $\alpha\beta$ TCR $^+$ NKT cells [41]. The same research group has also showed a decrease in the number of intraprostatic NK cells and a high NK T/T cell ratio were reported in rats with estradiol-induced prostate inflammation [42]. These studies take a special attention since the chronic prostatitis is the most common urologic disorder in men younger than age 50. Therefore, detailed investigations are needed in order to identify the phenotype and functions of NK and NK T cells and highlight the immunological aspects of the diagnosis and treatment approaches in chronic prostatitis.

NK cells have the immunotherapeutic potential for the treatment of several cancer types including prostate cancer which is the most common cancer among men across the world [43,44]. Previous reports indicated an association between different stages of prostate cancer and NK cells [45–48]. It was even indicated that NK cells alone can conduct prostate tumor immunosurveillance and mediate protection [49]. However, the isolation of tumor-infiltrating immune components is problematic due to the small prostate sample size and the infiltrative growth of the tumor within the gland. Pasero and colleagues, therefore, have investigated the NK cell phenotype and functions infiltrating control and tumor prostate tissues [13]. NK cell infiltrates in prostate tissues were mainly CD56 (NCAM1) positive showing an unexpected immature, but activated, a phenotype with low or no cytotoxic potential. The study reported that tumor breaks the balance between activating and inhibitory NK cell receptors in a way that prostate cancer cells alter the receptors involved in their recognition by inducing the expression of the inhibitory receptor (ILT2/LILRB1) and downregulating the expression of activating receptors Nkp46 (NCR1), NKG2D (KLRK1), and CD16 (FCCR3) by NK cells [13]. The cytokine milieu in the prostate tissue environment was suggested to impact the immunosuppressive effects on NK cells partly through TGF-$\beta$ (Fig. 1B). Therefore, it has been suggested that the strong immunosuppressive environment impairs NK cell function at multiple levels in prostate cancer providing a rationale for the consideration of therapies that could restore NK cell efficiency in the prostate tumor microenvironment [13].

It is well known that cytokines are significantly involved in tumor development and progression. It was originally demonstrated that interleukin (IL)-32$\beta$ inhibits prostate tumor growth via the activation of cytotoxic T cells and NK cells, and the inactivation of NF-kB and STAT3 [50]. However, it remains to be investigated how IL-32$\beta$ modulates STAT3 and NF-kB signals through these immune cells. On the other hand, in a very recent study, among a panel of NK cell-activating cytokines, IL-15 was suggested as the only cytokine that could stimulate the expansion of NK cells in the presence of prostate cancer cells [51] (Fig. 1B).

Xu and colleagues demonstrated that M2-type tumor-associated macrophages (TAMs) affect CRPC to decrease the susceptibility to NK cell mediated-cytotoxic action using the phorbol myristate acetate (PMA)/IL-4 treated THP-1 cells as macrophage source [52]. The JAK/Stat3 pathway was indicated as the critical IL-6 downstream signaling that triggers the THP-1 conditional media effect (Fig. 1C). Thus, targeting the JAK/Stat3 signaling may enhance the NK cell action to CRPC tumors [52–54]. However, whether TAMs also affect the susceptibility of androgen-dependent prostate cancer cells to NK cell cytotoxicity needs to be answered in future studies. Another study was also conducted by Xu and colleagues.

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Role of NK cells in the control of metastatic prostate cancer

Unfortunately, 25% of men with prostate cancer worldwide develop metastatic disease statistically, and the 5-year survival of patients with metastasis to a distant site is significantly reduced to 29% [64,65]. It has been previously demonstrated that the metastasis of prostate cancer is largely affected by NK cells [66]. In addition, highly effective NK cells were shown to be related to a good prognosis in patients with metastatic prostate cancer [67]. In fact, NK cells from metastatic prostate cancer patients with longer survival and response to castration displayed strong cytotoxic potential [67].
Studies indicated that deficiency in MIC-NKG2D immune surveillance may contribute to prostate cancer progression [68]. In vivo, using a prostate tumor model, a hypothesis was indicated whether or not shedding of the MIC may be one of the mechanisms by which tumors evade host immunosurveillance and progress [69]. In vitro cytotoxicity assay revealed the loss of NKG2D-mediated NK cell function suggesting that persistent levels of soluble MICB in the serum can impair NK cell function and thus allow tumor growth. Subsequently, another study was conducted with two novel lines of humanized bi-transgenic (bi-Tg) mice in which the native human NKG2D ligand MICB or the engineered membrane-restricted MICB (MICB-A2) was expressed in the prostate of the TRAMP model of spontaneous carcinogenesis [70]. The conclusion was presented as the perturbation of the NK cell peripheral homeostasis accelerates prostate carcinoma metastasis emphasizing the impact of soluble NKG2D ligands [70].

In line with these studies, the tumor microenvironment is proposed to increase MIC shedding in human prostate cancer cells, although activation of NO signaling inhibits hypoxia-mediated MIC shedding [71]. Another study reported that MICA was not shed at all but rather was secreted in exosomes in the prostate carcinoma cell line PC-3 [72]. Prostate tumor-derived exosomes have also been shown as downregulators of the NKG2D-mediated cytotoxic response in prostate cancer patients which may subsequently facilitate immune escape [73]. More recently, the secretome of PC3/nKR cells, which are a new subline of PC3 cells that were originally isolated from nude mice, that were implanted with PC3 cells without anti-NK cell treatment, was published [74]. The results indicated that the PC3/nKR cells have significantly higher NK resistance and migration/invasion activities as compared with PC3 cells with more HLA-A and less NKG2D ligand secretion by PC3/nKR cells [74]. Therefore, this study represents a new cell system for studying metastasis and progression of prostate cancer [74]. However, further studies are needed to establish whether or not secretion of prostate cancer-derived exosomes is a major immune-suppressive mechanism during tumor progression.

Other mechanisms have also been suggested to highlight the underlying mechanism related to the immune escape of prostate cancer cells from NK cells [75]. The reduced Nkp46 expression was reported in prostate cancer patients and prostate cancer cells downregulated the expression of Nkp46, thus preventing their recognition of tumor cells [13]. Nkp46 is one of the natural cytotoxicity receptors and is accepted as a main activating receptor of NK cells. Therefore, NKP46/NCR1 receptor seems to play a key role in controlling tumors metastasis [76]. Chen and colleagues showed that upregulated HIF-1α expression suppresses NCR1/Nkp46 pathway through upregulating miR-224, which affects the killing capability of NK cells on prostate cancer, thus inducing immune escape of tumor cells under conditions of hypoxia in prostate cancer [75].

Prostate tumor cell-surface carbohydrates, such as Core2 O-glycans, may also play roles in tumor metastasis [77]. MUC1 is modified by poly-N-acetyllactosamine on its O-glycan residues in Core2 β-1,6-N-acetylgalactosaminyltransferase (C2GnT)-expressing prostate cancer cells. An earlier report indicated that there is a longer survival of C2GnT-expressing prostate cancer cells in the host blood circulation, resulting in the promotion of prostate cancer metastasis [77].

In a study to highlight a potential role for interferon regulatory factor 7 (IRF7) in bone metastases of prostate cancer, lower expression of IRF7 in bone metastases of prostate cancer was demonstrated [78]. IRF7 overexpression in prostate cancer cells showed a marked effect on VI. NK Immunotherapy in Various Cancers
inhibiting bone metastases but not on tumor growth in xenograft nude mice [78]. Furthermore, in vitro studies revealed that human prostate cancer cell lines, PC-3 and LnCap, overexpressing interferon IFN-β) significantly enhanced the activity of NK cells, which resulted in the cytolysis of prostate cancer target cells suggesting a role for IFN-β in reducing bone metastasis of prostate cancer by IFN-β-mediated NK activity [78]. In another study, a tumor cell line, CTV-1 cells are also shown to have the ability to prime resting NK cells from healthy donors and enhanced their ability to lyse a variety of NK-resistant tumor cells including the DU145 metastatic prostate cancer cell line with alterations in the NK cell phenotype upon priming [79].

The prognostic significance of NK cells in prostate cancer

Previously, a reduction of CD56 (bright) cells in prostate cancer patients was reported, which may precede NK cell dysfunction, leading to impaired cytotoxicity against prostate cancer cells [47]. In addition, the elevated abundance of CD56+ NK cells was associated with a lower risk of human prostate cancer progression confirming that NK cells have a protective role against tumor progression of advanced prostate cancer [12]. In other words, intraprostatic NK cells seem to be associated with a lower risk of progression, and high count of NK cells in prostate tumors of men who received androgen deprivation therapy was associated with a good prognosis [12].

The expression of natural cytotoxicity receptors (NKp30 and NKp46) has also been demonstrated on two different human prostate cancer cell lines, DU145 and PC3/Luc grown in culture and primary derived tumors [80]. An effective inhibition of tumor growth was succeeded in vivo when animals were treated with NKp30-Ig fusion protein. The same study reported that the NKp30-Ig fusion protein selectively binds to benign prostate hyperplasia but not to primary prostatic adenocarcinoma samples derived from human patients [80]. This may also have indications to distinguish between these two common pathological conditions [80]. On the other hand, prostate tumor tissue was shown to have a 1.7-fold higher MICA expression relative to normal tissue suggesting MICA expression may be related to the aggressive nature of prostate cancer and, therefore, the relevance of MICA may be as a potential prognostic marker for prostate cancer patients [81]. Interestingly, a race-associated different expression of MICA in prostate cancer was demonstrated [81].

Kastelan and colleagues reported that NK activity recordings in treated prostate cancer patients might be indicative of the presence of tumor cells in the circulation [46]. The study discussed the idea that the relationship between NK activity and prostatic cancer may depend on some antigens released by tumor cells and their capacity to regulate the immunosurveillance system in the control of metastases. Since the inflammatory milieu has been shown closely related to affect cancer progression, more recently, the NK cell number and NK activity before and after prostatectomy were also studied elucidating the relationship between NK cells and prostate cancer status from 51 patients treated with robot-assisted laparoscopic radical prostatectomy [82]. In fact, patients with prostate cancer exhibited lower NK activity than healthy participants. In addition, patients who undergo prostatectomy showed lower NK activity preoperatively than postoperatively, and lower postoperative NK activity was
observed in higher-stage than lower-stage prostate cancer. Therefore, it can also be speculated that NK activity decrease may be resulting from prostate cancer cell alteration [82]. However, the study included a relatively small patient cohort size and did not have a direct comparison with sufficiently healthy participants as a control group. Therefore, the potential mechanism between their prostate tumor microenvironment and NK cells and their altered activity needs to be further studied with possibly longer follow-up periods.

An association between NK cell activity and prostate cancer using a simple blood test by in vitro diagnostic device in subjects selected for prostate biopsy revealed that subjects with low values of NK cell activity are more likely to have a positive outcome at prostate biopsy [83]. This pilot study included a totally 43 subjects undergoing a prostate biopsy and reported that those subjects with an NK activity <200 pg/mL were more likely to have prostate cancer at prostate biopsy (86%) compared to men with NK activity ≥200 pg/mL (31%) [83]. A more recent study suggested the optimal cutoff of NK activity level for the prediction of prostate cancer was 500 pg/dL, with a sensitivity of 68.0% and a specificity of 73.1% in 102 patients who underwent transrectal prostate biopsy [84]. Other studies also demonstrated similar results supporting the link between immune function and prostate cancer [47,85]. With the technological and scientific improvements, a very recent study demonstrated a machine learning model to accurately differentiate between the presence of low-/intermediate-risk disease and high-risk disease without the need for additional clinical data in 54 patients with prostate cancer [86]. Thus, it has been suggested that NK cells and NK cell activity may be a prognosis factor in prostate cancer patients. However, another study among Korean men found no associations between NK activity and prostate cancer diagnosis [87]. Thus, more studies are needed to determine optimal cutoffs and the ideal patient population with larger sample sizes.

"Hide-and-seek" between CSCs and NK cells: Could prostate CSCs be targeted with NK cells?

Tumors consisting of a rare subpopulation with stem cell characteristics are responsible for tumor initiation, progression, tumor metastasis, relapse, and resistance to therapy. These cells are referred to as "cancer stem cells" or "cancer-initiating cells" [88]. Cancer stem cells (CSCs) exhibit properties to shape an immunosuppressive microenvironment (CSC niche) by modulating certain immune subgroups to evade recognition by immunity and maintain integrity [89]. As the structure of their own dynamic ecosystems, CSCs are in multiple interactions with immune system cells, including macrophages, dendritic cells, T-cells, and NK cells, which determine the outcomes of CSC immunity. Considering that traditional treatments are not qualified enough to target and eliminate CSCs, CSCs immunology could offer unique opportunities for the development of effective treatment strategies [90].

