Targeting Lentiviral Vectors to Antigen-Specific Immunoglobulins

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Abstract

Gene transfer into B cells by lentivectors can provide an alternative approach to managing B lymphocyte malignancies and autoreactive B cell-mediated autoimmune diseases. These pathogenic B cell populations can be distinguished by their surface expression of monospecific immunoglobulin. Development of a novel vector system to deliver genes to these specific B cells could improve the safety and efficacy of gene therapy. We have developed an efficient method to target lentivectors to monospecific immunoglobulin-expressing cells *in vitro* and *in vivo*. We were able to incorporate a model antigen CD20 and a fusogenic protein derived from the Sindbis virus as two distinct molecules into the lentiviral surface. This engineered vector could specifically bind to cells expressing surface immunoglobulin recognizing CD20 (αCD20), resulting in efficient transduction of target cells in a cognate antigen-dependent manner *in vitro*, and *in vivo* in a xenografted tumor model. Tumor suppression was observed *in vivo*, using the engineered lentivector to deliver a suicide gene to a xenografted tumor expressing αCD20. These results show the feasibility of engineering lentivectors to target immunoglobulin-specific cells to deliver a therapeutic effect. Such targeting lentivectors also could potentially be used to genetically mark antigen-specific B cells *in vivo* to study their B cell biology.

Introduction

GENETIC REARRANGEMENT in B cells allows for the immense diversity of immunoglobulin. Individual B cell clones differ from one another because of the variable sequences within the antigen-binding site of the expressed immunoglobulin (Buhl *et al.*, 2002). Thus, surface immunoglobulin can serve as a unique molecular determinant to differentiate between clonal populations of B cells that can be targeted for gene therapy delivering either a toxic gene to eliminate specific B cells, or modulating genes to enhance the function of specific B cell populations. In fact, targeting specific immunoglobulin on B cells by protein-based delivery of either unconjugated or conjugated anti-idiotypic antibodies has been explored clinically to treat autoimmune B cells as well as cancerous B cells (Rankin *et al.*, 1985; Maloney *et al.*, 1992; Davis *et al.*, 1998). Unconjugated anti-idiotypic antibodies could cross-link surface immunoglobulin to initiate tyrosine kinase signaling, resulting in stimulation of B cell apoptosis (Vuist *et al.*, 1994; Reff *et al.*, 2002). Conjugation with toxins could be used to further increase the toxic effects of anti-idiotypic antibodies (Reff *et al.*, 2002).

Despite considerable progress in treating malignancies of the hematolymphoid system, new treatment modalities are required to treat patients more efficaciously and to target malignant cells more accurately. In addition, some types of B cell malignancies remain refractory to current treatment regimens (Moskowitz, 2006). Thus, the development of novel approaches, such as gene therapy, could contribute to the ability to manage these diseases (Wierda and Kipps, 2000). Lentivectors represent one of the most effective delivery vehicles for transferring genes to B cells, because of their ability to transduce not only highly proliferative cells but also quiescent cells and poorly proliferative cells such as those of an indolent B cell neoplasm, for example, B cell chronic lymphocytic leukemia (Bovia *et al.*, 2003; Janssens *et al.*, 2003). In the context of successful gene transfer into B cell neoplasms, the transduction of precursor B-lymphoblastic leukemia cells with lentiviral vectors to deliver cytokine stimulator, granulocyte-macrophage colony-stimulating factor (GM-CSF), along with an immune-modulating molecule, CD80, has been shown to elicit marked antileukemia immune responses, indicating the potential use of these vectors for treating B cell tumors (Stripecke *et al.*, 2000).

Most investigations using lentiviral vectors to deliver genes to B cells focus on using ecotropic, amphotropic, or vesicular stomatitis virus glycoprotein (VSVG) to envelope human immunodeficiency virus (HIV)-derived lentiviral vectors (Janssens *et al.*, 2003). Because of the broad tropisms of these pseudotyped vectors, prepurification of B cells *in*

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vitro, using B cell-specific markers such as CD19, is required before transduction. The development of methods to direct lentivectors to B cells to achieve cell type-specific transduction could enable *in vivo* delivery of genes to B cells. Although few attempts have been made to engineer lentivectors to achieve B cell-specific transduction (Mailly *et al.*, 2006), several efforts have been reported to incorporate B cell-specific promoters/enhancers into lentivectors to achieve B cell-lineage-specific expression of transgenes (Logan *et al.*, 2002; Lutzko *et al.*, 2003; Moreau *et al.*, 2004).

