Trends in Cancer

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Review

The clinical landscape of CAR-engineered unconventional T cells

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Unconventional T cells, such as invariant natural killer T (iNKT), γδ T, and mucosal-associated invariant T (MAIT) cells, play a pivotal role in bridging innate and adaptive immunity. Their capacity for rapid tumor targeting and effective modulation of the tumor microenvironment (TME) makes them promising candidates for cancer immunotherapy. Advances in chimeric antigen receptor (CAR) engineering have further highlighted their therapeutic potential, particularly for treating challenging cancers. Notably, these cells exhibit favorable safety profiles, enhancing their viability as off-the-shelf therapeutic options. We provide a comprehensive analysis of the clinical applications of CAR-engineered unconventional T cells, focusing on genetic modifications, manufacturing processes, preconditioning regimens, and dosing strategies. We discuss successful examples from recent clinical trials and explore future directions for utilizing these cells in cancer therapy and beyond.

Highlights

CAR-engineered unconventional T cells have so far demonstrated promising efficacy and safety in clinical trials for cancer therapy.

These cells possess diverse tumor-targeting mechanisms, exhibit robust infiltration into solid tumors, and modulate the tumor microenvironment (TME), making them a significant focus of clinical trials for solid tumor treatment.

Owing to their lack of graft-versus-host disease (GvHD) induction, these cells are well suited for allogeneic, off-the-shelf cell therapy applications.

Clinical trial designs for CAR-engineered unconventional T cells use innovative approaches, including advanced genetic modifications, optimized manufacturing processes, tailored preconditioning regimens, and refined dosing strategies.

CAR-engineered unconventional T cells offer a promising frontier in immunotherapy

Unconventional T cells, also referred to as innate-like T cells, iNKT cells, γδ T cells, or MAIT cells, play important roles in bridging innate and adaptive immunity by rapidly responding to a wide range of stimuli [1]. These cells are characterized by their ability to recognize non-peptide antigens, and this enables them to respond to infections, tissue damage, and malignancies in a manner distinct from conventional αβ T cells [2–4]. iNKT cells recognize lipid antigens presented by CD1d molecules [5], γδ T cells respond to stress-induced ligands or phosphoantigens via butyrophilin (BTN) proteins [6,7], and MAIT cells detect microbe-derived vitamin B metabolites presented by MR1 molecules [8]. Notably, CD1d and MR1 are non-polymorphic major histocompatibility complex (MHC) class I-like molecules, whereas BTNs belong to the immunoglobulin superfamily and are associated with the B7 protein family. These molecules present a limited variety of ligands to unconventional T cells. Together, these unconventional T cell subsets exhibit potent cytotoxicity, cytokine secretion, and immunoregulatory functions, and this positions them as key players in immune surveillance and potential targets for cancer immunotherapy and infectious disease management.

Given our enhanced understanding of unconventional T cell biology, advances in gene engineering technologies, innovative culture methods, and a wealth of clinical experience derived from CAR-engineered conventional αβ T cells, significant progress has been made in the development and clinical application of CAR-engineered unconventional T cells (Figure 1). Unconventional T cells possess unique advantages, including multiple tumor-targeting mechanisms, efficient tumor infiltration, ability to modulate the TME, and suitability for genetic engineering and immune enhancement (Box 1). Furthermore, these cells possess a T cell receptor (TCR) repertoire characterized by low diversity, which decreases the likelihood of crossreactivity with allo-MHC molecules. As a result, these cells are associated with a lower risk of **graft-versus-host disease** (**GvHD**; see Glossary). Because of the restricted TCR repertoire, there is no necessity to inactivate or disrupt the endogenous TCR locus to prevent alloreactivity in unconventional

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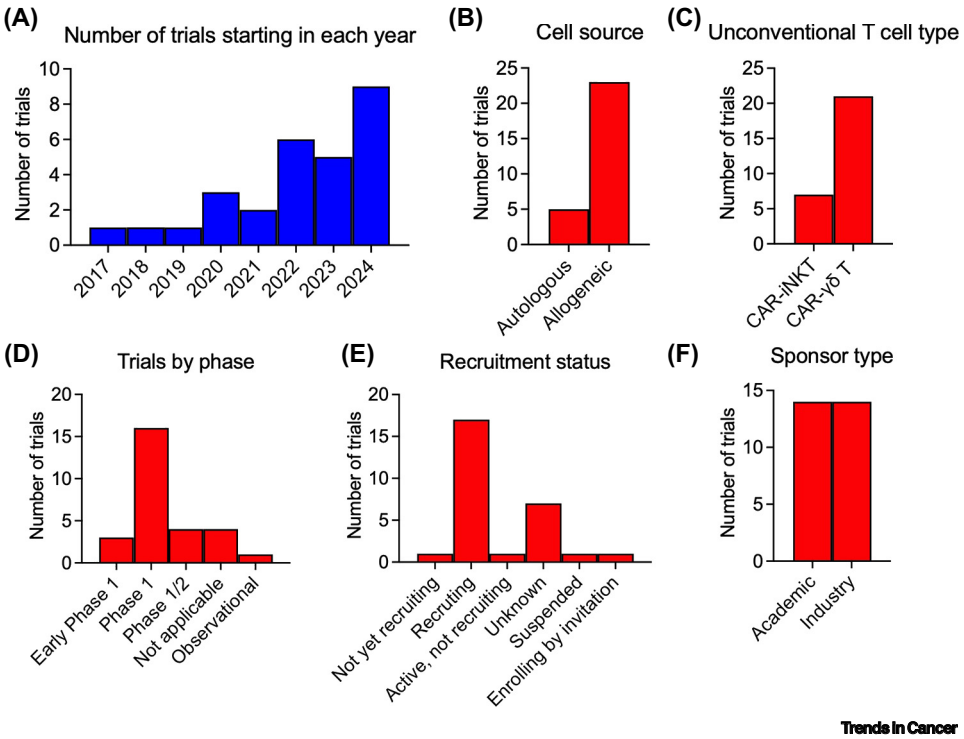

Figure 1. Summary of clinical trials involving chimeric antigen receptor (CAR)-engineered unconventional T cells. (A) Annual number of registered clinical trials over time. (B) Classification of clinical trials by cell source, categorized as 'autologous' or 'allogeneic'. (C) Classification of clinical trials by unconventional T cell type, categorized as 'CAR-iNKT' or 'CAR-γδ T'. (D) Distribution of clinical trials by phase, including those labeled 'not applicable'. (E) Recruitment status of clinical trials. (F) Classification of clinical trials by sponsor type, categorized as 'academic' or 'industry'. Abbreviation: iNKT cell, invariant natural killer T cell.

T cells. Another advantage of iNKT and MAIT cells is that their invariant TCRs can be serially stimulated by low levels of the restricting CD1d or MR1 molecules expressed by normal tissues, and this contributes to improved survival of these therapeutic cells [9–11]. In addition, the expression of CD1d or MR1 molecules by tumor cells can enhance cytotoxicity through TCR-mediated recognition [12–15]. Collectively, these characteristics render unconventional T cells particularly suitable for allogeneic, off-the-shelf therapies [16–18]. Notably, CAR-engineered iNKT (CAR-iNKT) cells (Table 1) and CAR-engineered γδ T (CAR-γδ T) cells (Table 2) have shown promise in clinical applications for allogeneic cancer therapy.

In this review we present a detailed analysis of the clinical trial landscape involving CAR-engineered unconventional T cells for the treatment of cancer and other diseases including autoimmune disorders. We examine diverse clinical strategies, encompassing genetic modifications, manufacturing protocols, preconditioning regimens, and dosing approaches. In addition, we highlight key successes from recent clinical trials and discuss potential future applications of these cells in cancer immunotherapy and other therapeutic contexts.

Overview of clinical trials with CAR-engineered unconventional T cells

CAR-iNKT cells

iNKT cells have been investigated in clinical trials for more than two decades as a therapeutic strategy for various cancers, including melanoma, lung cancer, head and neck squamous cell

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carcinoma (HNSCC), and other advanced solid tumors [19]. These trials have predominantly utilized two approaches: activation and expansion of endogenous iNKT cells through **α-galactosylceramide (αGC)** administration or αGC-pulsed antigen-presenting cells, and adoptive transfer of *ex vivo* expanded, non-gene-modified autologous iNKT cells. These treatments have demonstrated safety and tolerability in patients with advanced cancers [20–24]. However, their clinical efficacy has been modest, with limited therapeutic benefits. This lack of effectiveness is attributed to the extremely low frequency of iNKT cells in human peripheral blood, rapid depletion of αGC *in vivo*, and overactivation-induced exhaustion of iNKT cells following αGC stimulation [5,19,25]. In recent years advances in CAR engineering, together with robust *in vitro* expansion protocols for human iNKT cells, have paved the way for adoptive transfer of CAR-iNKT cells. This approach is being actively explored in both preclinical and clinical settings as a promising strategy to enhance the therapeutic potential of iNKT cells in cancer treatment.