There is a mysterious game of “hide and seek” between CSCs and immune cells, and the results of this game are sometimes surprising due to the escape mechanisms exhibited by CSCs. The duel between CSCs and immune cells is a new field of study in immune therapy, and the current picture of this situation reveals that we actually need to change the scenario in our fight against these cells and the immune system needs to be reeducated and equipped...
with various modifications. CSCs declare victory in the war on cancer by using a wide variety of strategies, such as low expression of major histocompatibility complex class I (MHC-I) class molecules, lack of expression of costimulatory molecules, and production of immunosuppressive molecules for evading immune surveillance and modulating immune cells [91–94]. In the study conducted by Yang and colleagues, it was reported that the expressions of MHC-I molecule and most parts of the antigen-processing machinery (APM) components were at low levels in glioblastoma stem cells (GSCs) compared to the bulk population (non-GSCs) [91]. It has been demonstrated that cytokines secreted by CSCs such as TGF-β, interleukin-4, and IL-10 inhibit immune cell proliferation and function [92,95].

NK cells, which are an important part of the innate immune system, are the most relevant candidate immune cells for killing CSCs that escape from host immune surveillance by downregulating self-antigen presentation. However, with some growing evidence, the participation of NK cells in anti-CSCs immune surveillance is controversial. The less immunogenic character of CSCs prevents these cells from being recognized by immune cells, which remains a major in cancer treatment. Interestingly, various studies have suggested that NK cells preferentially kill CSCs compared with the bulk population. Castriconi and colleagues reported that glioblastoma multiforme cells with stem cell characteristics express various ligands that trigger NK activation and that these cells are highly susceptible to lysis by both allogeneic and autogenic IL2 and/or IL15-activated NK cells [96]. Tallerico and colleagues demonstrated that allogeneic NK cells can recognize and kill colorectal adenocarcinoma CSCs compared to their differentiated counterparts due to the higher expression of ligands for NKp30 and NKp44 in CSCs that activate NK cytotoxicity receptor [94]. Similarly, primary oral squamous CSCs have been found to be significantly sensitive to NK-mediated cytotoxicity compared to their differentiated counterparts [97]. While the mechanisms underlying the targeting and elimination of CSCs by NK-cells continue to remain mysterious, death receptors and NKG2D ligands, which we can express as game changer, contribute to recognition processes in the NK-CSCs axis. Ames and colleagues reported that cell populations expressing certain CSCs markers in breast cancer (CD24+/CD44+), pancreatic cancer (CD24+/CD44+), and glioblastoma (CD133) are targeted by NK cells. These findings of the study revealed that NK cell-mediated lysis efficiency of CSCs is due to the high expression levels of NKG2D ligands (MICA/B) and death receptors Fas and DR5 in CSCs versus non-CSCs [98].

Although significant advances have been made in the treatment of prostate cancer in recent years, relapse of prostate cancer due to hormone resistance and metastasis is an important challenge in patients with advanced disease. Improved rates of treatment success in aggressive prostate cancer depend largely on the cellular and/or molecular mechanisms that can target prostate CSCs (PCSCs). There are different opinions about the cellular origin and putative biomarkers of PCSCs [99]. However, ongoing studies in prostate cancer continue to provide substantial evidence. It has been reported that cells with the CD44+/alpha2beta1hi/CD133+ phenotype in prostate tumors with different Gleason and metastatic properties have self-renewal and high proliferative potential in vitro [100]. Patrawala and colleagues isolated CD44+ and CD44− cell populations from multiple prostate cancer cell cultures and xenograft tumors. The results of the study revealed that CD44+ cells express genes related to stemness at high levels and their proliferative, clonogenic, tumorigenic, and metastatic properties are more compared to CD44− cells [101].

VI. NK Immunotherapy in Various Cancers

Benjamin_Bonavida, 978-0-12-824375-6
Intensive studies are continuing to target and kill stem cells in prostate cancer. There is a study showing an increase in the stem/progenitor cell population in prostate cancer patients receiving androgen deprivation therapy. It has been shown that prostate stem/progenitor cells could be targeted with 5-azathioprine and gamma-tocotrienol at the castration-resistant stage in human prostate cancer cell lines and in different mouse models [102]. In the study by Soner and colleagues, it has been shown that flavopiridol, a cyclin-dependent kinase inhibitor, inhibits cell viability and spheroid formation in CD133\textsuperscript{high}/CD44\textsuperscript{high} human PCSCs and induces apoptosis by the upregulation of caspase-3, caspase-8, and p53 [103]. Similarly, Acikgoz and colleagues demonstrated that trabectedin, a marine tetrahydroisoquinoline alkaloid, could be targeted to CD133\textsuperscript{+} high/CD44\textsuperscript{+} high PCSCs isolated from DU145 and PC-3 prostate cancer cell lines [104]. Many signaling pathways such as Wnt, Hedgehog, Notch, and Hippo, which are associated with embryonic development/stemness, are highly active in cancer stem cells and these pathways are mainly used as an important tool in eliminating therapy-resistant cell populations [105]. It has been revealed that blocking of the sonic hedgehog pathway via NVP-LDE-225/Erismodegib inhibits the spheroid formation and self-renewal ability of PCSCs by suppressing Nanog, Oct-4, Sox-2, and c-Myc, which are associated with pluripotency. NVP-LDE-225 has also been shown to inhibit the epithelial-mesenchymal transition (EMT) by inhibiting the Snail, Slug, and Zeb1 transcriptional factors associated with EMT by the induction of miR-128 [106].

Recently, targeting CSCs that develop drug resistance with adoptive cell therapies, dendritic cell-based vaccines, oncolytic viruses, immune checkpoint inhibitors, and/or combination therapies is considered a more effective strategy [107]. However, research on immune therapies targeting PCSCs has not yet been sufficiently documented. The most important obstacles in targeting and eliminating PCSCs with immunotherapy are as follows: (i) the immunological properties of PCSCs are not fully known and (ii) antigenic molecules specific to PCSCs that immune cells can target are not yet discovered.

It was revealed that sphere-forming DU145 and GFP-NANOG-overexpressing DU145 prostate cells have a strengthened ability to escape NK cell attack due to the suppression of intercellular adhesion molecule-1 (ICAM-1) expression in SCID mice [108]. In a study conducted by Jachetti and colleagues, it was reported that PCSCs can be targeted with both innate and adaptive immunities. It has been demonstrated that CSC lines generated from the prostate of transgenic adenocarcinoma express prostate cancer-associated antigens, MHC Class I and II molecules, and ligands for NK cell receptors, and PCSCs were targeted by both NK cells and cytotoxic T lymphocytes (CTLs) in vitro and in vivo [109]. It has recently been demonstrated that adoptive cytokine-induced killer (CIK) cells have cytotoxic activity against PCSCs. In the study, dendritic cells (DCs), which were isolated from peripheral blood mononuclear cells and loaded with PCSCs-related membrane molecules CD44 and EpCAM, were cocultured with peripheral blood lymphocyte-derived CIK cells. It has been shown that DC-activated CIK cells have specific cytotoxic properties against PCSCs [110].

NK cells are the first line of defense against tumor formation, tumor relapse, and metastasis and are highly effective immune cells in targeting and eliminating of CSCs. There are missing links in the immunological properties of PCSCs and their interaction with immune cells. Discovering new molecules that can target PCSCs and elucidating the mechanisms of escape from immune cells will make important contributions to the development of new therapeutic strategies. Basically, it should not be forgotten that the immune system is an enormous army...
that defends our body against all kinds of invaders, whose power depends on the action of all other immune cells.

**Conclusion**

A dynamic conversation between tumor cells and immune cells involves a highly complex puzzle—the positioning of the pieces determines who wins the game. Considering the ability of cancer cells to manipulate immune cells in the tumor microenvironment, it can be suggested that the immune system has dual functions in cancer immunotherapy. NK cells are immune cells that play an active role in eliminating the rebellious cells that invade our body. In this review, we present a perspective on the various roles and importance of NK cells in prostate cancer biology. Aside from the mystery of the interaction of NK cells in prostate cancer, deciphering the dynamics between NK cells and prostate cancer cells could make important contributions to the development of new therapeutic strategies.

**References**


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Involvement of natural killer cells in the pathogenesis of lymphomas: Therapeutic implications

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Abstract

Natural killer cells play a pivotal role in the control of various infectious diseases and malignancies. Their functional activities are impaired in many cancers and, both directly and indirectly, participate in the progression of cancer and escape from immune surveillance. Their role in the regulation of anticancer adaptive immunity is consequently compromised. This is the case also in lymphoid malignancies where they are modified phenotypically and functionally lose their antitumor cytotoxic activities and cytokine secretion. Based on these important roles of NK cells in lymphomas, several new strategies have been devised to either reactivate them in vitro and/or adaptively administer ex vivo generated activated NK cells with anticancer activities. These include, for example, the use of agonist antibodies directed against NK activating receptors or the use of antagonists directed against the NK inhibitory receptors or checkpoint inhibitors. In addition, the adaptation of NK cell lines that have been modified, such as CAR-NK cells, as donor cells are compatible with the allogeneic hosts and are not rejected, while exerting significant antitumor activities when used alone or in combination with other therapeutics. Such novel therapeutic approaches by NK cells in the treatment of lymphomas are currently in preclinical and clinical studies for their validation and approval.
Abbreviations

ACHT autologous hematopoietic cell transplantation
ADCC antibody-dependent cell-mediated cytotoxicity
CAR chimeric antigen receptor
CAR-T chimeric antigen receptor-modified T-cell
cHL classical Hodgkin lymphoma
CLL chronic lymphocyte leukemia
CTL A-4 cytotoxic T lymphocyte-associated antigen
EBV Epstein-Barr virus
ERKs extracellular signal-regulated kinases
HLA human leukocyte antigen
HLs Hodgkin lymphomas
HRS Hodgkin and Reed-Sternberg
HSCT hematopoietic stem cell transplantation
IFN-γ interferon-γ
ILCs innate lymphoid cells
ILC1s Group 1 innate lymphoid cells
ILC2s Group 2 innate lymphoid cells
ILC3s Group 3 innate lymphoid cells
ITAM immunoreceptor tyrosine-based activation motif
ITIM immunoreceptor tyrosine-based inhibitory motifs
KIRs killer immunoglobulin-like receptors
LAT linker for the activation of T cells
LIRs leukocyte immunoglobulin-like receptors
LTi lymphoid tissue inducer
MAPKs mitogen-activated protein kinases
MHC major histocompatibility complex
MHCII major histocompatibility complex II
NHLs non-Hodgkin lymphomas
NLPHL nodular lymphocyte predominant
NK natural killer
NNKTLs nasal NK/T-cell lymphomas
PBNC peripheral blood mononuclear cells
PD-1 programmed cell death-1
RORγ-T RAR-related orphan receptor gamma
RT radiation therapy
SHP-1 Src homology-containing tyrosine phosphatase 1
SHP-2 Src homology-containing tyrosine phosphatase 2
TME tumor microenvironment

Conflict of interest

No potential conflicts of interest were disclosed.

Introduction

Lymphomas

Lymphomas account for about 50% of all hematological cancers. They consist in a lymphoproliferative malignant process, which might involve different lymphoid cell lineages, including B, T, and natural killer (NK) lymphocytes [1]. They are cancers of the lymphatic system that are typically characterized by tumors surrounded by a reactive...
inflammatory environment (i.e., lymphocytes, eosinophils, neutrophils, histiocytes, and plasma cells) [1–3]. Their specific appearance or presentation, such as shape, size, and surrounding tissues, however, depends on the organ implicated as well as the nature of the lymphoma itself [4]. This means that the degree of aggressiveness of the lymphomas highly influences the typical development and presentations of the lymphomas.

Indolent lymphomas are typically observed in the form of lymph nodes. The lymph node form of lymphomas, in fact, is the most common form of lymphomas and it can form in any area. However, lymph nodes are only considered pathological if their short axis is more than 1 cm [4,5]. On the other hand, aggressive lymphomas are typically observed in the form of a high tumor mass and might cause side effects such as superior vena cava obstruction and medullary compression [4].