As opposed to transcriptional targeting, we were interested in developing transductional targeting of lentivectors. Various strategies have been attempted to direct gammaretroviral and lentiviral vectors to transduce specific cell types (Lavillette *et al.*, 2001; Sandrin *et al.*, 2003; Verhoeyen and Cosset, 2004; Yu and Schaffer, 2005). We have previously reported a method to target lentivectors to B cells via CD20 (Yang *et al.*, 2006). The method involves the incorporation of a surface antibody specific to CD20 and a fusogenic protein as two distinct molecules into the lentiviral surface. We have shown that this targeting method allows for efficient and specific delivery of genes to human B cells *in vitro* and *in vivo* (Yang *et al.*, 2006). In this paper, we engineer lentivectors capable of specifically transducing immunoglobulin-specific cells. Using anti-CD20 (αCD20) surface immunoglobulin as a model target, we demonstrate that targeting lentivectors displaying a cognate antigen and a fusogenic protein can deliver genes to cells expressing specific surface immunoglobulin both *in vitro* and *in vivo* with remarkable specificity and efficiency. This result also expands our targeting methodology and presents a first example of targeting lentivectors to immunoglobulin-specific cells.

Materials and Methods

Mice

Nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice (Jackson Laboratory, Bar Harbor, ME) were maintained in the animal facility of the University of Southern California (Los Angeles, CA) and cared for in accordance with institutional regulations. Treated mice were maintained on a mixed antibiotic (sulfamethoxazole and trimethoprim oral suspension; Hi-Tech Pharmacal, Amityville, NY).

Antibodies and flow cytometric analysis

The antibodies used in this study were phycoerythrin–cyanine 5 (PE–Cy5)-conjugated anti-human CD20, PE–Cy5-conjugated anti-human IgG, streptavidin–PE (all from BD Biosciences, San Jose, CA), and biotin-conjugated anti-hemagglutinin (HA) (Miltenyi Biotec, Bergisch Gladbach, Germany). Flow cytometric analysis was performed with a FACScan (BD Biosciences) and analyzed with FlowJo software (Tree Star, Ashland, OR).

Lentivector plasmids

Lentivector plasmids used in this study were FUGW (Lois *et al.*, 2002), FUW, FUWLuc, and FUWSR39tk. FUW is a self-inactivating third-generation lentivector. To make FUWLuc and FUWSR39tk, firefly luciferase cDNA amplified from pGL4.12Luc2P (Promega, Madison, WI) or SR39tk (Black *et*

al., 2001), respectively, was cloned downstream of the human ubiquitin-C promoter in the lentivector plasmid FUW.

Viral vector production

Human CD20 cDNA was cloned downstream of the cytomegalovirus (CMV) promoter in pcDNA3 (Invitrogen, Carlsbad, CA) to create pCD20. Lentivectors were generated by calcium phosphate precipitation transfection of 293T cells. 293T cells were transfected with the appropriate lentivector plasmid (5 μg), together with 2.5 μg each of pCD20, pSINmu (Yang *et al.*, 2006), and the packaging vector plasmids pMDLg/pRRE and pRSV-Rev (Dull *et al.*, 1998) when 80% confluent in 6-cm culture dishes. The viral supernatants were harvested 36 and 48 hr after transfection and filtered through a 0.45-μm pore size filter (Nalgene, Lima, OH). Titering was done by serial dilution of the virus on 293T or 293T/αCD20 cells with green fluorescent protein (GFP)-expressing virus. Flow cytometry was used to determine the linear range of infection as measured by GFP expression. For animal experiments, viral vectors were concentrated by ultracentrifugation as described previously (Yang *et al.*, 2006).

Cell line construction

To construct cell lines expressing CD20-specific surface immunoglobulin, we amplified cDNAs of the light chain (αCD20L) and the heavy chain (αCD20H) of mouse–human chimeric antibody (clone 2H7) from plasmid pαCD20 (Yang *et al.*, 2006) and cloned them into FUW to generate FUWαCD20L and FUWαCD20H. The cDNAs of human B cell coreceptor, Igα and Igβ, were cloned into FUW to yield FUWIgα and FUWIgβ. VSVG-pseudotyped viruses were prepared for FUWαCD20L, FUWαCD20H, FUWIgα, and FUWIgβ. These viruses were used to cotransduce target cells. After a few passages, the resulting cells were stained with anti-human IgG1 antibody and subjected to cell sorting to obtain a uniform population of αCD20$^+$ cells designated 293T/αCD20 or Jurkat/αCD20. To make Jurkat/αCD20Luc, FUWLuc was pseudotyped with VSVG and used to transduce Jurkat/αCD20 cells.