Since 2018 at least seven registered clinical trials have evaluated CAR-iNKT cells for cancer treatment, comprising four autologous and three allogeneic approaches (Table 1). These CAR-iNKT cell products include autologous GD2-targeting CAR-iNKT cells for treating glioblastoma, autologous CD70-targeting CAR-iNKT cells for treating relapsed or metastatic advanced renal cell carcinoma (RCC) and other solid tumors, and allogeneic CD19-targeting CAR-iNKT cells for treating B cell lymphoma and leukemia. Currently, all these trials remain in the patient recruitment phase. One clinical trial (NCT03294954) is specifically investigating autologous GD2-targeting CAR-iNKT cells in children with relapsed or refractory neuroblastoma. Interim analysis from this Phase 1 dose-escalation study has reported data from the initial cohort of three patients at dose level 1, showing the safety and feasibility of the cell product [26]. Recently updated interim results from this trial, encompassing 12 patients, reported an objective response rate of 25% (3/12) [27]. These findings further support the safety and therapeutic potential of CAR-iNKT cells coexpressing human IL-15 in cancer treatment.

Autologous CAR-iNKT cell therapy has been used to target solid tumors. CAR-iNKT cells possess an intrinsic ability to home to and infiltrate solid tumors effectively while also modulating the TME [1]. These characteristics provide a strong rationale for their application in solid tumor therapy. Moreover, given the remarkable success of conventional CAR-T cell therapies in treating hematological malignancies, particularly $CD19^+$ B cell malignancies and $BCMA^+$ multiple myeloma [28–30], research efforts are increasingly directed toward exploring alternative cell types for solid tumors, where conventional CAR-T cells have shown limited efficacy.

Three clinical trials have reported the use of universal or allogeneic CAR-iNKT cells for the treatment of B cell lymphoma and leukemia (Table 1). Because iNKT cells display a TCR repertoire with very low diversity, they are less likely to trigger GvHD, making them a promising candidate for allogeneic cell therapy with a better safety profile than conventional CAR-T cells [31–33]. However, a key challenge in the clinical application of allogeneic CAR-iNKT cells is overcoming allorejection because host T and natural killer (NK) cells may recognize and reject these foreign cells, thus impairing the *in vivo* antitumor efficacy [34]. To address this, several genetic engineering strategies have been used. In a recent clinical trial, an allogeneic CD19-targeting CAR-iNKT cell product was developed that incorporated **short hairpin RNAs (shRNAs)** targeting *B2M* and *CD74* to downregulate HLA class I and class II molecules, respectively [35]. Recent preclinical studies utilized CRISPR-Cas9 to knock out *B2M* and *CIITA* in CAR-iNKT cells, and this effectively abrogated both HLA-I and HLA-II expression and generated universal stem cell-derived CAR-iNKT cells [16,36]. These engineered cells exhibited robust resistance to host T cell-mediated allorejection. In addition, because of the absence of NK cell ligands, these CAR-iNKT cells were also resistant to host NK cell-mediated rejection [16,36]. Overall, in the presence of

Glossary

Adenine base editors: advanced genome-editing tools that enable precise conversion of adenine to guanine in DNA sequences without causing double-strand breaks, thereby facilitating the correction of point mutations associated with genetic diseases.

Apheresis: a medical procedure that involves the separation and removal of specific components of blood, such as plasma, platelets, or leukocytes, while returning the remaining blood cells back to the patient. Apheresis is often used for therapeutic purposes.

Bispecific T cell engager (BiTE): a type of biotherapeutic that simultaneously binds to a T cell and a target tumor cell, thereby facilitating T cell activation and promoting the targeted destruction of tumor cells.

Concanavalin A: a plant-derived lectin that binds specifically to mannose and glucose residues on glycoproteins and polysaccharides, and is commonly used to stimulate and expand γδ T cells.

Cytokine release syndrome (CRS): a systemic inflammatory response caused by the excessive release of cytokines, often triggered by immunotherapies such as CAR-T cell therapy, and characterized by fever, fatigue, and organ dysfunction.

Cytopenia: a reduction in the number of blood cells, including red blood cells, white blood cells, and platelets, which can lead to various health complications such as anemia, increased risk of infection, and bleeding disorders.

Dose-limiting toxicity (DLT): adverse effects of a drug or treatment that are severe enough to prevent an increase in dose or continuation of therapy, often used in clinical trials to determine the maximum tolerated dose of a medication.

α-Galactosylceramide (αGC): a glycolipid ligand that specifically activates iNKT cells and plays a crucial role in immune modulation and antitumor responses.

Glucocorticoid: a class of steroid hormones produced by the adrenal cortex that play crucial roles in regulating metabolism, inflammation, and immune response. Glucocorticoids are often used therapeutically to treat various inflammatory and autoimmune conditions.

Graft-versus-host disease (GvHD): a potentially severe immune condition that

host cells, these cells demonstrated prolonged *in vivo* persistence and potent antitumor efficacy in human multiple myeloma xenograft mouse models [16,36]. In addition, a study using canine iNKT cells showed that unedited allogeneic iNKT cells can persist for >78 days in MHC-mismatched recipients without requiring high-intensity preconditioning, while maintaining functional activity [37]. This finding highlights the inherent ability of allogeneic iNKT cells to resist host allorejection, thereby supporting the potential use of both allogeneic iNKT and CAR-iNKT cell therapies in cancer treatment.

Overall, CAR-iNKT cells are being actively investigated in both preclinical and clinical studies. A variety of CAR-iNKT cell products have been developed, including those derived from cancer patient-derived peripheral blood mononuclear cells (PBMCs), healthy donor-derived PBMCs, human hematopoietic stem and progenitor cells (HSPCs), and induced pluripotent stem cells (iPSCs) [12,16,36,38,39]. This represents a promising new approach, particularly for the treatment of solid tumors.

CAR-γδ T cells

γδ T cells have been explored as candidates for adoptive immunotherapy because of their potent responses against bacteria, viruses, and tumors. In contrast to αβ T cells, γδ T cells exhibit potent cytotoxicity without MHC restriction, enabling their broader and safer application in allogeneic transplantation and treatment in diseases beyond cancer including autoimmune disorders [6,40]. Clinical trials have been initiated to assess the clinical potential of γδ T cells [41–43]. However, achieving optimal clinical outcomes with γδ T cells remains challenging because of limitations in scalable and efficient expansion methods, as well as inconsistencies between their *in vitro* and *in vivo* phenotypes following expansion [41–43]. Strategies such as genetic engineering, particularly CAR engineering, have been developed to address these limitations. Findings suggest that, compared to the combinatorial administration of stimulatory γδ T ligands and cytokines for *in vivo* expansion, engineering and expanding γδ T cells *in vitro* before *in vivo* infusion yields a more robust and precise enhancement of their therapeutic efficacy [44–47]. For example, the implementation of CAR technology and antibody **bispecific T cell engager (BiTE)** enables engineered γδ T cells to be redirected into tumor-implanted tissues, thereby addressing their limited infiltration ability [42,46]. Such approaches are now being actively evaluated in both preclinical and clinical settings to optimize the therapeutic potential of γδ T cells.

At least 21 registered clinical trials are currently evaluating CAR-γδ T cells for the treatment of cancer and autoimmune diseases (Table 2). Their unique safety profile, characterized by potent cytotoxicity without MHC restriction, makes γδ T cells particularly suitable for broader applications in allogeneic therapies. The targets of these trials include hematological malignancies such as acute myeloid leukemia (AML), non-Hodgkin's lymphoma, and multiple myeloma. For example, UTAA06 cells – γδ T cells derived from leukopak of healthy donors and armored with B7-H3-targeting CAR – are being tested in two clinical trials for their efficacy and immunogenicity in treating AML. In addition to CAR engineering, these cells are further adjusted to coexpress IL-2 for a prolonged antitumor effect [48]. Moreover, because of the enhanced tumor infiltration conferred by CARs, solid tumors, including breast cancer, non-small cell lung cancer (NSCLC), and malignant brain gliomas, are also being explored as potential targets for CAR-γδ T cells in clinical settings (Table 2). γδ T cells coexpressing an HLA-G-targeting CAR and BiTE are able to target PD-L1 and/or HLA-G-positive solid tumor cells simultaneously, thereby reducing off-tumor toxicity and overcoming immune evasion [46]. Besides tumors, one clinical trial is investigating the therapeutic potential of CD19-targeting universal CAR-γδ T cells in treating systemic lupus erythematosus (NCT06106893). Successful application of these CAR-γδ T cells could mitigate

occurs when donor immune cells attack the tissues of the recipient following allogeneic cell or organ transplantation.
Immune effector cell-associated neurotoxicity syndrome (ICANS): a neurological complication that can occur following immunotherapy, particularly with CAR-T cell treatment, that is characterized by symptoms such as confusion, seizures, and motor dysfunction caused by inflammatory effects on the central nervous system.
Rimiducid: a small-molecule dimerizing agent used to selectively activate inducible caspase-9 safety switches in gene-modified cell therapies, thereby enabling controlled elimination of engineered cells in case of adverse effects.
Short hairpin RNA (shRNA): a synthetic RNA molecule designed to silence gene expression by forming a hairpin loop structure that is processed into small interfering RNA within cells.
Tachycardia: an abnormally fast heart rate, typically defined as >100 beats per minute in adults, which can result from various physiological or pathological conditions.
Tachypnea: an abnormally rapid breathing pattern, often indicative of underlying respiratory or metabolic conditions.