General properties and subtypes

Lymphomas are traditionally divided into two broad categories: Hodgkin lymphomas (HLs) and non-Hodgkin lymphomas (NHLs) [6,7]. Of the two, Hodgkin lymphomas are less common, comprising only approximately 10% of new lymphoma cases and 5% of lymphoma-related annual deaths in the United States [3,6]. The pathogenesis of HLs is not completely known, but previous research has linked the gamma herpes virus Epstein-Barr virus (EBV) to the rising incidence of HLs. It is hypothesized that EBV-positive individuals lacked immune surveillance, which is consistent with the symptoms observed in HL individuals [8]. The proposed connection is backed by the detection of small RNA encoded by the EBV in the Hodgkin and Reed-Sternberg (HRS) cells [3]. On the other hand, the pathology behind the NHL is more widely known: it is typically derived from the immune cells, including B cells, T cells, or natural killer (NK) cells, and NHL can occur at various stages of the development of these immune cells. To be specific, 85%–90% of NHLs are derived from B cells while the remaining are derived from either T cells or NK cells [2,6]. Existing research has observed an income-based pattern of B-cell NHL distribution, yet such distributions have shifted in recent investigations, which suggests that the lifestyle can potentially alter this preexisting pattern of distribution. Other factors that influence the development of NHLs include immune disorders, genetics, race, and infections.

Apart from the two broad categories, both HLs and NHLs can be further classified into different subgroups. HL is typically subdivided into classical Hodgkin lymphoma (cHL) and nodular lymphocyte predominant (NLPHL), while NHL is typically divided into B-cell NHL, T-cell NHL, and NK cell NHL. According to the WHO classification, over 80 types of lymphomas can be accounted and classified based on their specific morphology, immunophenotype, genetic mutational background, cellular derivation, and clinical features [9].

Unlike the literature describing the effects of NK-cell cytotoxicity on various lymphoma prognoses, there are currently consistent findings describing nasal NK/T-cell lymphomas. To begin, NK/T-cell lymphomas are most commonly found in the nose, nasopharynx, oropharynx, and the upper aerodigestive tract [10]. Nasal NK/T-cell lymphomas (NNKTLs), also known as lethal midline granulomas, are perhaps the most common site of infection for the extra nodal NK/T-cell lymphomas [10,11]. Despite the name, there are instances in which the nasal lymphoma originated from B cells. Like other lymphomas, there is a distinct
geographic distribution of NNKTL. To illustrate, it is 10 times more likely for individuals in Asia to have NNKTL than those in Europe [12].

Pathologically, NNKTLs express T-cell markers such as CD2, CD45, and the NK-cell marker CD56. Recent studies by Harabuchi et al. [13] and Nagata et al. [14] showed that NNKTL cell lines can be established without TCR rearrangement, which supported the current notion that NNKTL originated from either the NK- or γδ T-cell lineages [13]. In a previous study, Ooi et al. [11] observed that patients with NK/T-cell lymphomas typically have a worse prognosis compared with patients with B- and T-cell lymphomas.

Treatments

There are various forms of treatments available for lymphomas, including observation (also known as watch and wait), drug therapy, radiation therapy (RT), chemotherapy, induction therapy, hematopoietic stem cell transplantation (HSCT) and immunotherapy [15–19]. However, there are many considerations when it comes to treatment for lymphomas, such as the patient’s symptoms, age, and general health, but also the size, type, location, and aggressiveness of the tumor.

Individuals with asymptomatic, aggressive NHLs that only occupy one or two adjacent areas (i.e., no more than 10 cm in diameter) typically have a good prognosis. Localized NHLs may be treated with radiotherapy alone after careful laparotomy staging. Combination therapy using chemotherapy with various agents (e.g., cyclophosphamide, vincristine, and prednisone) is often introduced as well, since previous studies have observed a higher cure rate for combination therapies [15,17]. However, clinical staging is sometimes not suitable for older patients, and therefore the current protocol for asymptomatic, advanced-stage low-grade NHLs usually includes watchful waiting. With this, careful observations of the disease prognosis are required prior to the later administration of chemotherapy or radiotherapy treatments. Nevertheless, the low outcome of watchful waiting has called for an ample amount of research today focused on investigating the effects of early and late systemic therapy introductions [16,17].

Besides chemotherapy and radiation therapy, targeted therapies are one of the current treatments used for B- and T-cell lymphomas. Based on Chung’s [20] article, there are four targeted B-cell lymphoma drug therapies. The first uses antibodies that specifically target the CD20 lymphocyte antigen, such as Obinutuzumab and Rituximab [20]. The second uses gene transfer therapy, such as the popular chimeric antigen receptor-modified T-cell (CAR-T) therapy, which in this case targets the CD19 antigen. The third form of lymphoma drug therapy uses small-molecule inhibitors that attack the B-cell receptor signaling pathways. The last form of lymphoma drug therapy is focused on attacking the lymphoma microenvironment, such as using bortezomib and pembrolizumab [20].

Natural killer cells

The immune system is the first line of defense against pathogens and the innate lymphoid cells (ILCs) are part of the immune system that supports their functions in infection,
inflammation, and tissue repair [21]. There are three groups of ILCs: group 1 ILCs (ILC1s), group 2 ILCs (ILC2s), and group 3 ILCs (ILC3s). ILC1s typically express interferon-γ (IFN-γ), while ILC2s typically express type 2 cytokines such as interleukin-5 (IL-5), IL-9, and IL-13. Similarly, ILC3s typically express IL-22 and IL-17 [21–23]. ILC2s are highly associated with type 2 immunity and thus play an important role in inducing the expression of effector cytokines that ultimately assist in tissue repair. In addition, recent research has indicated that ILC2s directly regulate T-cell activation through their expression of the major histocompatibility complex II (MHCII). This explains the involvement of ILC2s in innate immunity. On the other hand, other than IL-22 and IL-17, ILC3s also express the RAR-related orphan receptor gamma (RORγ-T), which includes the lymphoid tissue inducer (LTi) cells. Together, these cytokines are essential for the development of lymphoid tissues, particularly at the mucosal surfaces [21]. Like ILC2s, ILC3s also express MHCII and were recently shown to play a role in regulating T-cell responses by assisting in the antigen-processing machinery of major histocompatibility complex (MHC) cells [24].

Although different for the classical ILCs, NK cells, also known as CD56+ CD3− cells, are a class of cytotoxic innate lymphocytes that is often deemed as key immune effectors [25,26]. Despite being classified as innate cells, NK cells, like adaptive B and T cells, undergo selection, clonal expansion and they are also able to differentiate in memory NK cells [27].

NK cells represent the first line of defense against pathogen infections (especially of viral origins) and cancer cells [28,29]. They develop within the bone marrow and make up approximately 10% of the total lymphocytes among the peripheral blood mononuclear cells (PBNC) [25,30]. Although part of both the body’s innate and adaptive immunities, NK cells do not express the MHC [25,30]. Instead, NK cells express activating and inhibitory receptors on their cell surface [31,32]. Although NK cells are well known for their role in eliminating and/or targeting a wide range of cancer cells, they are also responsible for mediating a myriad of both immune and nonimmune cells through their role in cytokine and chemokine productions [25,30].

The two NK cell subsets are defined by the relative expression of CD16 and CD56 cell surface antigens: CD16bright CD56dim and CD16low and CD56bright [25,30]. The CD16bright CD56dim is a mature NK cell, comprising about 95% of the total peripheral NK cells. Widely available in circulation, these NK cells have a high cytotoxic activity [25]. On the other hand, the CD16low and CD56bright subset is a more immature NK cell. It makes up about 5% of the total peripheral NK cells and has a regulatory function (i.e., cytokine-mediated immunity) and resides in the mucosa.

There are four stages in the life cycle of an NK cell [25]. The first stage is CD16bright CD56dim CD69+, which makes up 90% of the peripheral blood NK cells and is responsible for targeting cancer stem cells. The second stage is CD16low and CD56bright CD69bright, which is a low or noncytotoxic NK cell that is activated through induction by tumors. Additionally, this second stage NK cells also mediate the function of other cells, including the differentiation of tumor cells. The third stage of NK cell maturation involves the weakening of NK cytotoxic activity, such as losing the ability to secrete interferon (IFN)-γ or mediate cytotoxicity, while the fourth stage of NK cell maturation involves cell apoptosis [25].

As previously mentioned, NK cells contain activating and inhibitory receptors on their surface. These receptors are responsible for the functionality of NK cells, including the elimination of cancer metastasis [32]. More specifically, the balance between the signals from the
activating and inhibitory receptors as well as the ligands associated with these receptors all contribute to the ability of NK cells to cause cells infected with viruses or cancer cells to undergo cell death and apoptosis [31,33,34]. The unique aspect about NK cells is that although they only have a limited number of receptors and/or ligands, they are responsible for recognizing a wide range of bacteria, viruses, parasites, and even tumors [31,34,35]. This unique quality stems from the abundant receptor-ligand combinations that these NK cell receptors can express [34].

NK cell function depends on the activating and inhibitory receptors [35–37]. For the activating receptors, the mechanism begins through the phosphorylation of the immunoreceptor tyrosine-based activation motif (ITAM) [35,37]. Hence, the phosphorylation of ITAM by the Src family of tyrosine kinases would, in turn, result in the recruitment and activation of the tyrosine kinase Syk and Zap70. The tyrosine kinase Zap70 is associated with a downstream signaling pathway that involves the phosphorylation of different proteins, including the linker for the activation of T cells (LAT), the activation of mitogen-activated protein kinases (MAPKs) and extracellular signal-regulated kinases (ERKs). On the other hand, phosphorylation of Syk results in the recruitment of PLC-6, which in turn induces the activation of the transcription factors NF-κB and NFAT. Together, the two downstream pathways result in elevated levels of calcium and transcription of cytokine and chemokine genes, which in turn release cytokines, chemokines, and cytotoxic molecules [37] (see Fig. 1).

Similarly, for the inhibitory receptors, the mechanism starts with the phosphorylation of the immunoreceptor tyrosine-based inhibitory motifs (ITIM) that are present in their cytoplasmic tails [37–39]. Upon phosphorylation, phosphatases such as the Src homology-containing tyrosine phosphatase 1 (SHP-1) and SHP-2 are recruited to balance off the activating signals. This is because SHP-1 contains two SHP-2 binding sites, and the SHP-1-SHP-2 interaction relieves SHP-1 auto-inhibited conformation [37] (see Fig. 2).

![FIG. 1 Natural killer cell activating receptor signaling. The activating receptor signaling mechanism begins with the phosphorylation of the immunoreceptor tyrosine-based activation motif (ITAM). Upon ITAM activation, the tyrosine kinase Syk and Zap 70 are recruited and subsequently activated. The activation of Zap70 will, in turn, activate a downstream pathway that phosphorylates different proteins, including the linker for the activation of T cells (LAT), the activation of mitogen-activated protein kinases (MAPKs) and extracellular signal-regulated kinases (ERKs). Similarly, the activation of Syk will initiate a downstream pathway that activates the transcription factors NF-kB and NFAT through the recruitment of phospholipase-6 (PLC-6). Together, the phosphorylation of proteins and the activation of transcription factors activate cytokine and chemokine genes and increase the true calcium level. These two reactions then produce chemokines, cytokines, and cytotoxic molecules.](image)

VI. NK Immunotherapy in various cancers
Role of natural killer cells in lymphomas

NK cells have a pivotal role in contrasting both solid and hematological cancer genesis. As described above, upon cancer cell recognition they can switch to an activated killing status and mediate the elimination of target malignant cells [40]. Often, cancer cells may escape NK-mediated immune surveillance. In fact, the long-term exposure of NK cells to antitumor molecules produced by cancer cells may allow the switching of NK cells toward an immune-suppressive phenotype, which in turn favors cancer genesis and progression [40].

In lymphomas, immunodeficiency has been indicated as one of the defining characteristics and is perhaps the cause of lymphomas. Similarly, tumor evasion is another characteristic that Hodgkin lymphoma (HL) possesses. For HL, previous research has shown that there is a higher incidence of HL in patients with variable immune deficiencies, HIV, or other immune-related diseases. Previous research has also shown that immune deficiency typically precedes the HL diagnosis and suggested that this might be due to the increased susceptibility of immune-deficient individuals who have bacterial, fungal, or even viral infections [41]. The role of HL in the immune system, however, is still being investigated. Recent studies have highlighted the importance of regulatory T lymphocytes (Tregs) in the pathogenesis of HL. Marshall et al. [42] showed that Hodgkin lymphoma-infiltrating lymphocytes (HLILs) suppress the activation of peripheral blood mononuclear cells (PMBC) and are very responsive to antigens. Additionally, HL lymphocytes are found to inhibit the T-cell response, which suggests that a majority of the HLILs are Treg cells that give HLILs the ability to inhibit the host immune system [41]. Besides inhibiting the T-cell response, the remaining nonmalignant cells in HL continue to release cytokines that essentially support the proliferation of tumor cells.