Targeted transduction of cell lines *in vitro*

293T/αCD20, 293T, Jurkat/αCD20, or Jurkat cells (0.1 $\times$ 10^6) were plated with 2 ml of viral supernatant in a 24-well tissue culture dish (BD Falcon; BD Biosciences). Cells and supernatant were incubated for 8 hr at 37°C, 5% CO$_2$. Cells were then cultured for 4 days in fresh medium at 37°C, 5% CO$_2$. The percentage of GFP$^+$ cells was determined by flow cytometry.

Effects of soluble antibody and NH$_4$Cl on viral transduction

293T/αCD20 cells (0.1 $\times$ 10^6) and 1 ml of viral supernatant (FUGW/CD20+SINmu) were coincubated in the presence of soluble anti-human CD20 (clone 2H7; BD Biosciences), an isotype control [IgG2b(κ); BD Biosciences], or NH$_4$Cl (Fisher Scientific, Waltham, MA). The medium was replaced 8 hr later with fresh medium and incubated for another 4 days at 37°C, 5% CO$_2$. GFP expression was analyzed by flow cytometry.

Imaging for virus and viral cell binding

For labeling αCD20 expression on the cell surface, 293T and 293T/αCD20 cells preincubated in a 35-mm glass-bottom culture dish (MatTek, Ashland, MA) were fixed with 4% formaldehyde and immunostained with Alexa Fluor 594-labeled goat anti-human IgG (Invitrogen). These samples were also treated by 4',6-diamidino-2-phenylindole (DAPI) nuclear staining. For detection of individual viral particles, fresh viral supernatants were overlaid on polylysine-coated coverslips and centrifuged at $3700 \times g$ at 4°C for 2 hr. The coverslips were rinsed with cold phosphate-buffered saline (PBS) twice, and adherent virus was immunostained with Alexa Fluor 647 anti-human CD20 (Biolegend, San Diego, CA) and anti-HA–biotin (Miltenyi Biotec) followed by secondary staining with Texas red–streptavidin (Zymed Laboratories, South San Francisco, CA). The coverslips were then mounted in VECTASHIELD (Vector Laboratories, Burlingame, CA). For imaging viral cell binding, 5×10^5 cells were seeded into a 35-mm glass-bottom culture dish and grown at 37°C overnight. The seeded cells were rinsed with cold PBS twice and incubated with concentrated virus for 1 hr at 4°C to allow binding but inhibit endocytosis. Cells were washed with cold PBS to remove unbound virus, fixed, and then imaged in PBS. Fluorescence images were taken with a Zeiss LSM 510 laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany). A Plan-Apochromat $\times 63$/1.4 oil immersion objective was used for imaging. Images were analyzed with the use of Zeiss LSM Image Browser software.

Targeted transduction of cell lines in vivo

Mice were anesthetized with 2.5% isoflurane (Abbott Animal Health, Abbott Park, IL) and injected subcutaneously via the right flank with 10×10^6 Jurkat/αCD20 cells. Eight hours later the mice were injected subcutaneously via both flanks with concentrated lentivector expressing firefly luciferase (FUWLuc/CD20+SINmu or FUWLuc/VSVG). To analyze targeting efficiency, mice were anesthetized and 3 mg of D-luciferin (Xenogen/Caliper Life Sciences, Hopkinton, MA) in PBS was injected intraperitoneally. After 4 min, the mice were imaged with an IVIS 200 system (Xenogen/Caliper Life Sciences). Images were analyzed with Living Image 2.50.1 software (Xenogen/Caliper Life Sciences). This experiment was performed in triplicate.

Suicide gene delivery in vivo

Mice were anesthetized and injected subcutaneously via the right flank with 5×10^6 Jurkat/αCD20Luc cells. Eight hours later the mice were injected subcutaneously with concentrated lentivector (multiplicity of infection [MOI] of 10) expressing herpes simplex virus-1 thymidine kinase mutant SR39tk (FUWSR39tk/CD20+SINmu or FUWSR39tk/VSVG) or no virus as a control on the right flank. On days 7 through 12, mice were injected intraperitoneally daily with the prodrug ganciclovir (GCV, 50 mg/kg; Sigma-Aldrich, St. Louis, MO). To analyze tumor size, mice were imaged with the IVIS 200 imaging system as described previously. This experiment was performed in triplicate.