Box 1. Unique features of CAR-engineered unconventional T cells.

CAR-engineered unconventional T cells exhibit unique attributes that make them highly promising for cancer immunotherapy (Figure I). These cells leverage multiple tumor-targeting mechanisms, including CAR, TCR, and NKR-mediated tumor cytotoxicity, that enable them to recognize and eliminate a diverse array of tumor cells while mitigating tumor antigen escape [36,38]. Furthermore, they demonstrate superior infiltration into solid tumors, thereby addressing a key limitation of many conventional T cell therapies [13]. In addition, unconventional T cells possess intrinsic abilities to modulate the TME, thereby enhancing antitumor immune responses [15,103].

The amenability of these cells to genetic modifications, such as cytokine engineering and checkpoint gene knockout, allows optimization of their functional properties, including prolonged persistence, enhanced cytotoxicity, and resistance to the immunosuppressive milieu of the TME [68,69]. Notably, these cells recognize non-polymorphic MHC molecules or, in some cases, are MHC-independent. This feature significantly reduces the risk of GvHD, making them particularly well suited for allogeneic, off-the-shelf therapies that utilize donor-derived cells for multiple recipients without requiring patient-specific customization [17,18,104].

These advantages have catalyzed the clinical exploration of CAR-engineered unconventional T cells, such as CAR-iNKT and CAR-γδ T cells, which are under investigation in numerous clinical trials targeting refractory cancers. Early results are encouraging, and these therapies demonstrate antitumor activity, favorable safety profiles, and scalability [26,27,35,92]. Such attributes position them as transformative tools in cancer immunotherapy. Continued research and clinical optimization are anticipated to further enhance their efficacy and broaden their application, and could potentially revolutionize therapeutic strategies for patients with limited treatment options.

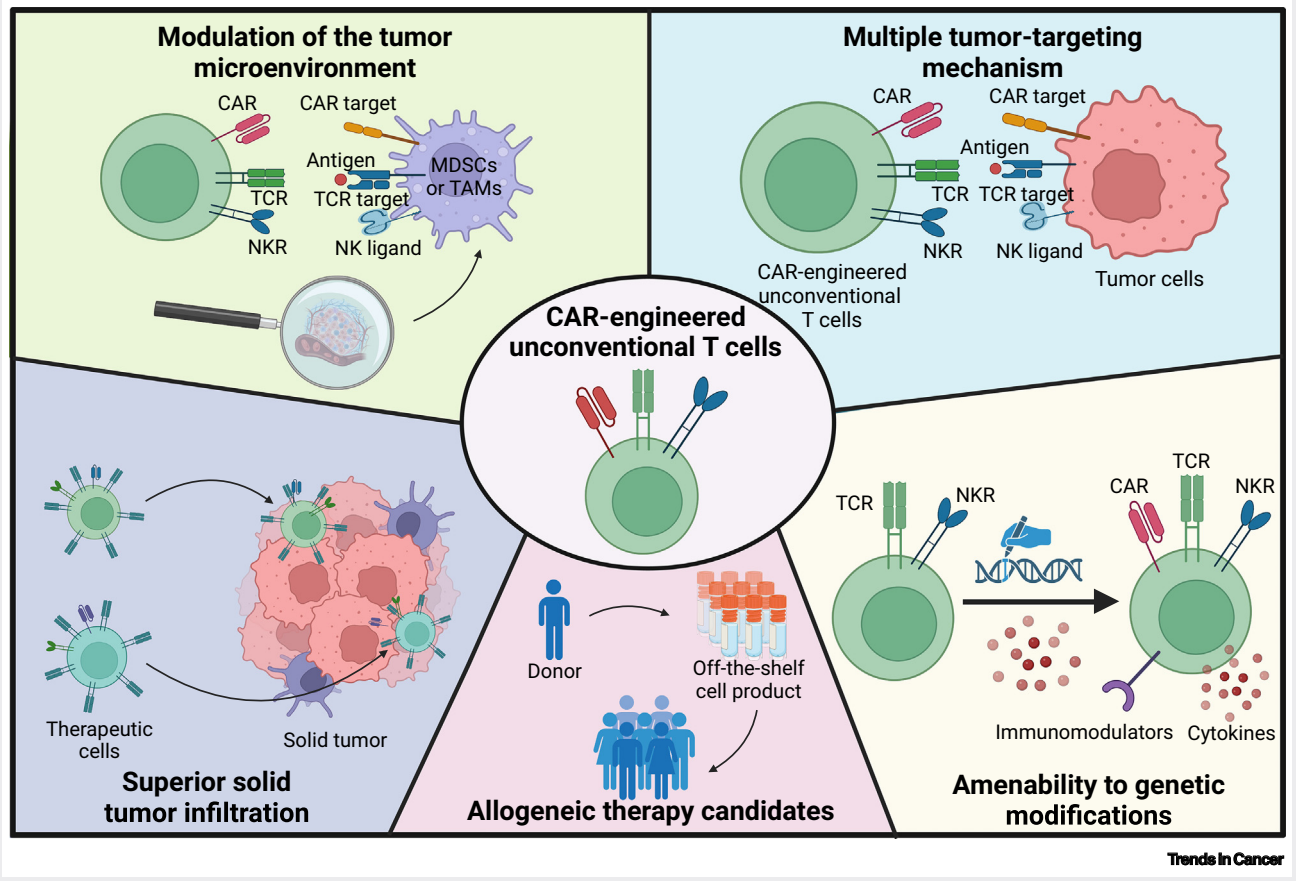

Figure I. Unique features of CAR-engineered unconventional T cells in cancer therapy. Abbreviations: CAR, chimeric antigen receptor; MDSC, myeloid-derived suppressor cell; NK, natural killer; NKR, NK receptor; TAM, tumor-associated macrophage; TCR, T cell receptor. Figure created with BioRender.

side effects such as **glucocorticoid**-related diabetes which often arises from prolonged glucocorticoid dependence during treatment [49,50].

In conclusion, γδ T cells hold promise as candidates for adoptive immunotherapy because of their potent, MHC-independent cytotoxicity and ability to target a wide range of diseases, including

Table 1. Clinical trials utilizing CAR-iNKT cells

Autologous or allogeneic	CAR target	Additional genetic engineering	Disease indication	Current stage	ClinicalTrials.gov ID
Autologous	GD2	IL-15 overexpression	Relapsed/refractory high-risk neuroblastoma	Recruiting	NCT03294954
	CD70	NA[a]	Relapsed/metastatic advanced renal cell carcinoma	Recruiting	NCT06182735
	CD70	NA	Advanced malignant solid tumor	Recruiting	NCT06394622
	CD70	NA	Advanced malignant solid tumors	Recruiting	NCT06728189
Allogeneic	CD19	IL-15 overexpression	Refractory/relapsed B cell lymphoma or leukemia	Recruiting	NCT05487651
	CD19	IL-15 overexpression	Refractory/relapsed B cell lymphoma or leukemia	Recruiting	NCT03774654
	CD19	IL-15 overexpression	Refractory, relapsed, or high-risk B cell tumors	Unknown status	NCT04814004

[a]NA, not applicable.

cancer and autoimmune disorders. However, γδ T cells still pose a potential risk of generating autoimmunity because they can secrete proinflammatory cytokines (e.g., IFN-γ, TNF-α, and IL-17) which promote autoimmune diseases such as rheumatoid arthritis [51–54]. Therefore,