Due to its broad set of receptor-ligand combinations, several research investigations have suggested the role of NK cells in predicting the susceptibility to disease [43]. A number of studies have tried to identify the role of NK cells in cancer. Previous studies have suggested that there are higher frequencies of Stage 3 and 4 NK cells in cancer patients. This means that cancer patients typically have NK cells with a lower cytotoxic activity and worse prognosis [25]. Moreover, a study by Klanova et al. [44] showed that the baseline peripheral NK cell levels might potentially act as a predictor of an anti-CD20-based immunochemotherapy outcome on B-cell NHL patients. In particular, Klanova et al. [44] showed that individuals with
low baseline peripheral NK cells are linked to shorter progression-free survival and overall survival. In addition, Gattringer et al. [45] found that NHL affected tissues have impaired cytotoxicity and five times less NK cell density. However, the certainty behind this finding is still controversial, as Mundy-Bosee et al. [46] showed that highly cytotoxic NK cells are also linked to poorer outcomes in individuals with skin T-cell lymphomas. Furthermore, Ayoub et al. [47] found that despite the lower peripheral blood NK cells in individuals with lymphoma, there was no correlation to their prognosis or clinical stage.

Chiu et al. [48] have reported the poor activity of NK cells in the tumor microenvironment (TME) of cHL. In the TME, the NK cells are inhibited from proliferation and their activity is impaired leading to tumor escape. This defect in NK cells is initiated at the tumor site and progresses systematically in patients with advanced disease. Several factors are implicated in the NK defect including the expression of ligands for the inhibitory receptors on NK cells, the secretion of soluble IL-2 receptor, TGF-beta, IL-10 and chemokines CXCL9 and CXCL10, all of which mediate immunosuppression. The NK defect can be reversed. For example, the NK count rapidly returns after 2 weeks post autologous hematopoietic cell transplantation (AHCT).

Hence, multiple studies have tried to identify the function of NK cells in patients with lymphoma [49–52]. Bachnova et al. [49] showed that the NK cells in NHL patients possess different characteristics: poorer cytotoxic function, lower CD16 expression, higher T-cell immunoglobulin and mucin domain-3 (TIM3) inhibitory receptor, and lower levels of the immunosuppressive receptor T-cell immunoreceptor with Ig and ITIM domains (TIGIT). These findings suggest that active NK cells might be able to effectively treat individuals with severe lymphomas.

**Use of natural killer cells in the immunotherapy of lymphomas**

Tonn et al. [53] reported the treatment of patients with various malignancies including leukemia/lymphoma and found that the infusion of NK-92 cells (which is a cell line with features of NK cells) was well tolerated and the development of anti-HLA antibodies directed against NK-92 was rare. There were some encouraging clinical responses.

Many studies have tried to leverage NK’s antitumor activity in the immunotherapy against cancer [36,54,55]. As mentioned above, NK cells do not require any prior sensitization to target foreign antigens. Additionally, NK cells need a strong trigger that can overcome inhibitory signals to be fully activated. NK cells achieve this by identifying stress-induced ligands using its activating receptors, such as NKG2D, NKp44, CD226, and the low-affinity receptor CD16 [36]. Moreover, NK cells also produce proinflammatory cytokines and chemokines such as IFN-Ɣ, TNF, and IL-6 to indirectly promote innate and adaptive immunities. Nevertheless, when it comes to lymphomas, there is a change in the NK cell’s receptor ligand expression which decreases its activity. This is contributed by the immunosuppressive cells and the interference caused by the tumor-associated cells. Moreover, the tumor environment also diminishes NK cells’ ability by inhibiting its antimetastatic function and maturation through platelet activation and the release of cytokines and metabolites [38]. Therefore, recent studies have tried to identify ways to overcome the immunosuppressive tumor microenvironment.
by leveraging the immune checkpoints and adaptive cellular therapy [36]. In particular, recent technological advances have increased efforts in improving cellular therapy, particularly in engineering T cells to express a chimeric antigen receptor (CAR) against tumor cells.

The majority of B-cell malignancies are resistant to killing by NK cells. The introduction of CAR into NK cells may overcome the resistance. Romanski et al. [56] used the NK-92 cell line transduced with anti-CD19 CAR and demonstrated cytotoxic activity against CD19 positive cell lines and primary leukemia cells resistant to native NK-92 cells. The antiCD19 CAR NK cells also lysed lymphoblasts from leukemia patients. These findings implicated the use of the engineered NK-92 cells for clinical use in patients.

CAR-CD19 T cells have been approved for the treatment of B-cell cancers and shown a significant clinical efficiency. However, this treatment is associated with toxicity and the generation of such cells is complex, expensive, and individualized. Extending this approach with allogeneic T cells to facilitate the development and cost is not practical as it will elicit graft-versus-host (GVH) disease. However, the use of modified NK cells can be a practical approach. Liu et al. [57] have transduced cord blood-derived NK cells with a retroviral vector incorporating the genes for CAR-CD19, IL-15, and an inducible caspase-9-base suicide gene (iC9). These transduced cells were shown in vitro to kill CD19-expressing cell lines and primary chronic lymphocytic leukemia (CLL) cells and a significant prolongation of survival in a xenograft Raji lymphoma model in vivo.

The same group has recently reported the use of human leukocyte antigen (HLA)-mismatched anti-CD19 CAR-NK cells in phase 1 and 2 clinical trials and shown how to overcome the above limitations (NCT03056339) [58]. HLA-mismatched anti-CD19 CAR-NK cells were derived from cord blood and administered to 11 patients with relapsed or refractory CD-19 positive cancers (non-Hodgkin’s lymphoma or CLL). Of the 11 patients who were treated, eight had a response, seven had a complete remission (four with NHL and three with CLL), and one had persistent CLL. The responses were rapid and observed within 30 days after infusion. The infused CAR-NK cells expanded in vivo and persisted at low levels for at least 1 year. There were no major toxic effects.

Ravi et al. [59] reported the mechanism of anti-CD19 CAR NK92 cells on lymphoma cell lines and patient-derived Rituximab and Obinutuzumab resistant cells as well as a human xenograft model. The CD19-CAR. NK92 cells induced a significant cytolytic activity against all cells above. The cytotoxicity was accompanied by the induction of apoptosis, induction of IFN-gamma, and the secretion of granzymes and FASL, CCl3, and IL-10.

According to Khan et al.’s [38] comprehensive review, immune checkpoint inhibition refers to “the exploitation of the NK cell inhibitory receptors by tumors for immune evasion.” Traditionally, when it comes to immune checkpoint inhibition therapy, killer immunoglobulin-like receptors (KIRs, also known as CD 158), leukocyte immunoglobulin-like receptors (LIRs), and NKG2As (also known as CD159) are the targeted NK cell receptors [39]. However, more NK cell receptors have recently been implicated, such as cytotoxic T lymphocyte-associated antigen (CTLA-4), programmed cell death-1 (PD-1), TIM-3, CD200, and CD47 [38]. These immune checkpoint receptors are targeted by the immune checkpoint inhibitors (e.g., monalizumab, pembrolizumab, and lirilumab) and have shown promising results in terms of prolonging progression-free survivals of 24 months [60,61]. However, the phase 1 clinical trial was conducted on multiple melanoma patients, not lymphoma patients.
Xie et al. [62] reviewed the prognostic and clinicopathological significance of the PD-1/PD-L1 expression in the TME of malignant lymphomas. The increased infiltration of TILs is a favorable prognostic factor in diffuse large B-cell lymphoma (DLBCL) but not in HL. The infiltration also showed a trend in overall survival in NNKTL and adult T-cell leukemia/lymphoma. The peripheral PD-1 T cells in the blood samples correlated with disease progression in DLBCL and CLL. PD-L1 expression predominated in DLBCL. Elevated serum levels of soluble PD-L1 represented adverse prognostic factors. In conclusion, they showed that the clinical roles of PD-1/PD-L1 vary among the subtypes of lymphoma.

A recent report by Sasse et al. [63] examined the role of checkpoint inhibitors in cHL and compared with classic standard chemoradiotherapy in phase 1 and 2 clinical trials. A clinical response was obtained in 60%–70% of patients, who were refractory to chemotherapy, following a PD-1 blockade. The overall survival was improved, and some patients achieved a persistent response.

Nevertheless, there are other molecules that are currently being clinically tested to investigate the effectiveness of leveraging NK cell cytotoxicity in lymphoma immunotherapies [64–66]. The heat shock protein-90 inhibitor BIIB021 has concluded preclinical testing of heat shock protein-90 inhibitor BIIB021 and showed promising results in terms of blocking the NF-kB pathway and repressing MHC class 1 molecules while concurrently inducing the NKG2D ligands to trigger and activate the corresponding NKG2D NK activating receptors [66]. This, in turn, resulted in the enhancement of NK cell-directed lysis. Besides immune checkpoint inhibitors, NK cells adoptive therapy have also been clinically investigated [67,68]. Maki et al. [68] used the malignant NK cell line NK-92 in a clinical phase 1 trial in several patients, including a lymphoma patient. However, the result of the trial has not been reported.

Yang et al. [69] have reported a specific approach to improve the cytotoxic activity of NK cells against cancer cells. They wished to make the NK cells more specific for targeting cancer cells. They used synthetic CD-30 specific aptamers that are anchored on the cell surface of NK cells. Aptamers are short single-stranded oligonucleotides that specifically recognize their targets with high affinity similar to antibodies. Apten-engineered primary human NK cells were found to bind to CD30 expressing lymphoma cells but not to off-target cells. This specific binding induced higher apoptosis and cell death compared with native NK cells.

Essa et al. [70] analyzed the expression of the NK activating receptor, NKp44, in patients with DLBCL and correlated the findings with clinical pathological data. They examined 30 new cases of DLBCL and 20 healthy controls. They did not detect any differences between the patients and healthy controls. However, stimulation with PHA revealed in DLBCL a significant increase of NKp44 on the NK cells compared with controls. The increased expression of NKp44 in patients was associated with earlier stages of DLBCL. They suggest that improvement of NKp44 function is an approach for immunotherapy of DLBCL.

Bachanova et al. [49] reported a phase 2 clinical trial in patients with poor prognosis NHL by testing the efficacy of haploidentical NK-cell therapy with rituximab and IL-2 (NCT01181258). The therapy was tolerated. Of the 14 patients evaluated, four had objective responses and two had complete responses lasting 3–9 months. The responding patients had lower frequencies of Tregs and myeloid-derived suppressor cells (MDSCs) in the circulation. They proposed that this therapy is a novel treatment for advanced and chemoresistant NHL.
Chester et al. [71] examined the 4-1BB (CD137) receptor, which is an inducible costimulatory receptor expressed on activated T and NK cells. On NK cells, triggering CD137 increases antibody-dependent cell-mediated cytotoxicity (ADCC). Therefore, two agonistic antibodies, Urelumab and Utomilumab, directed against CD137 were developed. Clinical trials with two such antibodies are ongoing. Both antibodies have shown promising results in patients with lymphoma and are being tested in combination therapy trials. To avoid liver toxicity, they suggest targeting the agonists in the TME.

Although the majority of clinical trials reported here demonstrated the safety and efficacy of NK infusions in lymphoma patients, a trial showed a high rate of GVHD reaction in leukemia and lymphoma patients upon activated allogenic NK transplantation, likely due to an increase of T-cell alloreactivity. This suggests that a GVHD prophylaxis might be of benefit to better tolerate this NK cell administration (NCT01287104) [72].

Clinical trials involving the administration of rhIL-15 showed a huge increase in the number of activated NK cells (specifically a 38-fold increase in the 10-day number of circulating NK cells, a 3580-fold increase in CD56 bright NK cells, and a 5.8-fold increase in CD8 T cells). However, using this for monotherapy was ineffective due to lack of tumor specific targeting by NK cells and actions of immunological checkpoints. To explain this further, the increase in activated NK cells was still inhibited by the MHC Class 1 molecules expressed by tumor cells that interact with KIRs and NKG2A [73].

To address the above issue, additional clinical trials were initiated such as a combination of antibodies (e.g., antibodies against CTLA-4, PD-L1) and IL-15 administration [73]. They also tested a combination of IL-15 with anticancer monoclonal antibodies and found that IL-15 enhanced the ADCC and therapeutic efficacy of both antibodies. They found that NK cells and macrophages were critical elements of interacting effectors involved in the augmented ADCC and augmented therapeutic responses.