Results

General targeting scheme and design of a functional lentivector envelope

The two necessary functions of the lentiviral envelope protein include binding to the host cell and fusion of the viral membrane with the cell membrane to release the contents of the lentivirus into the cytoplasm (Dimitrov, 2004). By separating these functions into two separate proteins on a gammaretroviral or lentiviral surface, cell-specific targeting can be achieved while maintaining viral titers (Lin et al., 2001; Chandrashekran et al., 2004; Yang et al., 2006). There are several features to consider in order to create an effective targeting lentiviral particle by this strategy. First, the binding of the lentivectors to the cell should induce endocytosis, and must not interfere with fusion. Second, the fusion molecule should be incorporated into the surface of the lentivector, should not interfere with binding, and should induce fusion at low pH. We set out to test whether this general method could be extended to target B cells expressing a specific surface immunoglobulin. We chose surface immunoglobulin specific to CD20 as the molecular target and designed an experiment to evaluate whether CD20 antigen could be incorporated on the viral surface to target cells expressing its cognate αCD20 surface immunoglobulin (Fig. 1). Immunoglobulins on B cells are known to endocytose when bound to an antigen, an anti-idiotypic antibody, or an anti-immunoglobulin antibody (Drake et al., 1989), making them a good target for lentivectors in accordance with the intended targeting method. For fusion, we chose the glycoprotein derived from Sindbis virus (denoted as SIN), which has been shown to be able to efficiently pseudotype HIV-1-derived lentiviral vectors. SIN contains two transmembrane proteins (E1, responsible for fusion; and E2, responsible for binding), which form a heterodimer when displayed on the viral surface (Phinney et al., 2000). By making mutations in the E2 protein to inactivate the receptor-binding sites, we and other have shown that SIN can be engineered into a binding-deficient but fusion-functional form (termed SINmu) (Morizono et al., 2005; Yang et al., 2006).