Table 2. Clinical trials utilizing CAR-γδ T cells

Autologous or allogeneic	CAR target	Additional genetic engineering	Disease indication	Current stage	ClinicalTrials.gov ID
Autologous	GPC3/mesothelin	NA[a]	Advanced cancer that expresses GPC3 or mesothelin	Recruiting	NCT06196294
Allogeneic	NKG2DL	NA	Advanced solid tumor or hematological malignancies	Recruiting	NCT05302037
	NKG2DL	NA	Relapsed or refractory solid tumor	Unknown status	NCT04107142
	HLA-G	PD-L1/CD3ε bispecific T cell engager (BiTE) coexpression	Relapsed/refractory triple-negative breast cancer, non-small cell lung cancer, colorectal cancer, or glioblastoma	Recruiting	NCT06150885
	CD20	NA	Relapsed/refractory B cell malignancies	Active, not recruiting	NCT04735471
	B7-H3	IL-2 overexpression	Advanced malignant solid tumors	Recruiting	NCT06372236
	B7-H3	IL-2 overexpression	Relapsed/refractory acute myeloid leukemia	Recruiting	NCT05731219, NCT05722171
	CD123	NA	Relapsed acute myeloid leukemia	Unknown status	NCT04796441,NCT05388305
	CD19	NA	Relapsed/refractory B cell malignancies	Unknown status, not yet recruiting	NCT02656147, NCT06092047
	CD7	NA	T cell malignancies	Unknown status	NCT04702841
	B7-H3	NA	Malignant brain glioma	Suspended	NCT06018363
	CD19	NA	B cell acute lymphoblastic leukemia	Recruiting	NCT06056752
	CD19	NA	Relapsed/refractory B cell non-Hodgkin's lymphoma	Recruiting	NCT06503211, NCT05554939
	B7-H3	NA	Meningeal metastases	Recruiting	NCT06592092
	BCMA	NA	Relapsed/refractory multiple myeloma	Recruiting	NCT06279026
	CD20	NA	Diffuse large B cell lymphoma, follicular lymphoma, mantle-cell lymphoma, non-Hodgkin lymphoma, marginal zone lymphoma, primary mediastinal B cell lymphoma	Enrolling by invitation	NCT04911478
	CD19	NA	Systemic lupus erythematosus	Recruiting	NCT06106893

[a]NA, not applicable.

further research will be necessary to ensure the safe application of γδ T cells in clinical settings. Despite challenges in achieving optimal expansion and *in vivo* efficacy, combining the unique properties of γδ T cells with the specificity of CAR technology has provided more effective therapies for a wider range of cancers [55–57]. The ongoing development of CAR-γδ T cells equipped with enhanced safety and efficacy features further supports their feasible and scalable role in immunotherapy. With ongoing research and clinical trials, CAR-γδ T cells have the potential to improve treatment approaches for a wide range of diseases.

CAR-engineered MAIT (CAR-MAIT) cells

MAIT cells represent a recently characterized population of immune cells with promising therapeutic potential in cancer and other diseases such as autoimmune, infectious, and metabolic disorders [58,59]. CAR-MAIT cells have been developed in preclinical studies; however, no clinical trials have so far explored their application in cancer treatment. Preclinical evidence highlights the feasibility and antitumor activity of CAR-MAIT cells. For example, healthy donor PBMC-derived mesothelin-targeting CAR-MAIT cells can be generated by stimulating MAIT cells with 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU), a MAIT cell agonist, followed by lentiviral transduction for CAR engineering. These CAR-MAIT cells demonstrated potent cytotoxicity against mesothelin-positive ovarian cancer cells and effectively targeted tumor-associated macrophages (TAMs) through MR1/TCR recognition [15]. This dual-targeting mechanism suggests the ability of CAR-MAIT cells to act on both tumor cells and the TME. Similarly, CD19- and HER2-targeting CAR-MAIT cells were generated by stimulation with high doses of a related molecule, 5-amino-6-(D-ribitylamino)uracil (5-A-RU), in the presence of antigen-presenting cells [60]. These CAR-MAIT cells exhibited robust cytotoxicity against multiple tumor cell lines via CAR- and TCR-mediated mechanisms. In addition, hepatitis B virus (HBV)-specific TCR-engineered MAIT cells were developed to demonstrate anti-HBV functions without compromising their endogenous antimicrobial mechanisms or hepatotropic characteristics, thereby providing new insights into liver-directed immunotherapies [61]. Despite these promising findings, the majority of reported CAR-MAIT studies primarily present *in vitro* results without accompanying *in vivo* evidence. This limitation likely stems from challenges in achieving robust CAR-MAIT cell expansion and the lack of reliable methods for generating CAR-MAIT cells with high yield and purity, thereby hindering their translational development.

In addition to engineered CAR-MAIT cells, studies of primary tumor samples from patients with hepatocellular carcinoma (HCC), NSCLC, and high-grade serous ovarian cancer (HGSOC) suggest that tumor-infiltrating or peritumoral MAIT cells, despite exhibiting an exhausted phenotype (e.g., PD-1^{+} and/or LAG3^{+}), are strong prognostic markers for positive responses to immune checkpoint blockade and platinum-based chemotherapy [62–64]. These findings highlight the potential of MAIT cells and their CAR-engineered derivatives to target solid tumors, with particular relevance to those originating in mucosal tissues.

Given the innovative therapeutic potential of MAIT cells, further research will be necessary to optimize their performance. Although various expansion methods have been explored, including 5-OP-RU stimulation, sorting of MAIT cells followed by anti-CD3/IL-7/IL-15 stimulation, and activation with IL-12/IL-18, the most effective approach for enhancing their functional properties remains under investigation [65–67]. To unlock the full therapeutic potential of CAR-MAIT cells in the clinic, it is essential to overcome current challenges, including the development of robust and efficient strategies for their genetic engineering. Furthermore, integrating CAR-MAIT cell therapy with immune checkpoint inhibitors, such as anti-PD-1 or anti-LAG3, offers a promising avenue to amplify their clinical impact and pave the way for future cancer treatments.

Genetic modifications of CAR-engineered unconventional T cells in clinical trials

A diverse range of genetic engineering strategies have been used to optimize the efficacy and safety of CAR-engineered conventional T cells for cancer treatment in clinical settings (Figure 2). These strategies encompass the incorporation of cytokines and other immunostimulatory molecules, deletion of MHC molecules to mitigate allorejection, and integration of safety switches. These advances build upon the successful implementation of conventional CAR-T cell therapies and insights gained from preclinical research on CAR-engineered unconventional T cells.

Cytokine engineering

Cytokine engineering is increasingly being utilized in CAR-engineered unconventional T cells in clinical trials to enhance their *in vivo* persistence and antitumor efficacy (Tables 1 and 2). Notably, IL-15 is being tested in at least four CAR-iNKT cell clinical trials, encompassing both autologous and allogeneic cell therapies (NCT03294954, NCT05487651, NCT03774654, and NCT04814004). IL-15 has demonstrated efficacy in improving the *in vivo* performance of CAR-iNKT cells in both preclinical and clinical studies [27,38,68]. In the context of CAR-γδ T cell therapy, IL-2 has been incorporated with a B7-H3-targeting CAR to enhance the antitumor activity of these cells against both hematologic and solid tumors (NCT06372236, NCT05731219, and NCT05722171). Preclinical studies have also shown that coexpression of IL-2 with the B7-H3-targeting CAR resulted in sustained antitumor responses of CAR-Vδ1 T cells both *in vitro* and *in vivo* across multiple solid tumor models [56].

In addition to clinical trials, numerous preclinical studies have explored cytokine engineering in unconventional T cells. For instance, IL-12 has been engineered in CAR-iNKT cells, demonstrating that it reprograms these cells into long-lived, type 1 T helper (Th1)-polarized, CD62L-expressing memory cells with potent antitumor activity [69]. The coexpression of IL-21 enhanced STAT3 signaling, mitigated exhaustion, and prolonged the proliferation of B7H3-targeting CAR-iNKT cells *in vivo*, thereby augmenting their therapeutic efficacy [70]. Another study developed IL-15-engineered mesothelin-targeting CAR-γδ T cells and identified CD16 as a biomarker for enhanced cytotoxicity in the treatment of ovarian cancer [55]. Similarly, GPC3-targeting

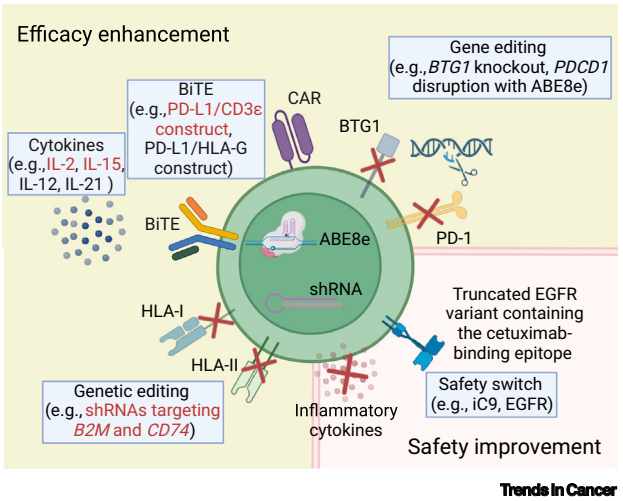

Figure 2. Genetic engineering strategies to enhance the antitumor efficacy and safety of chimeric antigen receptor (CAR)-engineered unconventional T cells. Various strategies have been used to enhance the antitumor efficacy and safety of CAR-engineered unconventional T cells. Key approaches include cytokine engineering, where cytokines such as IL-15 are incorporated to improve cell persistence and antitumor activity. Bispecific T cell engagers (BiTEs) are utilized to redirect therapeutic cells into tumor-implanted tissues, thereby facilitating tumor infiltration. Gene editing for efficacy enhancement involves techniques such as inhibitory gene disruption through techniques such as gene knockout and adenine base editors. Gene-editing strategies, such as knockout of *B2M* and *CD74*, have been used to render allogeneic cells resistant to host cell-mediated allorejection, thereby enhancing the *in vivo* persistence and antitumor efficacy of CAR-engineered cells. In addition, safety switches for safety improvement play a crucial role in mitigating adverse effects. Note that the strategies labeled in red indicate those that have been applied in clinical trials. Abbreviations: ABE8e, an adenine base editor enhanced by eight additional mutations; iC9, inducible caspase-9; shRNA, short hairpin RNA. Figure created with BioRender.