Several clinical trials are currently ongoing with the goal of assessing NK cell-based therapy safety and efficacy in lymphoma patients. The NK cells may derive from the same patient or from a healthy donor. To improve the killing capacity, the NK cells in some of the studies listed below are subjected to in vitro expansion and activation or genetic manipulation (through the CAR technology). Moreover, in some studies, the NK cells are infused together with small immunostimulatory molecules or with immunomodulating mAb (such as the bispecific anti-CD30/CD16) [74].

A Phase II clinical study, enrolling 100 patients with pediatric leukemia and lymphoma, aimed to test the safety and efficacy of treating such cancers at their first relapse with a combination of chemotherapy, Rituximab, and infusion of haploidentical NK cells (NCT01700946).

A Phase II trial is currently studying the efficacy of allogeneic NK-92 cell transplantation, combined with radiation therapy and anti-CD20 immunotherapy, in extending the progression-free survival time of 100 leukemia and lymphoma patients (NCT02727803).

A Phase I study on 54 multiple cancer patients, including lymphomas, is aimed at studying the therapeutic efficacy of HLA-unmatched NK-cell transplantation together with ALT803 NK-activating molecule administration (NCT02890758).

A Phase I study is assessing in 13 hematological patients, including lymphoma, the feasibility and safety of ex vivo expanded cord blood donor-derived NK-cell transplants (NCT01619761).
A pilot Phase I study (24 patients) is currently testing the safety of HLA-haploidentical or HLA-mismatched related donor nicotinamide expanded-NK cell-based therapy in patients with relapsed or refractory multiple myelomas or CD20 positive NHLs (NCT03019666).

A pilot Phase I study (30 participants) is testing the safety of coadministering a CD56-enriched donor lymphocyte infusion with haploidentical peripheral blood allogeneic stem cell transplantation, including reduction of GVHD development posttransplant (NCT03524235).

A Phase II clinical study, enrolling 40 patients with refractory B-NHL, is establishing the potentially improved efficacy of combining cord blood-derived expanded NK-cell allogenic transplantation with chemotherapy, immunotherapy, and allogenic stem cell transplantation (NCT03019640).

An interventional study enrolling 30 patients affected by recurrent B-NHL will compare both the safety and efficacy of injecting autologous expanded NK cells in combination with anti-CD20 immunotherapy (NCT02843061).

A Phase I/II pilot study (nine participants) will evaluate both the efficacy and safety of combined therapy of allogenic NK-cell infusion and Rituximab (NCT03778619).

A Phase I pilot study will assess the maximum tolerated dose of haploidentical donor-derived NK-cell infusion in combination with chemotherapy in 18 leukemia and lymphoma patients (NCT00640796).

A Phase II study of 162 leukemia and lymphoma patients will test the successful engraftment of a combined allogenic stem cell and NK-cell transplantation upon myelodepletion (NCT01807611).

A Phase I/II study of 41 refractory lymphoma patients aims to assess whether allogenic stem cell transplantation combined with donor NK-cell therapy infusion improves the outcome in such patients (NCT00799776).

A Phase I study of 48 lymphoma patients will examine the dose-limiting toxicity and the overall response rate of CAR-NK cells infusion (NCT03774654).

A pilot study (18 participants) is currently evaluating the biological activity of anti-CD30/CD16A monoclonal antibody (mAb) AFM13 in CD30-positive lymphomas. Such a molecule is an inducer of NK-mediated tumor cell killing (NCT03192202).

A Phase II clinical study (23 patients) will also assess the objective response rate of administering AFM13 mAb in relapsed or refractory HL patients (NCT02321592).

A Phase I study enrolling 30 refractory lymphoma patients will additionally test the efficacy of cord blood-derived NK cells preloaded with AFM13 Ab transplant, followed by AFM13 mAb infusion (NCT04074746).

All the reported ongoing trials will help to uncover the potentials of NK-based cell therapy in ameliorating the outcome of lymphoma patients.

**General conclusions and future directions**

It is clear that the majority of patients with lymphomas have poor NK activities in vivo and this defect plays a pivotal role in the management of the tumor and its eradication by both the innate and the adaptive immune responses. Many preclinical studied and clinical studies...
have reported the immunotherapeutic value of NK cells in many cancers, and clearly it can be extended to lymphomas, particularly those that do not respond to conventional therapies. Several such approaches have been examined and include the in vivo and in vitro activation of the patient’s NK cells, the ex vivo expansion and genetic modification of allogeneic NK cell lines, the genetic engineering of CAR-NK cells, the use of agonists directed against the NK activating receptors and antagonists directed against the NK inhibitory receptors, the use of checkpoint inhibitors, and combination therapies.

The above new therapeutic strategies are clearly being evaluated clinically and considered as a new armamentarium in the treatment of various lymphomas that are resistant to conventional therapies. In addition, the prognostic significance of NK cells in lymphomas is an area of intensive research investigations.

Acknowledgments

We (FK, BB) acknowledge the Department of Microbiology, Immunology & Molecular Genetics and the David Geffen School of Medicine at UCLA for their support.

References


VI. NK Immunotherapy in various cancers

Benjamin_Bonavida, 978-0-12-824375-6
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References


21. Natural killer cells and lymphomas


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References


VI. NK Immunotherapy in various cancers
NK cells in brain tumors: From biology to treatment

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Abstract

Natural killer cells are key elements of the innate immune defense system. Its inherent therapeutic advantages include not requiring prior sensitization to target transformed malignant cells, decreased incidence of graft-versus-host disease, and activation via antibody-dependent cell-mediated cytotoxicity. These biological properties of NK cells have favored its exploration and use in adoptive immunotherapy for brain tumors over recent years. Even though the last few years have witnessed an explosion of biological data, targeted therapy including adoptive cellular therapy for highly aggressive brain tumors such as GBM and DIPG, survival outcome continues to remain dismal. This is being increasingly attributed to an immunosuppressive cross talk between tumors and the immune microenvironment, which interferes with NK cell tumor homing, activation, and cytotoxicity. Improved understanding of biological mechanisms underlying therapeutic failure and resistance to NK cell therapy will allow development of combination therapy that will circumvent or overcome immunosuppression and improve outcomes in patients with brain tumors. Genetic engineering of NK cells is also being considered to improve targeting of brain tumors. These strategies could involve either NK cells alone or in combination with other immune strategies, to address multiple immunosuppressive mechanisms that have contributed to therapeutic failure of immunotherapy against brain tumors.

Abbreviations

\begin{itemize}
  \item ABCC3 \quad \text{ATP-binding cassette subfamily C member 3}
  \item ADCC \quad \text{antibody-dependent cell mediated cytotoxicity}
\end{itemize}

* Both the authors (VG and SK) have contributed equally and are senior coauthors.
22. NK cells in brain tumors: From biology to treatment

VI. NK immunotherapy in various cancers
Introduction

Brain tumors are intracranial neoplasms that account for approximately 1.4% of all cancers [1]. Treatment is based on neurosurgical procedures, chemotherapy, and radiotherapy [2]. Patients with malignant brain tumors continue to suffer from dismal survival and poor outcomes due to inevitable recurrence and progression [3]. During the last decades, an important amount of data about the genetic basis of brain tumors has been generated, providing a better understanding of key molecular pathways involved in their pathogenesis [4]. This has helped in the elucidation of mechanisms of tumor growth and resistance to therapy.

Brain tumors are characterized by their ability to evade immune checkpoint and suppress antitumoral immunity [5]. One major limitation to effective therapy is the blood-brain barrier, which blocks the access of targeted drugs into tumor sites [6]. Furthermore, the lack of lymphatic drainage and antigen-presenting cells protects brain tumors from the immune system. However, preclinical and translational studies in the past decade have changed the perception and shown an important role for immune cells in brain tumors [7–10].

Effectector cells, such as natural killer (NK) cells, comprised an integral constituent of the innate immune defense system long before T and B cells arrived on the adaptive immune scene about 500 million years ago [11]. Today, the three lymphocytic lineages survive, with NK cells outnumbering B cells in circulation by a 3:1 ratio. The newly discovered functional complexity of NK cells rivals their antigen-specific memory-bearing B cell counterparts in antitumor activity and potential [12].

NK cells are large granular lymphocytes that play an important role in anticancerous immunity [10]. Upon activation, cytotoxic NK cells induce apoptotic death in target cells through the secretion of perforin, granzyme B [13–15], and proinflammatory cytokines, such as tumor necrosis factor-α and interferon-γ (IFN-γ) [12, 16, 17]. Early preclinical studies have demonstrated the efficacy of NK cells in brain tumor settings, in vitro [18–20] and in vivo [21–23]. As such, NK-cell immunotherapy appears as a promising, novel treatment approach for brain tumors [4].

This chapter explores the role of NK cells in the brain tumor microenvironment, highlights preclinical investigations with NK cells, and discusses clinical outcomes of NK-cell–based therapies in adult and pediatric patients with brain tumors. The chapter further deliberates on the challenges of NK-cell therapy and future directions in the continued fight against brain tumors.

VI. NK immunotherapy in various cancers
NK cells in the brain tumor microenvironment

NK cells play a fundamental role in the intratumoral immune response in the brain microenvironment. It has been shown that NK cells are present in metastatic brain tumors, craniopharyngiomas [24], and meningiomas [25], and they frequently infiltrate glioblastomas [26]. Current advances and the development of more precise detection methods offer the opportunity to better study the infiltration of NK cells in various other brain tumors [10].

Impaired immune function and increased antiinflammatory molecules are common in patients with brain tumors. This antitumoral milieu often affects the functioning of NK cells due to the immunosuppressive factors released by brain cancer cells (Fig. 1). Glioblastoma cells, for example, express high levels of a special form of major histocompatibility complex class I molecules related chains A and B (MICA and MICB) [27, 28], which act as ligands for the NKG2D activating receptor on NK cells. Tumor cells that express MICA potentially provide stimulating signals to NK cells. Tumor-derived inflammatory mediators, such as cyclooxygenases (COX)

![Image of NK cell deactivation by cancer cells in the brain microenvironment](image-url)

**FIG. 1** Mechanisms of NK cell deactivation by cancer cells in the brain microenvironment. Brain tumor cells that express MHC-I and PD-1 proteins can bind to inhibitory receptors on NK cells that lead to NK cell deactivation. CD73, encoded by NT5E in cancer cells, drives adenosine-mediated immunosuppression. The release of cytokines, such as IL-6, IL-8, and TGF-β, and mediators of inflammation, such as COX and PGE2, further promote NK cell deactivation. NKG2E proteins expressed on NK cells and encoded by KLRC3 have been associated with brain tumor maintenance, aggressiveness, and recurrence. Silencing of the KLRC3 gene has been shown to deprive cancer cells of progressive abilities. The binding of CD155 to TIGIT on NK cells promotes NK cell immunosuppression.

VI. NK immunotherapy in various cancers
and prostaglandin E2, impair NK-cell viability and chemokine production [29]. This inhibits dendritic cell accumulation in the tumor microenvironment and decreases antitumor immune activity [29].

The NK-cell number in the brain tumor microenvironment is often suppressed. Patients with glioblastoma have a decreased number of NK cells in isolated tumor specimens after being treated with radiation and temozolomide [30]. The NK-cell frequency and cytotoxic activity are downregulated as a result of antiinflammatory molecules like transforming growth factor-β (TGF-β). Moreover, the decrease in the level of expression of NK Group 2-member-D (NKG2D) in patients with glioblastoma further reflects the common immune evasion mechanisms exhibited by brain tumors [31]. Metastatic brain tumor cells also develop immunological escape mechanisms through the NKG2D receptor-ligand (NKG2DL) system [32]. Notably, the majority of NKG2DL-positive cells are positive for dormancy markers in patients with cerebral metastases [32]. As such, the NKG2DL-positive cell population may represent a promising future therapeutic target.