Preparation of recombinant lentivectors

To produce the recombinant lentivectors, 293T cells were transiently cotransfected by the standard calcium phosphate method (Pear et al., 1993) with a self-inactivating and replication-incompetent lentiviral backbone that contains a human ubiquitin-C promoter driving the expression of a GFP reporter gene, FUGW (Fig. 2A) (Lois et al., 2002); plasmids encoding viral *gag*, *pol*, and *rev* genes; the plasmid encoding human CD20 protein (denoted as pCD20); and the plasmid encoding the fusogenic protein SINmu (denoted as pSINmu). The resultant viral vector from these transfected cells was designated FUGW/CD20+SINmu. Transfection without the plasmid encoding either CD20 or SINmu was performed to generate viral vectors FUGW/SINmu and FUGW/CD20, respectively, which were used as controls. As a positive control we generated a lentivector pseudotyped with VSVG and designated it FUGW/VSVG. Virus-producing cells were analyzed 3 days posttransfection by fluorescence-activated cell sorting (FACS) analysis (Fig. 2B and C). GFP expression was observed in all transfected cells (Fig.
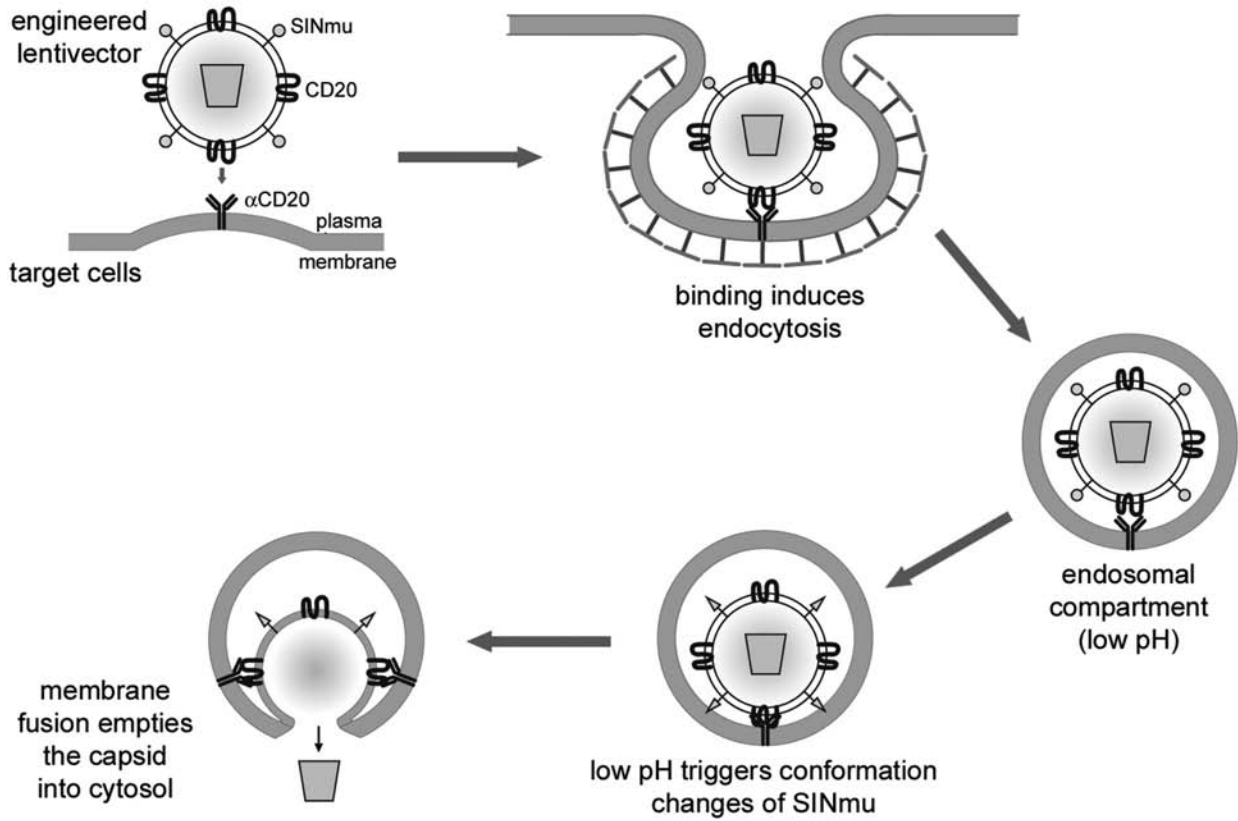
FIG. 1. Schematic diagram of the general targeting strategy used for this study. A lentivector is engineered to codisplay a binding determinant (CD20 antigen) and a fusogenic protein (SINmu). Specific binding of CD20 on the viral surface to the surface αCD20 antibody expressed on the target cells induces endocytosis into the endosomal compartment. In the endosomal compartment, low pH triggers conformational changes of the pH-sensitive fusogen SINmu into a fusion-active form. The activated SINmu then mediates the fusion of viral and endosomal membrane to empty the viral capsid into the cytosol.
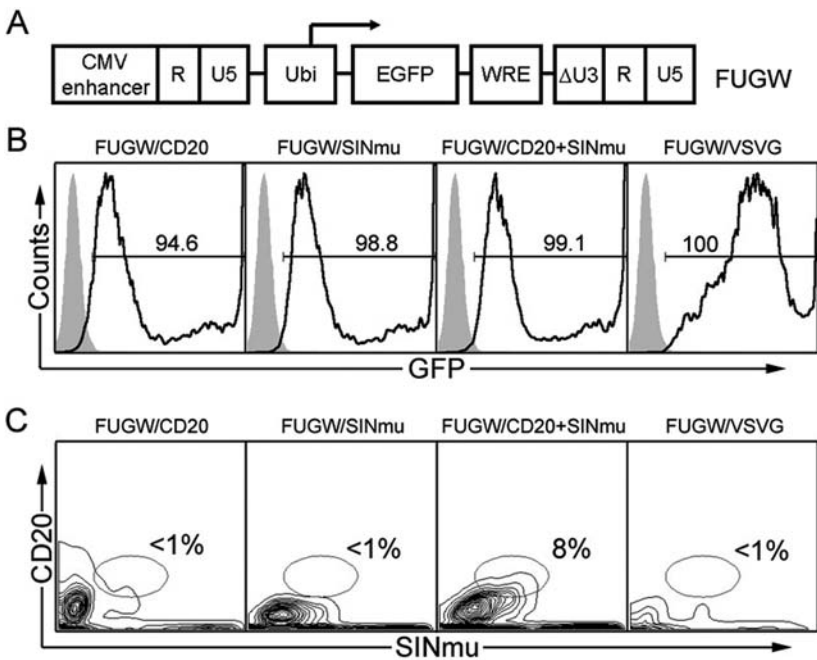