CAR-Vδ1 T cells engineered to express IL-15 effectively inhibited liver cancer progression [57]. Based on advances in cytokine engineering of conventional CAR-T cells, including IL-10, IL-12, IL-15, IL-18, IL-21, and IL-23, these cytokines and their combinations hold potential for application in CAR-engineered unconventional T cells [71–73].

In conclusion, various cytokine engineering strategies have been implemented to enhance the efficacy of CAR-engineered unconventional T cells. Clinical trials have demonstrated the safety and improved persistence of IL-15-engineered CAR-iNKT cells [26,27]. However, another recent clinical trial evaluating IL-15-engineered GPC3-targeting conventional CAR-T cells for HCC reported enhanced antitumor efficacy, but this was accompanied by increased **cytokine release syndrome (CRS)** toxicity [74]. The differential outcomes between conventional CAR-T cells and CAR-iNKT cells may be attributed to the innate-like NK cell properties of CAR-iNKT cells, as well as their potential ability to deplete CRS-associated macrophages [38]. Further investigations will be necessary to identify the optimal cytokine candidates and their compatibility with specific unconventional T cell types. These cytokines should be prioritized for future development and rigorous evaluation in preclinical and clinical settings.

Other immune-enhancement molecules

In addition to cytokine engineering, other immune-enhancement strategies have been applied to the CAR-engineered unconventional T cells in clinical trials, including the incorporation of a locally secreted BiTE into a CAR-γδ T cell platform [46]. In this approach, Vδ2 γδ T cells are engineered via mRNA electroporation to express a nanobody-based HLA-G-targeting CAR and secrete a PD-L1/CD3ε BiTE construct [46]. The BiTE bridges PD-L1 on tumor cells with CD3ε on T cells, thereby recruiting untransduced bystander T cells to amplify the antitumor response [46]. Preclinical studies have demonstrated that these BiTE-secreting γδ T cells can effectively eliminate PD-L1 and HLA-G-positive solid tumors, where the BiTE addresses tumor antigen heterogeneity and enhances immune cell recruitment while minimizing systemic toxicity by restricting its activity to the tumor site [46]. The results from this clinical trial (NCT06150885) have yet to be published.

Recent advances in preclinical studies have also identified numerous immune-enhancement molecules to improve the efficacy and *in vivo* performance of CAR-engineered unconventional T cells. One promising approach involves knocking down *BTG1*, a gene that regulates the transition of T cells from a naïve to an effector state following antigenic stimulation [27]. CAR-NKT cells with *BTG1* knockdown exhibited enhanced IFN-γ production and superior tumor control in a neuroblastoma mouse model, and effectively bolstered antitumor responses while preventing exhaustion, thereby ensuring sustained efficacy both *in vitro* and *in vivo* [27]. Similarly, for γδ T cells, combinatorial gene editing has been utilized to tackle the challenges of limited persistence and durability [75]. Knocking out *SOCS* family genes enabled prolonged cytokine signaling and sustained cytotoxic activity, whereas deletion of proapoptotic genes improved cell survival and increased their resilience in the TME [75]. Armed with CAR constructs targeting tumor-associated antigens, these engineered γδ T cells exhibited robust and long-lasting tumor killing in both B cell- and myeloid-malignancy preclinical models [75]. Additional genetic modifications of γδ T cells have focused on enhancing intrinsic cytolytic activity [76]. Disruption of inhibitory genes such as *PDCD1*, *CISH*, and *FAS* using the **adenine base editor** ABE8e, either alone or in combination, interrupted inhibitory signaling pathways and significantly amplified their antitumor efficacy [76].

Beyond these strategies, advances from conventional CAR-T cell therapy can further inform the development of unconventional T cells [1]. For instance, overexpression of chemokine receptors such as CSF-1R, CCR4, and CCR2b has been shown to enhance CAR-T cell tumor homing and

infiltration [28]. Similarly, transcription factors such as FOXO1 and CJUN have been leveraged to enhance T cell stemness and resistance to exhaustion, respectively [77,78]. Epigenetic reprogramming through SUV39H1 has also demonstrated improved T cell expansion and tumor rejection [79]. In addition, the secretion of autocrine anti-PD-1 antibodies has shown promise in mitigating the suppressive effects of the TME, further enhancing therapeutic efficacy [80]. It is essential to investigate whether the genetic strategies developed for CAR-T cells can also be applied to unconventional T cells to enhance their therapeutic efficacy. Such research could provide valuable insights into the development of next-generation CAR-engineered unconventional T cell therapies.

In conclusion, these genetic engineering strategies have significantly advanced the functionality, persistence, and antitumor potential of CAR-engineered unconventional T cells. Although preclinical findings are encouraging, clinical trials remain crucial to evaluate their safety, durability, and therapeutic efficacy in human patients before these promising approaches can ultimately be translated into effective cancer treatments.

Allo-evasion strategies

Unconventional T cells possess the distinctive ability to maintain a TCR repertoire characterized by low diversity while recognizing non-polymorphic MHC molecules. This unique feature is associated with a reduced risk of inducing GvHD in allogeneic settings. Clinical data from patients undergoing allogeneic hematopoietic stem cell transplantation (alloHSCT) further support this notion because there was a positive correlation between the presence of unconventional T cells and reduced GvHD incidence [32,81–83]. Consequently, numerous clinical trials are investigating the allogeneic potential of CAR-engineered unconventional T cells. However, a key challenge in this context is their susceptibility to allorejection by host immune cells, including T and NK cells [34]. To address this limitation, allo-evasion strategies are being incorporated into clinical trials to enhance the persistence and efficacy of these CAR-engineered unconventional T cells.

A recent clinical trial demonstrated the potential of allogeneic CD19-targeting CAR-iNKT cells in the treatment of patients with relapsed or refractory B cell malignancies (NCT03774654). In this study, researchers engineered CAR-iNKT cells to coexpress IL-15 and shRNAs targeting *B2M* and *CD74* to downregulate the expression of HLA class I and class II molecules, respectively [35]. B2M plays a crucial role in stabilizing and maintaining the surface expression of HLA class I complexes, whereas CD74 is essential for the assembly and trafficking of HLA class II complexes to the cell surface. These engineered CAR-iNKT cells were well tolerated and elicited objective clinical responses in patients with relapsed or refractory non-Hodgkin lymphoma (NHL) or acute lymphoblastic leukemia (ALL), even at low doses [35]. These findings underscore the promise of CAR-iNKT cells as a versatile platform for 'off-the-shelf' cancer immunotherapy

Similarly, a clinical trial investigating iPSC-derived CD19-targeting CAR-NK cells in patients with relapsed or refractory CD19-positive B cell malignancies (NCT05336409) showcased the efficacy of advanced allo-evasion technologies. These CAR-NK cells incorporated *B2M* and *CIITA* knockouts to eliminate MHC class I and II expression, coupled with an HLA-E knock-in to prevent NK cell-mediated rejection [84]. HLA-E, a non-polymorphic MHC molecule, interacts with inhibitory NK cell receptors, thereby shielding the engineered cells from NK-mediated destruction. The trial demonstrated acceptable safety and clinical benefit, and positions iPSC-derived CAR-NK cells as a promising platform for scalable and effective allogeneic therapies [84].