Brain tumor cells are equipped with the ability to secrete numerous chemokines, cytokines, and growth factors that can target the antitumor activity of NK cells in the brain. Altogether, these components are crucial for cancer proliferation, spread, and response to treatment. For instance, the survival and differentiation of gliomas are controlled through two distinct phenotypes of cytotoxic and split anergized NK cells, respectively [33]. Split anergized NK cells promote glioma differentiation, which results in the resistance of differentiated glioma cells to NK-cell–mediated cytotoxicity. Resistance to cytotoxicity is mediated by sustained release of interleukin (IL)-6 and IL-8 along with decreased IFN-γ, postdifferentiation [34]. As such, immunotherapies designed to retain the tumor killing function as well as IFN-γ secretion by the NK cells, while suppressing tumor-induced IL-6 and IL-8 secretion, can be effective. Furthermore, priming NK-cell–derived extracellular vehicles with IL-15 enhances their ability, as an immunotherapy, to kill human cancer cells in vitro and in vivo, including glioblastoma [35]. These IL-15-primed vesicles show a significantly higher cytolytic activity and increase the expression of molecules associated with NK-cell cytotoxicity [35]. Such strategies can improve the application and the immunotherapeutic effects of NK-cell–derived extracellular vesicles in the future. Moreover, treating NK cells with IL-2 and heat shock protein 70 (HSP70) improves homing around and within the tumor site in vivo [36]. Systemic injection confirms migration of activated NK cells over the blood-brain barrier and subsequent targeting of glioblastoma tumor cells [36]. This suggests that the administration of HSP70/IL-2-treated NK cells may be a promising therapeutic approach to be considered in the treatment of glioblastoma (Fig. 2). In addition, COX-deficient tumors are extensively infiltrated by dendritic cells. It has been shown that NK cells recruit dendritic cells to the tumor microenvironment by releasing chemokine ligand 5 (CCL5) and X-C Motif Chemokine Ligand 1 (XCL1) [29]. This NK-cell–mediated activity governs dendritic cell antitumor function in various human cancers, and amplifying its role can impact survival positively [29].

Gene expression patterns of NK cells can contribute to brain tumor recurrence and aggressiveness. Identifying these genes can help in developing new therapeutics. By screening glycosylation-related genes to characterize specific genes involved in glioma stem cell maintenance, killer cell lectin like receptor C3 (KLRC3) was found to be overexpressed [37]. KLRC3 gene codes for NKG2E, a protein initially identified in NK cells. Silencing of KLRC3 decreases self-renewal capacity, invasion, proliferation, radioresistance, and
tumorigenicity of the U87-MG glioblastoma cell line [37]. Furthermore, the CD73 marker that is overexpressed in glioblastoma contributes to the generation of immunosuppressive adenosine. This suppresses NK cells through the interplay between expression of NT5E, which encodes CD73, in glioblastoma and specific NK genes [38]. Functional analysis demonstrates that CD73 is a negative prognostic factor for glioblastoma [38]. Thus, the presence of NK cells may associate with improved prognosis. CD155 is also overexpressed on glioblastoma cell surfaces and contributes to increased tumor migration and aggressiveness [39]. It is an immunomodulatory receptor that is able to both activate NK cells through interactions with membrane proteins, CD226 and CD96, that play a role in adhesive interactions, and inhibit them through interaction with the T cell immunoreceptor with Ig and ITIM domains (TIGIT) [39]. In the setting of glioblastoma, NK cell TIGIT expression has been shown to be upregulated, establishing CD155 as a predominantly inhibitory receptor.
within the context of glioblastoma, rendering it of interest as a potential target for antigen-specific NK-cell–based immunotherapy [39]. Moreover, it has been shown that NK cells expressing CD16 (a marker of NK cell maturation) predominate in patients surviving more than 12 months after surgery without disease progression [40]. This subtype of NK cells coexpress high levels of the multidrug resistance protein ATP-binding cassette subfamily C member 3 (ABCC3) and IFN-γ, which are related to a strong, long-term NK cell response and a better prognosis of patients [40]. In fact, temozolomide in combination with dendritic cell immunotherapy can upregulate the expression of ABCC3 in NK cells in patients with glioblastoma. This permits NK cells to escape apoptosis and favors their role as antitumor effector cells.

Preclinical advancements in NK cell therapy

Cytotoxic agents against brain tumor cells can promote the NK-cell immune response. N6-isopentenyladenosine (iPA) is an isoprenoid-modified adenosine that possesses a well-established anticancer activity [41]. Suboptimal doses of this drug are able to induce a significant upregulation of cell surface expression of NKG2D ligands on glioma cells in vitro and in vivo [41]. This facilitates NK-cell–mediated recognition and activation against the glioma cells through a mechanism that depends on the p53 status of malignancy. Furthermore, the combination of NK cells and temozolomide may prove to be a promising immunochemotherapeutic approach in patients with glioblastoma. Growth inhibition assays revealed that genuinely induced NK cells enhance temozolomide-induced inhibition and apoptosis in U87MG glioma cell lines that are sensitive or resistant to temozolomide [42].

Antitumor immunity in the brain is restrained by multiple immunosuppressive pathways. Therapies involving immunotherapeutic agents and NK cells can be vital in overcoming resistance to immune checkpoint inhibitors in brain tumors. It has been shown that programmed death 1 (PD-1)/PD-L1 blockade elicits a strong NK cell response that is indispensable for the full therapeutic effect of immunotherapy [43]. Ultimately, this can increase the portion of patients that can benefit from immunotherapy. Furthermore, blocking the PD-1/B7-H1 pathway in NK cells promotes the NK-cell–mediated immune response and the subsequent targeting of glioma stemness [44]. Functional assays and gene expression profiling shows that NK cells in glioblastoma are characterized by TGF-β–mediated inhibition [45]. This NK cell inhibition is accompanied by expression of multiple immune checkpoint molecules on T cells [45]. As such, combination immunotherapies directed against multiple immunosuppressive pathways can be an effective strategy against brain tumors.

Oncolytic viral therapy is often limited by rapid viral clearance by innate immune NK cells and poor intratumoral viral spread. Engineering oncolytic Herpes Simplex Virus (oHSV) to encode E-cadherin, an adherent molecule and a ligand for KLRG1, an inhibitory receptor expressed on NK cells, induces high surface E-cadherin on infected glioblastoma cells [46]. This selectively protects virus-infected cells from KLRG1+ NK cell killing and prolongs survival in glioblastoma-bearing mouse models, due to improved viral spread and adherence [46]. Thus, virus-induced overexpression of E-cadherin may be a generalizable strategy for improving cancer virotherapy.
Bortezomib is a proteasome inhibitor that possesses antitumor activity. Pretreatment of glioblastoma with bortezomib sensitizes tumor cells to NK cell lysis by inducing stress antigens recognized by NK-activating receptors [47]. As a result, NK cells release IFN-γ, perforin, and granzyme A cytolytic granules disrupt mitochondrial function and kill 24%–46% of glioblastoma cells by apoptosis [47]. Bortezomib further increases stress ligands, induces TRAIL-R2 expression, and enhances glioblastoma lysis to 33%–76% through augmented IFN-γ release [47]. Adding oHSV to bortezomib and NK cell therapy shows that the synergetic treatment of oHSV-infected glioblastomas with bortezomib induces necroptotic cell death to enhance NK-cell immunotherapy [48, 49]. The triple combination further prolongs survival against human glioblastoma [48, 49].

Chimeric antigen receptor (CAR)-modified NK cells have become of interest in recent years. In breast cancer brain metastases, the combination of CAR NK cells and oHSV was tested in vitro and in a breast cancer intracranial mouse model. NK-92 cells and primary NK cells were engineered to express the second generation of EGFR-CAR [50]. In vitro, the combination of EGFR-CAR NK-92 cells and oHSV-1 demonstrates a synergistic cytolytic effect compared to monotherapies [50]. EGFR-CAR-engineered NK-92 cells and primary NK cells display enhanced cytotoxicity and increased IFN-γ production. In vivo, the combination therapy against EGFR-expressing MDA-MB-231 cells mitigates tumor growth and significantly improves survival [50]. NK cells have also been employed to express ErbB2-specific CAR, after ErbB2 was found to be elevated in 41% of primary glioblastoma patient samples and in a majority of cell lines investigated [51]. In vitro, ErbB2-CAR NK-92 cells lyse all ErbB2-positive glioblastoma cells [51]. Potent in vivo antitumor activity of ErbB2-CAR NK-92 cells eventually leads to a marked improvement in survival and the development of long-term antitumor immunity [51]. This shows the potential of CAR NK-92 cells as an adoptive immunotherapy for glioblastoma.

NK cell therapy trial for adult brain tumors

The presence of NK cells in the brain tumor microenvironment led to the development of novel therapies that aim to increase NK cell recruitment and boost their function. An important obstacle lies in the immunosuppressive environment of brain tumors that can downregulate NK-cell activation and upregulate inhibition. Nevertheless, adoptive immunotherapy of brain tumors with NK cells that are activated, expanded ex vivo, and then injected into the tumor site after local resection has made it into clinical trials (Table 1).

In patients with glioblastoma, NK cells were able to kill human glioblastoma cells that exhibit stem cell-like properties [19]. Pure autologous NK cells were shown to be safe and partially effective in patients with recurrent malignant gliomas, whereby tumor regression was recorded in four of nine patients [57]. The tumor-induced immune suppression and immune escape mechanisms might be behind the partial efficacy of NK cells [10]. In a phase II trial, haploidentical transplant and donor NK cell immunotherapy followed by mTOR inhibition maintenance is well tolerated in patients with a high-risk set of solid tumors, including brain tumors [53]. Of 15 patients, four developed graft-versus-host disease but no patients died from transplant-related causes [53]. With a median follow-up of 1.3 years, the disease control
<table>
<thead>
<tr>
<th>NCTID</th>
<th>Trial</th>
<th>Phase</th>
<th>Location</th>
<th>Start date</th>
<th>Number enrolled</th>
<th>Inclusion criteria</th>
<th>Primary outcome</th>
<th>Notes</th>
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<tr>
<td>NCT02271711</td>
<td>Expanded Natural Killer cells infusion treating younger patients with recurrent/refractory brain tumors</td>
<td>I</td>
<td>US</td>
<td>2015</td>
<td>9</td>
<td>Age &lt;21 years, recurrent/refractory medulloblastoma, AT/RT, ependymoma</td>
<td>Tolerability, side effects</td>
<td>There was no dose limiting toxicity. At higher dosing, NK cells increased in the CSF during treatment with repetitive infusions. The study demonstrated feasibility [52]</td>
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<tr>
<td>NCT02100891</td>
<td>Phase 2 STIR trial: haploidentical transplant and donor natural killer cells for solid tumors (STIR)</td>
<td>II</td>
<td>US</td>
<td>2014</td>
<td>15</td>
<td>All ages; high-risk solid tumors, including brain tumors</td>
<td>Disease-control rate (DCR)</td>
<td>All donor NK infusions were well-tolerated without cytokine release syndrome. Four patients developed acute graft-versus-host disease. No patients died from transplant-related causes. With a median follow-up of 1.3 years, 6-month DCR was 72%. At 2 years, overall survival for the entire cohort was estimated at 40%, while progression-free survival was 22% [53]</td>
</tr>
<tr>
<td>NCT01235845</td>
<td>Dendritic cell (DC) activated cytokine-induced killer cell (DCIK) combined with DC treatment for glioma</td>
<td>I/II</td>
<td>China</td>
<td>2011</td>
<td>30</td>
<td>Adults (18–70 years); malignant gliomas</td>
<td>Overall survival</td>
<td>Combination of cytokine-induced NK cells and IL-2</td>
</tr>
<tr>
<td>NCTID</td>
<td>Trial</td>
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<tr>
<td>NCT01588769</td>
<td>A phase I study to investigate tolerability and efficacy of ALECSAT administered to glioblastoma multiforme patients (ALECSAT-GBM)</td>
<td>I</td>
<td>Denmark</td>
<td>2011</td>
<td>23</td>
<td>Age &gt; 18 years; glioblastoma</td>
<td>Tolerability, side effects</td>
<td>Anti-EGFRvIII CAR transduced lymphocytes lymphocytes homed to the tumor, with tumor regression ongoing in 3 patients for 14, 22, and 27 months, respectively. No treatment-related adverse effects were observed [54]</td>
</tr>
<tr>
<td>NCT01213407</td>
<td>Dendritic cell cancer vaccine for high-grade glioma (GBM-Vax)</td>
<td>II</td>
<td>Austria</td>
<td>2010</td>
<td>87</td>
<td>All ages; glioblastoma</td>
<td>Progression-free survival</td>
<td>Audence immunotherapy based on dendritic cells had no effect on overall and progression-free survival [55]. The treatment led to a significant up-regulation of the Th1-related immunovariables ELISPOT IFNγ, the transcription factor T-bet in the blood and ELISPOT IL-2 in a dose-dependent manner upon vaccination. Post-vaccination levels of ELISPOT IFNγ and CD8 + cells in the blood were indicative of a significantly better survival [56]</td>
</tr>
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Lymphokine-activated killer cells or gliadel wafer in treating patients with newly diagnosed glioblastoma that can be removed by surgery.