FIG. 2. Coexpression of binding molecule CD20, and fusogenic molecule SINmu, on the surface of virus-producing cells. 293T cells were transiently transfected with plasmids encoding lentivector FUGW, binding protein CD20, and fusogen SINmu, along with other standard packaging plasmids. Three days later, the transfected cells were detected with αCD20 antibody to stain for the binding protein and anti-HA tag to stain for the fusogen. (**A**) Schematic representation of the lentiviral backbone FUGW. Ubi, human ubiquitin-C promoter; EGFP, enhanced green fluorescent protein; WRE, woodchuck regulatory element; ΔU3, deleted U3 region that results in the transcriptional inactivation of the integrated viral LTR promoter. (**B**) Flow cytometric analysis of GFP expression in packaging cells. Shaded areas, cells without exposure to virus; solid line, cells exposed to the indicated viral vector. (**C**) Gating on GFP-positive cells; coexpression of CD20 and SINmu is shown.

2B), indicating the presence of lentiviral backbone in virus-producing cells. When we gated on GFP+ cells, we found that approximately 8% of 293T cells coexpressed CD20 and SINmu, which presumably were able to produce FUGW/CD20+SINmu (Fig. 2C). The apparently low percentage of coexpression is partially due to the insensitivity of anti-CD20 staining antibody, which can detect only highly expressed CD20.

Coincorporation of fusogen and binding protein into lentivectors

Because both binding and fusion are required for lentivectors to productively transduce target cells, it was important to investigate whether the engineered viral particles express both CD20 and SINmu in a single virion. We designed a confocal imaging experiment to examine this question. We constructed a plasmid that expresses GFP fused to the N terminus of HIV-1 Vpr (designated as GFP–Vpr). It has been shown that GFP–Vpr was incorporated into the virion when the GFP–Vpr plasmid was supplied *in trans* during viral preparation; the resulting virus was labeled by GFP and could be detected by green fluorescence (McDonald *et al.*, 2002). We prepared GFP-marked recombinant viral vector FUW-GFPvpr/CD20+SINmu under similar transfection conditions as were used to make FUGW/CD20+SINmu, except that the lentiviral backbone FUW lacking the GFP transgene replaced FUGW, and an additional plasmid encoding GFP–Vpr was used. The resultant viral supernatant was exposed to a clean glass coverslip. Costaining experiments
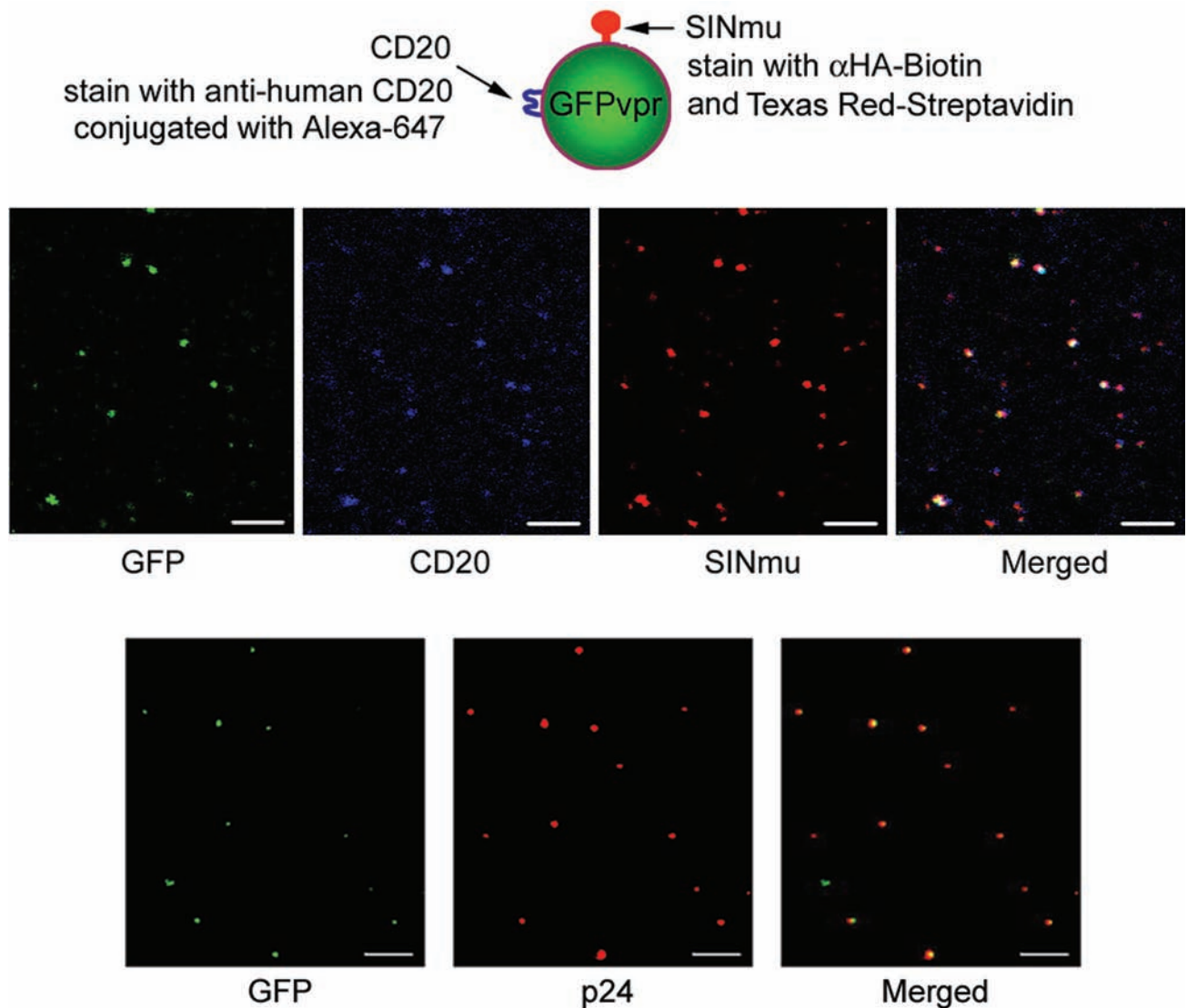