Preclinical studies have further refined allo-evasion strategies to enhance the scalability and efficacy of engineered T and NK cells. One notable study focused on iPSC-derived CAR-γδ T

and CAR-NK cells for autoimmune diseases [85]. These cells were edited to include *B2M* and *CIITA* knockouts to remove MHC class I and II expression, and HLA-E and HLA-G knock-ins to evade immune rejection. HLA-G, an immune checkpoint molecule, provides additional protection from T cell- and NK cell-mediated attacks. In a humanized mouse model, both CAR-γδ T cells and CAR-NK cells achieved near-complete B cell depletion in blood and bone marrow, underscoring their potential as off-the-shelf therapies for autoimmune and hematologic disorders [85]. Another preclinical study addressed the challenges of persistence and immune evasion in iPSC-derived CAR-γδ T cells [75]. These cells were engineered with *B2M* and *CIITA* knockouts to reduce immune surveillance, whereas HLA-E knock-ins minimized NK-mediated rejection. Enhanced signal pathways and gene edits allowed these iPSC-derived CAR-γδ T cells to exhibit prolonged *in vitro* cytotoxicity and sustained *in vivo* tumor clearance in models of B cell and myeloid malignancies, further validating their clinical potential [75]. A third study investigated HSPC-derived BCMA-targeting CAR-NKT cells using a scalable, feeder-free culture method [16,38]. These cells naturally express low levels of HLA-I and undetectable levels of HLA-II, thus minimizing immunogenicity. Additional gene edits via CRISPR-Cas9 complexes with *B2M*/*CIITA* guide RNAs (gRNAs) further ablated HLA-I/II expression and enabled the overexpression of HLA-E, providing robust allo-evasion properties [16]. These advances position HSPC-derived CAR-NKT cells as a scalable, safe, and effective platform for allogeneic cell therapies.

Collectively, these clinical and preclinical advances in allo-evasion strategies underscore the potential of engineered unconventional T cells as scalable and accessible off-the-shelf therapies. By addressing host immune rejection and ensuring consistent cell persistence, these innovations pave the way for next-generation cellular immunotherapies.

Safety switches

Unconventional T cells are generally associated with reduced toxicity, including lower risks of CRS and neurotoxicity [1]. However, the integration of immune-enhancing elements, such as engineered cytokines, may raise potential safety concerns. To address these challenges, safety switches have been incorporated into cell therapy products to mitigate risks and enhance therapeutic safety in preclinical studies [36,38,86]. Among the registered clinical trials utilizing CAR-iNKT or CAR-γδ T cells, none so far have incorporated safety switch mechanisms (Tables 1 and 2). This may be for two primary reasons. First, these unconventional T cells are inherently considered to be safer than conventional T cells and have a lower risk of CRS and neurotoxicity. Second, the majority of clinical trials involving these cells use an allogeneic approach. CAR-iNKT and CAR-γδ T cells do not pose a risk of GvHD and are typically rejected by the host immune system following the therapeutic window, thus minimizing prolonged immune-related risks [87]. Consequently, current designs of CAR-engineered unconventional T cells do not include safety switches. We highlight in the following section key and validated safety switch strategies used in clinical trials of CAR-T and CAR-engineered NK (CAR-NK) cell therapies, particularly those utilizing similar genetic engineering tactics such as IL-15 engineering. We believe that these experiences can be extended to the use of CAR-engineered unconventional T cells in future clinical testing.

A recent clinical trial investigating IL-15-enhanced GPC3-targeting CAR-T cells for HCC incorporated an inducible caspase-9 (iC9) safety switch within the CAR-T cell products. In this trial the iC9 safety switch was activated in three patients who experienced varying severities of adverse events, including CRS accompanied by fever, **tachycardia**, and **tachypnea**. Each patient received a single intravenous dose of **rimiducid**, a chemical inducer of dimerization for iC9, which led to rapid symptom resolution, a significant reduction in circulating CAR-T cells, and normalization of inflammatory cytokine levels [74]. Similarly, an allogeneic IL-15-engineered CAR-NK

cell product derived from iPSCs has been designed with an epidermal growth factor receptor (EGFR) safety switch for the treatment of patients with relapsed, refractory CD19-positive B cell malignancies (NCT05336409). This truncated EGFR variant, which contains the cetuximab-binding epitope, effectively functions as a safety mechanism because it enables the efficient *in vivo* ablation of adoptively transferred cells when necessary [84].

In conclusion, safety switches have been shown to be effective in mitigating potential adverse effects. For CAR-engineered unconventional T cells, particularly those incorporating cytokine modifications such as IL-15, the administration of higher doses in cancer patients may increase the risk of side effects. The implementation of safety switches should be considered in future applications, as needed, to ensure a balance between therapeutic efficacy and safety.

Preconditioning strategies in clinical trials

Lymphodepletion is a crucial preparatory intervention in CAR-based cell therapy because it significantly enhances the therapeutic efficacy of these treatments [88]. By administering chemotherapy or other lymphodepleting agents before the infusion of CAR-engineered cells, this approach reduces the baseline lymphocytes of the patient. This fosters an environment conducive to the engraftment and expansion of the adoptively transferred CAR-engineered cells. In the context of allogeneic cell therapies, lymphodepletion is particularly beneficial by eliminating host T or NK cells that are responsible for graft rejection, thereby improving the *in vivo* persistence and functional longevity of the infused allogeneic CAR-engineered cells [34,87].

Cyclophosphamide and fludarabine are commonly employed lymphodepleting agents in clinical trials utilizing CAR-iNKT or CAR-γδ T cell therapies (Table 3). Cyclophosphamide, an alkylating agent, induces DNA crosslinking, whereas fludarabine, a purine analog, interferes with DNA synthesis, resulting in cell death [89]. For instance, in the autologous GD2-targeting CAR-iNKT cell clinical trial (NCT03294954), patients underwent lymphodepletion with cyclophosphamide (500 mg/m^2 per dose on days −4, −3, and −2) and fludarabine (30 mg/m^2 per dose on days −4 and −3) before receiving CAR-iNKT cell infusion on day 0 [27]. For the conventional CAR-T cell therapy, cyclophosphamide was typically administered intravenously at a dose range of 300–500 mg/m^2 over a 3 day regimen, with lower doses when used in combination with fludarabine. The standard dose of fludarabine was 25–30 mg/m^2 per day, delivered intravenously for 3–5 consecutive days [88]. Other lymphodepleting agents explored in these trials include bendamustine, cytarabine, decitabine, etoposide, and melphalan [90,91]. Although lymphodepletion is essential for augmenting the efficacy of cellular therapies, it is associated with several adverse effects, including an elevated risk of infections as a result of immune suppression, prolonged immunodeficiency necessitating prophylactic interventions, hematologic toxicities such as anemia and leukopenia, and non-hematologic side effects such as nausea and alopecia [88]. Therefore, careful monitoring and management of preconditioning regimens is essential to minimize these risks.

Dosing regimens and strategies in clinical trials

Dosage size

The dosages of CAR-engineered unconventional T cell therapies in clinical trials are determined by the specific cell type and target (Table 3). For GD2-targeting CAR-iNKT cells with IL-15 overexpression, dose levels have ranged from 3 × 10^6 cells/m^2 at the lowest level to 1 × 10^8 cells/m^2 at the highest for treating relapsed or resistant neuroblastoma. Although the overall safety profile of this cell product was favorable, and no severe dose-limiting toxicities were reported, there is room for improvement. Notably, some patients experienced systemic inflammatory responses, manifesting as fever or CRS, highlighting the need for strategies to mitigate these adverse effects

Table 3. Design and manufacturing strategies in the reported examples of clinical trials[a]

Unconventional T cell types	Engineering strategy	Manufacturing strategy	Preconditioning strategy	Dosing regimen and strategy	Clinical results	Year and Refs
CAR-iNKT	GD2-targeting CAR and IL-15 transduction	PBMCs were isolated from patient **apheresis (**leukapheresis) products, and iNKTs were enriched with anti-iNKT microbeads. The iNKTs cells were stimulated with αGC-pulsed PBMCs, cultured with IL-2 and IL-21, transduced with GD2-targeting CAR and IL-15 retroviruses and expanded for 9–15 days. For CAR-iNKT products with insufficient expansion by day 10, restimulation with αGC-pulsed PBMCs was performed	Cyclophosphamide and fludarabine	Dose level 1, 3×10^6 cells/m^2 Dose level 2, 1×10^7 cells/m^2 Dose level 3, 3×10^7 cells/m^2 Dose level 4, 1×10^8 cells/m^2	12 Neuroblastoma patients Efficacy: progressive disease, five patients; SD, four patients; PR, three patients Safety: no acute toxicities or DLTs were observed; grade 3–4 cytopenias related to the lymphodepletion chemotherapy were commonly observed; three patients had fevers of unknown origin, and one patient had grade 2 CRS	2020 [26], 2023 [27]
	CD19-targeting CAR and IL-15 transduction, and shRNA targeting *B2M* and *CD74*	PBMCs were isolated from leukapheresis product of one HLA-unmatched healthy individual. Isolated iNKT cells were transduced with CD19-targeting CAR and IL-15 viruses, as well as with shRNA targeting *B2M* and *CD74*, and expanded for 14 days.	Cyclophosphamide and fludarabine	Dose level 1, 1×10^7 cells/m^2 Dose level 2, 3×10^7 cells/m^2 Dose level 3, 1×10^8 cells/m^2	Nine patients in total (seven NHL and two ALL patients) Efficacy: NHL, PR in one patient; CR in two patients. ALL, CR in one patient Safety: no early adverse events observed; nausea and grade 3–4 hematologic toxicities related to the lymphodepletion chemotherapy were commonly observed; one patient had grade 1 CRS	2024 [35]
	CD70-targeting CAR transduction	Not mentioned	Cyclophosphamide and fludarabine	Dose level 1, 5.0×10^6 cells/m^2 Dose level 2, 1.5×10^7 cells/m^2 Dose level 3: 4.5×10^7 cells/m^2	NA	NCT06182735, NCT06394622 (no dose level 3), NCT06728189