Trial was withdrawn due to lack of support.

Overall survival

NC10814593

Age > 18 years; glioblastoma

US

2008

0

2004

9

Malignant glioma

Tumor response, side effects

Three patients partially responded, 2 had minor responses, 4 had no change, and 7 exhibited progressive disease in a total of 16 courses of treatment. Severe neurological toxicity was not observed in any of the patients [57].

Autologous natural killer cell therapy for human recurrent malignant glioma

Japan

Benjamin Bonavida, 978-0-12-824375-6
rate at the 6-month interval was 72% [53]. A 1- and 2-year overall survival for the entire cohort was estimated at 64% and 40%, respectively, while progression-free survival was 29% and 22%, respectively [53].

Tumor vaccines with antigen-presenting cells are capable of priming antitumor immune responses with cytotoxic effector cells, including NK cells. Tumor lysate-charged autologous dendritic cells were used in the treatment of adult patients with newly diagnosed glioblastoma [55]. Vaccinations were given for 7 months on average. Despite no severe toxicity, progression-free survival at 12 months did not differ significantly between the control (28.4%) and vaccine groups (24.5%). In addition, median overall survival was similar with 18.3 months [55]. Immunologically, the treatment led to a significant upregulation of IFN-γ, the transcription factor T-bet in the blood, and IL-2 in a dose-dependent manner upon vaccination [56]. Postvaccination levels of IFN-γ and CD8+ cells in the blood were indicative of a significantly better survival [56]. Upon activation with a DNA-demethylating agent, CD4+ T helper cells can also be used as antigen-presenting cells to generate autologous cytotoxic T lymphocytes and NK cells [54]. In a phase 1 trial of 25 adult patients with recurrent glioblastoma, cytotoxic lymphocytes homed to the tumor, with tumor regression ongoing in three patients for 14, 22, and 27 months, respectively; no treatment-related adverse effects were observed [54]. This shows that tumor-reactive effector cells can be generated ex vivo by exposure to antigens induced by DNA demethylation, which provides a novel, minimally invasive therapeutic strategy for treating adult brain tumors.

NK-cell therapies have their drawbacks. Immune-related reactions, such as graft-versus-host syndrome, can occur [58]. Furthermore, expanding NK cells can be very expensive, particularly when many purification and stimulation steps are needed [59]. In addition, there is a lack of a common large-scale clinical grade expansion method that yields uniform NK cell numbers, phenotypes, genotypes, and functions from individual donors [10].

The evolving era of NK cells in pediatric neurooncology

NK cells are a major component of antitumor immunity and play a key role in tumor immunosurveillance. They are effector lymphocytes of the innate lymphoid system, which can recognize cancer cells, bind to them, and induce cytotoxicity. This phenomenon is facilitated by activating receptors on the NK cells (the most potent being the CD16), which adhere to the ligands on the cancer cells [60]. This initiates NK cell activation, degranulation, and release of cytokines such as perforin and granzyme B (core molecules required for NK-cell–mediated tumor killing) that are directly toxic to the target cells, inducing apoptosis via various mechanisms including the FasL and TRAIL pathways [61, 62]. The resulting cytokine/chemokine surge is not only lethal to the target cells, but also promotes innate and adoptive responses, including influx of T cells and the dendritic cells, which enhances the cytotoxicity [63].

NK-based cell therapies are rapidly gaining attention for adoptive immunotherapy in pediatrics [64, 65]. NK cells do not require prior sensitization to target transformed malignant cells, while permitting self-tolerance. This enables them to recognize autologous cells that express HLA class I molecules, which prevents it from attacking the host tissues, and results
in decreased incidence of graft-versus-host disease (GVHD), posing a distinct advantage over T cells [65]. This phenomenon has been used in pediatric leukemia, enabling use of haploidentical NK cells to generate antileukemic effects with clinical efficacy and decreased risk of GVHD [62, 66]. Also, NK cells can be activated via antibody-dependent cell-mediated cytotoxicity (ADCC) through engaging CD16 (FcγRIIIA) with monoclonal antibodies, resulting in its use in hematological malignancies like T-ALL and lymphoma [67]. These biological insights of NK cells have made it more appealing and therapeutically efficacious against pediatric hematological malignancies, though clinical outcome/efficacy in children with solid tumors remains suboptimal, due to a hostile immunosuppressive tumor microenvironment.

### Immune targeting of pediatric solid tumors—The challenges

The tumor microenvironment is rich in immunosuppressive cytokines and metabolites such as TGF-β, adenosine, prostaglandin E2 (PGE2), and indoleamine 2,3-dioxygenase (IDO), which have been linked to NK cell dysfunction [68]. These are important limiting factors in the therapeutic success of immune targeting of pediatric solid tumors. Infiltration of tumor-associated macrophages (TAMs) are more abundant in these tumors than in their adult counterparts, conferring a poor prognosis [69]. Like TAMs, infiltration of myeloid-derived suppressor cells (MDSC) into the tumor incites the release of various immunosuppressive factors like cytokines and chemokines, including macrophage derived IL-10, vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and matrix-metallopeptidase-9, which induces therapeutic resistance. Also, TAM promote metastasis through the CCL2/CSF-1 signaling axis. These biological impediments are often seen in pediatric gliomas, sarcomas and in poor prognosis neuroblastoma [69–71].

The pediatric brain, in particular, is an intensive energy-consuming organ that fosters an efficient metabolic coordination through intercellular shuttling of metabolites and nutrients (glucose, pyruvate, and lactate) between neurons and astrocytes [72]. In brain tumors, this well-orchestrated metabolic interplay is disrupted, facilitating its growth and aggressiveness [73, 74]. The high-demanding tumor cells compete for these nutrients and compromise the antitumor activity of the immune cells by starving them of the essential nutrients. This significantly undermines the function of macrophages, NK cells, and NK-T cells, which forms an important layer of nonspecific innate immunity, and plays a pivotal role in suppressing tumor growth and progression [75]. This fractured metabolic symbiosis allows the tumor to manipulate and initiate a hostile tumor microenvironment, which in turn renders an influx of immunosuppressive cells such as TAM, MDSC, and regulatory T (T_{reg}) cells [76]. Another undermining factor in immune targeting of pediatric CNS tumors is the low mutational burden that these tumors harbor in contrast to adults. This results in expressing fewer potential neo-antigens, limiting their susceptibility to targeted therapy with immune cells [77].

Multiple studies have now consistently shown that the high density of tumor-infiltrating NK cells has been linked with a good prognosis in human solid tumors. This is reaffirmed in preclinical models, where depletion of the NK-cell population prior to tumor transplantation has been shown to induce a more aggressive phenotype with metastatic tumors [78]. Although studies have now more comprehensively described an immunosuppressive...
microenvironment in brain tumors, the majority of studies to date have used adult GBM models. This may not accurately represent the tumor microenvironment (TME) of pediatric brain tumors. In general, pediatric brain tumors are thought to have reduced NK-cell–mediated immune surveillance and harbor a less immunosuppressive TME compared with adults. The TME phenotype also varies between histological types of pediatric brain tumors [79, 80]. These suggest that immunotherapy strategies against pediatric brain tumors should be tailored not only based on their molecular signatures but also on the phenotype of the tumor microenvironment (inhibited immune surveillance) and its interplay with the tumor. The lack of understanding of this phenomenon could result in therapeutic resistance.

### Pediatric brain tumors

**Medulloblastoma**

Medulloblastoma is the most frequent malignant brain tumor in children. Although overall survival has improved, recurrent tumors are mostly considered incurable. The long-term side effects remain a major cause of concern, undermining clinical efficacy with conventional treatment [81]. Various genomic, epigenomic, and transcriptomic data have now identified four distinct molecular subgroups with distinct biological characteristics and clinical outcomes, and further defined intertumoral heterogeneity within each of these subgroups [82, 83]. However, a precise understanding of the function of medulloblastoma and its interplay with the microenvironment is essential as we embark on profiling optimal immunotherapy for these challenging neoplasms. One of the largest and most comprehensive studies of the microenvironment in medulloblastoma showed a molecular subgroup-specific immune and stromal microenvironment. Distinct differences in the clustering pattern of medulloblastoma subgroups according to their microenvironment were discerned. The SHH subtype showed a strong signature of fibroblasts, T cells and macrophages, while cytotoxic lymphocytes were noted in the Group 4 variant. PDL-1 gene expression was elevated in the WNT and SHH subtypes, though protein expression was minimal. This study also identified two diverse immune-stromal patterns with distinct types of immunosuppression, dominated by macrophages, T-cell–mediated mechanisms or immunosuppressive checkpoints and cytokines, respectively [84]. A study showed SHH tumors have a unique tumor microenvironment among medulloblastoma subgroups. The interactions of TAMs with SHH medulloblastoma cells may contribute to tumor growth, suggesting TAMs as a potential therapeutic target [85]. These revelations of medulloblastoma/TME interplay reaffirm the need to better understand the possible influence of the tumor and immune microenvironment on NK activity to foresee potential roadblocks in developing and improving therapeutic outcomes of NK cell therapy in medulloblastoma.

Earlier studies showed clinical efficacy and tolerability with adoptive immunotherapy in medulloblastoma patients, using lymphocyte-activated killer cells (LAK cells), which constituted mainly NK cells [86, 87]. From a biological perspective, medulloblastomas are appealing for therapeutic application of NK cells, as they express higher levels of ligands for NK cell activating receptors such as NKG2D and natural cytotoxicity receptors (NCRs) [88]. Also, the expression profile of diverse antigens like CD1d, UL16 binding protein-2 (ULBP-2), and
variable MHC expression by medulloblastoma favors NK-cell–based therapy, as the later can recognize and induce death of tumor cells with broad antigen specificity, without requiring prior antigen identification [89–91]. Various immunosuppressive cytokines have been identified in the TME of medulloblastomas, which can decrease clinical efficacy and enhance tumor resistance. Hypoxia in the TME has been shown to downregulate the expression of NKG2DL, a ligand for the primary activating receptor NKG2D of NK cells. The consequent immune evasion in medulloblastomas and other CNS tumors is unsurprising, and is thought to contribute to a therapy-refractory phenotype [92]. TGF-β is one of the most potent and widely investigated immunosuppressive molecules secreted by medulloblastomas, with negative effects on NK cell function [93]. In a recent study, engineered cord blood-derived NK cells expressing a TGFβ dominant negative receptor-2 (TGF-β DNRII) were used to demonstrate a functional advantage in overcoming the TGF-β rich medulloblastoma TME. This is an important tool and could provide the basis for its further development and evaluation as a novel “off-the-shelf” NK cell product against TGF-rich medulloblastoma tumors [94]. Despite the recent increase in preclinical data supporting the application of NK cells for medulloblastoma therapy, its clinical translation has been slow. Thus far, the trials described above with LAK cells and the recently completed first-in-human intraventricular infusions of NK cells in pediatric malignant brain tumors, including medulloblastoma, are the only two clear clinical investigation of NK cells in children. It is important to note that these trials employed autologous NK cells, which could also have impacted efficacy [52]. Future studies should must test allogeneic cell products alone or in combination with agents that modulate the TME to make the tumor more hospitable for NK cells and other immune cell therapy products, which in turn will decrease the potential for therapeutic resistance and promote sustained clinical efficacy [84].