(continued on next page)

Table 3. (continued)

Unconventional T cell types	Engineering strategy	Manufacturing strategy	Preconditioning strategy	Dosing regimen and strategy	Clinical results	Year and Refs
CAR-γδ T	CD20-targeting CAR transduction	The following procedure is described in the preclinical study paper: PBMCs were isolated from apheresis material collected from CMV-negative donors, activated with anti-Vδ1 monoclonal antibody, transduced with a gammaretroviral CD20 CAR construct, and expanded in a Xuri perfusion bioreactor in X-VIVO 15 medium with 10% FBS and IL-2. Post-expansion, αβ T cells were depleted using biotinylated anti-TCRαβ antibodies	Cyclophosphamide and fludarabine	Dose level 1, 3×10^7 cells/m^2 Dose level 2, 1×10^8 cells/m^2 Dose level 3, 3×10^8 cells/m^2	Six patients in total (five with large B cell lymphoma and one mantle cell lymphoma patient) Efficacy: CR in four patients Safety: no GvHD and DLTs observed; one 1 patient had grade 1 CRS, one patient had grade 2 CRS, and one patient had grade 1 ICANS	2022 [92], 2022 [44]
	B7-H3-targeting CAR transduction	Not mentioned	No lymphodepleting chemotherapy	Monthly infusions Dose level 1, 1×10^7 cells/m^2 Dose level 2, 3×10^7 cells/m^2 Dose level 3, 10×10^7 cells/m^2	Seven recurrent glioblastoma patients in total Efficacy: objective response (PR or above), four patients; disease control (SD or above), seven patients Safety: no GvHD and DLTs observed; no patient had grade 3 or higher CRS or ICANS	2024 [93], NCT06018363
	HLA-G-targeting CAR; transduction, and PD-L1/CD3ε BiTE	Not mentioned	Not mentioned	Group 1: low dose for a single administration Group 2: low dose for two administrations over 2 weeks Group 3: low dose for four repeated administrations over 4 weeks Group 4: medium dose for four repeated administrations over 4 weeks Group 5: high dose for four repeated administrations over 4 weeks	NA	NCT06150885

[a]Abbreviations: ALL, acute lymphoblastic leukemia; BiTE, bispecific T cell engager; CMV, cytomegalovirus; CR, complete response; CRS, cytokine release syndrome; DLT, dose-limiting toxicity; FBS, fetal bovine serum; αGC, α-galactosylceramide; GvHD, graft-versus-host disease; ICANS, immune effector cell-associated neurotoxicity syndrome; NA, not available; NHL, non-Hodgkin lymphoma; PBMC, peripheral blood mononuclear cells; PR, partial response; SD, stable disease.

in future applications [26,27]. By contrast, CD19-targeting CAR-iNKT cells, engineered with IL-15 and shRNA targeting *B2M* and *CD74*, were administered to patients with relapsed or refractory B cell malignancies at a narrower dose range of 1×10^7 to 1×10^8 cells/m^2. This therapy also exhibited a favorable safety profile, and only one patient experienced grade 1 CRS. Moreover, it demonstrated encouraging efficacy and achieved complete responses (CRs) in some patients [35].

CAR-γδ T cells have also been used at comparable dosage levels. For example, CD20-targeting CAR-γδ T cells were administered at doses from 3×10^7 to 3×10^8 cells/m^2 to treat B cell malignancies in cell therapy ADI-001 (Table 3). This therapy demonstrated no GvHD or **dose-limiting toxicities (DLTs)**, although some patients experienced mild to moderate CRS and one patient experienced **immune effector cell-associated neurotoxicity syndrome (ICANS)**. Despite these side effects, the therapy showed promising efficacy, and CRs were observed in some patients [44,92]. These dosing strategies highlight the adaptability of CAR-engineered unconventional T cells to optimize therapeutic outcomes while maintaining a favorable safety profile.

Dosing schedules

Clinical trials involving CAR-engineered unconventional T cells implement carefully designed dosing schedules to balance safety and efficacy. Most clinical trials require lymphodepletion using cyclophosphamide/fludarabine (Cy/Flu) before the administration of therapeutic cells (Table 3). In the trial investigating GD2-targeting CAR-iNKT cells, Cy/Flu chemotherapy was administered on days −4, −3, and −2, followed by CAR-NKT cell infusion on day 0 [26,27]. Similarly, Cy/Flu-based lymphodepletion was required in the trial involving CD19-targeting CAR-iNKT cells engineered with IL-15 and shRNA targeting *B2M* and *CD74*, although the specific dosing regimens for this combination were not detailed [35]. In both studies, biopsies of affected sites were performed at comparable timepoints: patients receiving GD2-targeting CAR-iNKT cells underwent core biopsies of a targeted site 2 weeks after infusion, whereas biopsies were collected from those receiving CD19-targeting CAR-iNKT cells 2–5 weeks after infusion. Adverse events were monitored from the start of lymphodepletion until 28 days after infusion and were classified according to the Common Terminology Criteria for Adverse Events. Therapeutic responses were evaluated in both trials at 4 weeks post-infusion. The dosing strategy for CAR-γδ T cells followed a similar pattern to CAR-iNKT cells. In the ADI-001 trial, therapeutic cell administration was followed by a 3 + 3 dose-escalation scheme in which patients received one of three flat dose levels: DL1 (3×10^7 cells), DL2 (1×10^8 cells), or DL3 (3×10^8 cells). Adverse events were closely monitored during the first 28 days post-administration to ensure that the safety profile of this therapy was thoroughly evaluated [92]. These meticulously planned dosing schedules and monitoring protocols underscore the effort in balancing therapeutic efficacy with patient safety in clinical trials of CAR-engineered unconventional T cells. By systematically evaluating adverse events and therapeutic responses, these studies provide crucial insights into optimizing dosing plans and enhancing the clinical utility of these innovative immunotherapies.

Safety and efficacy results from clinical trial examples

Clinical trials utilizing CAR-iNKT cells and CAR-γδ T cells have demonstrated encouraging efficacy and manageable safety profiles compared to conventional CAR-T cell therapies, underscoring their potential as novel cell-based therapies for various malignancies (Table 3). In the autologous GD2-targeting CAR-iNKT cell clinical trial (NCT03294954), 12 neuroblastoma patients were treated, resulting in three cases of partial response (PR), four cases of stable disease (SD), and five cases of disease progression (PD). The therapy was generally well tolerated, and no acute toxicities or DLTs were observed. However, grade 3–4 **cytopenias** related to lymphodepletion chemotherapy were frequently reported. Additional adverse events included fevers of unknown origin in three patients and grade 2 CRS in one patient, which was effectively

managed [26,27]. Similarly, allogeneic CD19-targeting CAR-iNKT cells were evaluated in nine patients, seven with NHL and two with ALL (NCT03774654). In this trial, three NHL patients achieved PR or CR, and one ALL patient achieved a CR. No early adverse events were noted, although nausea and lymphodepletion-related grade 3–4 hematologic toxicities were commonly observed. Grade 1 CRS were reported in one patient [35].

Trials involving CAR-engineered γδ T cells have also yielded promising results. In a study of allogeneic B7-H3-targeting CAR-γδ T cells in seven patients with recurrent glioblastoma (NCT06018363), four patients achieved objective responses (PR or CR), and disease control was observed in all seven patients (SD, PR, or CR). Notably, no GvHD, DLTs, or severe CRS (grade 3 or higher) or ICANS were reported [93]. In addition, in a trial of allogeneic CD20-targeting CAR-γδ T cells involving six patients (five with large B cell lymphoma and one with mantle cell lymphoma), four patients achieved CR (NCT04735471). Although no GvHD or DLTs were reported, mild CRS (grade 1–2) was observed in two patients and grade 1 ICANS was observed in one patient [44,92].

Collectively, these trials demonstrate the therapeutic potential of CAR-engineered iNKT and γδ T cells in both solid and hematologic malignancies. The absence of severe immune-related toxicities such as GvHD or DLT, alongside the observed efficacy in achieving disease control and objective responses, underscores their promise as candidates for further clinical development. Notably, the lack of severe GvHD in allogeneic trials highlights the suitability of NKT and γδ T cells as sources for allogeneic cell therapy. These results emphasize their potential to address existing challenges in adoptive cellular therapy, particularly in the context of allogeneic approaches.

Concluding remarks and future perspectives

CAR-engineered unconventional T cell therapy represents an emerging frontier in cancer immunotherapy that offers distinct efficacy and safety advantages compared to the conventional CAR-T cell therapy (Box 1). Despite the growing number of clinical trials investigating CAR-iNKT and CAR-γδ T cells, there remains a significant gap in the comprehensive evaluation of their safety, efficacy, manufacturing methodologies, and patient treatment protocols. This review discusses data from 28 registered clinical trials focusing on CAR-iNKT and CAR-γδ T cells (Tables 1 and 2) and the preliminary findings from six reported studies (Table 3). It also highlights distinct manufacturing strategies for each type of CAR-engineered unconventional T cell used in clinical trials (Box 2). To advance the development of CAR-engineered unconventional T cell therapies, key questions must be addressed in future research and clinical applications (see Outstanding questions).

First, the manufacturing process for unconventional T cell-based therapies must be standardized and optimized. Unlike conventional T cells, which are present in large quantities in cancer patients, unconventional T cells constitute only a minor subset. This challenge is exacerbated in cancer patients who have undergone pretreatments such as chemotherapy, resulting in insufficient numbers of unconventional T cells for CAR engineering and subsequent expansion. Hence, alternative strategies are crucially needed. Stem cell engineering offers a promising solution by utilizing stem cell differentiation and genetic modification to produce large quantities of unconventional T cells. Notably, a recent study introduced a clinically guided culture method that enables the generation of allogeneic CAR-iNKT cells from HSPCs, and has demonstrated enhanced antitumor efficacy and safety profiles [16,38]. In addition, advances in iPSC technology have shown potential to revolutionize cancer immunotherapy by providing a scalable and versatile source for generating engineered therapeutic cells [39,94–96].

Second, distinct disease indications should be matched with specific unconventional T cell subsets based on their biodistribution and unique biological properties. For instance, MAIT

Outstanding questions

How do CAR-engineered unconventional T cells compare to conventional CAR-T cells in terms of targeting solid tumors, especially those with immunosuppressive microenvironments?

What strategies can optimize antigen specificity to reduce off-target effects while maintaining robust efficacy in diverse tumor settings?

What are the factors that influence the *in vivo* persistence and exhaustion of CAR-engineered unconventional T cells, and how can they be addressed?

Can modifications to CAR constructs or engineering approaches enhance the long-term durability of these cells?

What are the challenges in developing off-the-shelf allogeneic CAR-engineered unconventional T cells, and how can immune rejection be minimized?

How can genome-editing techniques improve the compatibility and functionality of these cells in allogeneic settings?

What is the potential for combining CAR-engineered unconventional T cells with checkpoint inhibitors, cytokine therapies, or vaccines? How can these combinations be tailored to enhance therapeutic outcomes while reducing adverse events?

What are the current bottlenecks in translating preclinical successes with unconventional CAR-T cells to clinical trials?

How do the manufacturing challenges for unconventional T cells differ from those for conventional T cells, and what innovations are needed to overcome these?

How will regulatory frameworks need to adapt to accommodate the unique aspects of unconventional CAR-T cell therapy? What preclinical data will be essential to ensure the safety and efficacy of these therapies before clinical trials?

Which subsets of unconventional T cells (e.g., γδ T cells, iNKT cells, MAIT cells) are most suited for specific cancer types, and how can subset-

Box 2. Manufacturing strategies in clinical trials

Compared to the current manufacturing protocols for conventional CAR-T cells that primarily rely on anti-CD3/CD28 antibodies, the generation of CAR-iNKT and CAR-γδ T cells demonstrates greater versatility. Various strategies have been developed to achieve high-yield production of these cell types. These include the use of anti-CD3/CD28 antibodies, artificial antigen-presenting cells (aAPCs), specific TCR agonists such as αGC for selectively stimulating CAR-iNKT cells, and zoledronate for selectively stimulating CAR-γδ T cells [105,106].

In the reported clinical trial utilizing autologous GD2-targeting CAR-iNKT cells (NCT03294954), the manufacturing protocol involved several key steps [27]. First, iNKT cells were isolated from cancer patient PBMCs using anti-iNKT microbeads. These isolated iNKT cells were then stimulated with αGC-pulsed PBMCs and cultured in the presence of human IL-2 and IL-21. Following stimulation, the cells were transduced with retroviral vectors encoding the GD2-CAR.15 construct, and cultured for 9–15 days. For products that did not achieve sufficient expansion by day 10, restimulation with αGC-pulsed PBMCs was performed to facilitate further expansion, thereby ensuring the necessary cell numbers for the assigned dose level were reached [27].

In the clinical trial involving allogeneic CD20-targeting CAR-γδ T cells (NCT04735471), the manufacturing process followed a detailed protocol as described in their preclinical study [44]. PBMCs were obtained from apheresis material sourced from cytomegalovirus (CMV)-negative donors and activated using an agonistic anti-Vδ1 monoclonal antibody, followed by transduction with a gammaretroviral vector encoding the CD20 CAR construct. The transduced cells were then expanded using X-VIVO 15 medium supplemented with IL-2. After expansion, αβ T cells were selectively depleted using biotinylated anti-TCRαβ antibodies to generate a highly enriched population of CAR-γδ T cells for clinical application [44].

Preclinical studies have extensively reported various manufacturing strategies to achieve clinical-scale production of CAR-engineered unconventional T cells. A recent study described a clinically guided culture method for generating allogeneic CAR-iNKT cells with high yield and purity [38]. In this approach, cord blood-derived $CD34^+$ hematopoietic stem and progenitor cells (HSPCs) were genetically engineered to express an iNKT TCR and subsequently cultured in a feeder-free system for six weeks. This method facilitated the differentiation of HSPCs into CAR-iNKT cells. For CAR-γδ T cells, various strategies have also been developed to optimize their expansion. These include the use of anti-CD2 monoclonal antibodies [107], **concanavalin A** [108], zoledronate [55], and aAPCs [109]. In summary, these optimized protocols suggest that clinical-scale production of CAR-engineered unconventional T cells is achievable, and this provides a promising foundation for their application in cancer immunotherapy.

specific advantages be exploited? Are there underexplored unconventional T cell subsets that hold promise for CAR engineering?

What biomarkers can predict the efficacy or safety of CAR-engineered unconventional T cells in individual patients?

cells constitute ~40% of the total T cell population in healthy and malignant liver tissues [62,97], reflecting their natural homing capacity and functional relevance within the liver microenvironment. This suggests that CAR-MAIT cells could be particularly suited for targeting liver cancers. In addition, the high expression of CD1d molecules on myeloid malignancies highlights the potential of CAR-iNKT cells for these cancers [98] because they can engage tumor cells via a CAR/TCR/NKR triple-targeting mechanism. Although CAR-T cells have demonstrated significant efficacy in treating hematologic malignancies, CAR-engineered unconventional T cells offer distinct advantages for addressing solid tumors (Box 1). Nevertheless, rigorous evaluation will be necessary to determine the optimal pairing of cell types with specific cancer indications.

Third, combination therapies should be explored for CAR-engineered unconventional T cell therapies. Strategies such as checkpoint blockade, cancer vaccines, bispecific antibodies (e.g., BiTEs), and oncolytic viruses have the potential to enhance the efficacy of CAR-engineered unconventional T cell therapies [99]. The feasibility and therapeutic benefit of these approaches should be investigated in preclinical studies and carefully evaluated in clinical trials to assess their clinical applicability.

In conclusion, although CAR-engineered unconventional T cells are currently in the early phases of clinical development, their favorable safety profile and promising therapeutic efficacy, particularly in solid tumors, underscore their potential as a transformative approach to cancer immunotherapy. These cells also exhibit the capacity for broader applicability, extending to various malignancies as well as to autoimmune disorders [100–102]. Continued research, technological innovation, and clinical testing will be essential for refining these therapies and unlocking their full potential as a next-generation immunotherapy strategy.

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Declaration of interests

L.Y. is a scientific advisor to AlzChem and Amberstone Biosciences, and a cofounder, stockholder and advisory board member of Appia Bio. None of the declared companies contributed to this study, and Appia Bio did not provide any input regarding this study. The other authors declare no competing interests.

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