**Pediatric high-grade gliomas**

Children with pediatric high-grade glioma (HGG), including diffuse midline glioma (DMG) and glioblastoma multiforme (GBM), have a dismal 5-year overall survival of <20% [95]. Surgical resection, except in diffuse intrinsic pontine glioma (DIPG), radiation therapy, chemotherapy, or molecularly targeted therapy, has yet to reveal sustained efficacy [96]. Immunotherapy is an appealing therapeutic strategy for these cancers. However, major challenges exist. An inherent reduction in numbers of NK, T cells and the enhanced presence of immunosuppressive myeloid cells, reduced availability of neoepitopes due to a low mutational burden (immunologically cold tumors), and a hostile immunosuppressive TME induced by cytokines/metabolites continue to be key factors with the potential to impede immunotherapy against pediatric HGG [97, 98]. A sixfold elevation CD68-expressing and alternatively activated CD163-positive macrophages has been reported in pediatric HGG compared with normal tissues, suggesting that the immune microenvironment may not be permissive for immune activity [99]. Higher levels of immunosuppressive factors like programmed death ligand (PD-L1), B7-H3, and TGF-β and proinflammatory mediators like IL-8 are also seen in pediatric HGG [99]. Other studies suggest that the persistence of glioma stem cells (GSCs), lured by the TGF-β pathway and increased vascularization, could substantially contribute to tumor aggressiveness in pediatric gliomas [100]. As stated previously, TGF-β pathway activation is highly immunosuppressive. These challenges can also be extended to NK cell therapy in pediatric GBM. In addition, TGF-β reduces the expression of
NK cell activating receptors NKp30, NKG2D, DNAM-1, and antagonizes IL-15–induced proliferation and gene expression associated with NK cell activation. IL-15 is also known to activate m-TOR signaling and prime stress-activated gene expression leading to the prolonged antitumor capacity of NK cells [101]. The above lines of evidence suggest that TGF-β-mediated evasion of NK cell cytotoxicity may diminish the efficacy of NK cell therapy [79, 102, 103]. Indeed, glioma-infiltrating NK cells have a unique surface phenotype, which favors TGF-β–mediated immunosuppression in humans, and could contribute to suboptimal clinical efficacy [45]. These observations clearly indicate the need to explore strategies to dampen the effects of TGF-β activity in the TME to enhance NK cell efficacy. In preclinical studies, engineered cord blood (CB)-derived NK cells expressing a dominant negative TGF-β receptor-DNRII have shown improved lytic activity. This therapeutic approach demonstrated in preclinical models now needs to be translated into the clinical arena [104].

Oncolytic viruses are uniquely designed both to lyse tumor cells through their replication and the ability to recruit immune responses against virally infected cells. However, therapeutic efficacy has been undermined due to the immunosuppressive TME, leading to various studies looking into approaches to overcome this limitation in brain and other solid tumors [105]. A recent study demonstrated that the HDAC inhibitor valproic acid increased the expression of activating ligands for NK cell recognition and innate NK-cell killing. This showed that the efficacy of oncolytic herpes simplex virus (oHSV) therapy can be enhanced by NK cell killing of tumors when primed with the HDAC inhibitor [106]. Also, a combined treatment strategy comprising oncolytic measles virus (MeV) and activated NK cells resulted in enhanced oncolysis of pediatric sarcoma cell lines [107]. Although various studies have demonstrated the enhanced efficacy using oncolytic viral therapy (herpes, myoma, polio virus) with NK or CAR/NK therapy in brain tumors or brain metastasis, none to date have moved into the clinics in childhood brain tumors—an unmet need in pediatric neuro-oncology [50, 108, 109]. The use of CAR-redirected NK cells, which have been used in adults, should now be studied in pediatric GBM using specific inhibitory molecules [104, 110, 111]. Currently, two ongoing trials in adults (age 18 and above) are using placental NK cells (NCT: 04489420) in recurrent GBM or intracranial injection of NK-92/5.28.z cells in patients with recurrent HER2-positive GBM (NCT: 03383978). However, the design of these trials precludes recruitment of pediatric patients, highlighting the need for pediatric-centric studies and clinical trials using unmodified or modified (CAR NK) NK cells.

Diffuse intrinsic pontine glioma (DIPG)

Diffuse intrinsic pontine glioma is a uniformly fatal tumor with a median survival of 11 months and remains a leading cause of death among pediatric brain tumor patients. To date, even with therapeutic advances, no increase in survival has been achieved [112]. These tumors are characterized by a point mutation in the genes encoding histone 3.3, causing a K27M alteration, and are accompanied by significant changes in the tumor epigenome and transcriptome [112]. Mouse models have shown that this mutation by itself is insufficient for tumorigenesis and requires other molecular events such as PDGFR hyperactivity and p53 mutation to accelerate gliomagenesis [113]. While these studies focused on identifying drivers of tumorigenesis, their effects on the TME are early yet, as described below.

The DIPG microenvironment has fewer inflammatory cells and is immunologically more inert than adult or pediatric GBM. As stated earlier, adult HGGs are characterized by a highly

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Immunosuppressive microenvironment, whereas DIPGs do exhibit a limited influx of immune cells (myeloid or lymphoid infiltrate), suggesting that the DIPG-TME may be less hostile for immune cells [114]. Unlike adult HGGs, infiltration of immunosuppressive CD68- and CD163-positive macrophages appears to be less prevalent in pediatric HGGs and is virtually absent in DIPGs. Only minimal PD-L1 and NKG2D expression is described in pediatric HGGs, including DIPGs, in contrast to adult tumors [115]. These studies allude to pediatric HGGs being more receptive to immunotherapy, particularly NK cell therapy. Consistent with this supposition, studies by Lieberman et al. demonstrated superior in vitro NK cell-lytic activity against DIPGs compared with T-cells [99].

Clinically, the route of administration of immune cells has been a major unknown in DIPGs. The BBB penetration of systemically infused NK cells and trafficking to the tumor site remains to be evaluated in preclinical models. In parallel, techniques such as convection enhanced delivery (CED) could be pursued for intracranial administration of immune cells to patients with DIPG. Another important area of study that must be examined is how the histone H3.1 versus H3.3-K27M mutational status affects immunotherapy, as emergent studies have shown the transcriptome of H3.1. A previous study demonstrated gene signature difference between the two variants of K27M mutated tumors, with the H3.1-K27M mutated tumors having a gene signature similar to that seen in the mesenchymal subtype type of GBM. These H3.1-K27M-mutated tumors exhibit a mesenchymal/astrocytic phenotype and a pro-angiogenic/hypoxic signature, greater immune cell infiltration, unlike the H3.3-K27M-mutated DIPG who have a proneural/oligodendroglial phenotype and a pro-metastatic gene expression signature with PDGFRα activation. These differences in the gene expression profile and immune cell infiltration could explain a favorable outcome, less metastatic recurrence in the H3.1-K27M subtype [112]. Thus, a better understanding of tumor-TME cross talk in DIPGs is needed. These studies will determine if immune cell activity needs to be augmented by combination treatments to circumvent a potentially underreceptive TME.

Autologous versus allogenic source of NK cells in brain tumors

It is becoming increasingly clear that autologous NK cell therapy has significant limitations for solid tumors, including brain tumors. Impediments to the use of autologous cells include the need to achieve optimal ex vivo expansion of NK cells, because their inherent low numbers in peripheral blood mononuclear cells (PBMC) will not support achievement of sufficient potency. The consequent variability in expansion efficiency and quality from one patient to another, and the associated increase quality control steps make it not only somewhat unpredictable as a therapy, but also cost-prohibitive. The time for expansion contributes to significant delays in therapeutic product availability. Other potential problems of using autologous NK cells include repetitive freezing and thawing, which diminishes its efficacy. In addition, low function and exhaustion of autologous NK cells driven by exposure to transforming growth factor beta, tryptophan metabolites, adenosine, and chronic stimulation are matters for concern [116]. These logistical challenges are driving the field toward a major shift in the NK-cell therapeutic paradigm and the use of allogeneic “off-the-shelf” NK-cell products derived from peripheral blood or cord blood (CB). There are added advantages to the development of allogenic NK cells. First, they can be tailored to have mismatches against tumor HLA and thus circumvent immune evasion caused by MHC expression. Second, product availability and time to therapeutic delivery are
significantly improved because of the existence of CB banks, with increased donor repertoire and HLA-compatible and KIR-mismatched lines at many institutions.

Imaging the fate of therapeutic delivery of NK cells

Immunotherapy using adoptive transfer of ex vivo expanded natural killer (NK) cells is emerging as a new modality in the treatment of solid tumors [117]. This increased focus on immunotherapy has drawn attention to the need for a tool to monitor the fate of therapeutically delivered immune cells. In particular, when therapeutic efficacy is limited, a clear understanding of where the problem lies needs to be examined. For example, inefficient delivery, homing, and trafficking to and within the tumor, differentiation state of immune cells, and, along with it, persistence and activity in the tumor are all contributory factors. Studies have been conducted along these lines and have shown heterogeneity of NK cell populations, migratory behaviors at different stages of development, and the immune suppressive signals of the TME to be associated with lowered NK cell migration and activation at tumor sites, resulting in limited clinical efficacy [118, 119]. In light of this early knowledge, imaging technologies are being investigated as a means to inform on the real-time fate of adoptive transfer of NK cells to tumors. Over the last few years, various methods have been developed for the noninvasive in vivo tracking of NK by molecular imaging [120]. These technologies can provide visualization of biological, pathophysiological processes including characterization and quantification of these processes [121]. As development of NK-cell–based immunotherapies is now pursued and in the early stages of development, molecular imaging of NK cells needs to be developed in parallel to understand the trafficking, persistence of these immune cells to the tumor [121]. To date, these methods as discussed below are restricted to preclinical models of brain tumors. The stage is now being set for the gradual translation of these findings to the clinic from in vitro to in vivo settings in animals and humans [122].

Various imaging modalities that are now being applied for tracking immune cells include optical imaging using fluorescence imaging (FLI) or bioluminescent imaging (BLI), single photon emission computed tomography (SPECT), positron emission tomography (PET), and magnetic resonance imaging (MRI) [123]. Many of these techniques have used direct labeling of the NK cell surface with fluorophores or loading of the NK cells with cell-permeable fluorophores, various contrast agents, and radioisotopes [124, 125].

Optical imaging uses direct or indirect labeling of NK cells to track them. These cells can be directly labeled with exogenous fluorescent tracers for FLI, or indirectly by transfecting a reporter gene that induces a protein luciferase for BLI, or a green fluorescent protein (GFP) for FLI [126, 127]. These optical markers have demonstrated high sensitivity and specificity in small animal studies. In addition to the benefits of a very high signal-to-noise ratio, this modality has shown the potential of providing multiplex imaging using various probes with diverse spectral characteristics and low-cost instrumentation [128]. Although appealing and widely used, the major limitations of optical imaging include the small depth of penetration, which restricts its use to small animal models, precluding its evaluation in larger animals and humans [125].

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The two nuclear imaging modalities commonly used (PET and SPECT) are well integrated in clinics. These imaging modalities can be used in humans to visualize NK cells due to their capability of visualizing in vivo cell migration anywhere in the human body and provide three-dimensional (3D) imaging data. PET and SPECT have the advantage of high sensitivity, depth penetration, robust signal-to-background ratios, and the availability of a wide range of imaging probes. However, their high cost, limited anatomical information, and radiation exposure present limitations of concern [129–131].

MRI is also widely available in clinics and can easily be translated from preclinical to clinical applications. Advantages include high-resolution anatomical information due to its ability to penetrate deep into tissue and lack of ionizing radiation. Major constraints of this imaging modality is the low sensitivity for molecular detection [132]. 19F MRI is a cell tracking technique which is gaining interest because of its 100% natural abundance, high MRI sensitivity, background free images, and its recent approval by the FDA [133, 134]. 19F perfluorocarbon, specifically, has been successfully applied in preclinical models of medulloblastoma to demonstrate intracranial NK cell delivery. These 19F-labeled NK cells not only suppressed medulloblastoma growth without undermining cytotoxicity, but also enabled 19F MRI to provide adequate imaging feedback [135]. A separate body of work supported these findings and showed that labeling with this isotope had no detrimental effect on NK cell function and maintained cytotoxicity of leukemia cells [136].

Despite the promise of diverse noninvasive imaging tools, each of them is saddled with limitations, constraining their development into a gold standard for monitoring in vivo trafficking of immune cells such as NK cells. Multimodal hybrid imaging (MRI and PET, optical imaging and MRI), consisting of two or more imaging technologies, may be the way for the future and should be explored. Improving signal visualization in cell therapy and for extended periods will necessitate a reduction in label leaks from cells, accounting for diminution of signal, because of cell proliferation using biological reporters [125]. In summary, the last few years have successfully seen significant preclinical advances in the expansion, labeling, and in vivo tracking of NK cells [132]. The future should see refined, optimal, and robust imaging modalities to precisely quantify the homing, bio-distribution, and persistence of adoptively transferred NK cell trials in humans.

**Conclusion**

The last few years have seen a surge in efforts to evaluate the potential role of NK cells in adult and pediatric brain tumors, though mostly confined to the preclinical arena. Improved knowledge of the tumor-microenvironment interplay and the immune status could help in undermining therapeutic failures and profiling optimal clinical trials. In parallel, robust imaging modalities need to be developed, which could provide the most desirable characteristics for measuring responses to NK cell therapy in humans. The next few years should see strategies of loco-regional infusions of unmodified allogenic or next-generation engineered NK cells incorporated into the armamentarium of NK-cell-based therapeutics in neuro-oncology.
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VI. NK immunotherapy in various cancers

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22. NK cells in brain tumors: From biology to treatment


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