FIG. 3. Confocal imaging of engineered lentiviral particles. Viral supernatant collected from virus-producing cells transfected with GFP–Vpr, CD20, SINmu, lentiviral backbone FUW, and other necessary constructs was deposited on a coverslip precoated with polylysine by centrifugation. The resultant coverslip was rinsed with PBS and stained for CD20 with an Alexa Fluor 647-conjugated anti-human CD20 antibody (blue) and for SINmu with a biotinylated anti-HA tag antibody and Texas red-conjugated streptavidin (red). GFP-positive particles that had both CD20 and SINmu appeared to be white after merging. Viral particles were also stained with a mouse anti-p24 antibody and Texas red-conjugated anti-mouse antibody. Scale bars: 2 μm.

showed that ?40% of GFP-marked viral particles were positive for both CD20 and SINmu (Fig. 3, top). The colocalization of GFP–Vpr, CD20, and SINmu suggested that our method of vector preparation can produce a good fraction of viral particles displaying both binding and fusogenic molecules. We further stained the GFP–Vpr-containing particles (FUW-GFPvpr/CD20+SINmu) with an anti-p24 antibody. It showed that >80% of GFP$^+$ particles were p24$^+$ (Fig. 3, bottom), suggesting that the dots we detected were the true viral particles.

Binding of viral particles to specific cells

To facilitate our study of specific binding and transduction using engineered lentivectors, we made 293T and Jurkat cell lines expressing αCD20 by lentivirus-mediated transduction and cell sorting (293T/αCD20 cells, Fig. 4A and C; Jurkat/αCD20 cells, Fig. 4B). Flow cytometric analysis showed that the resultant αCD20 cell lines had uniform expression of αCD20 (Fig. 4A and B). We compared the human IgG expression level between the cell lines and human peripheral blood mononuclear cells (PBMCs) and found that Jurkat/αCD20 expressed a physiologically relevant level of human IgG (lower than the IgG expression in human B cells) (Fig. 4B). To confirm that CD20 displayed on the viral surface was able to maintain its binding specificity, we coincubated the concentrated viral particles (FUW-GFPvpr/CD20+SINmu) with 293T/αCD20 or 293T cells at 4°C for 1 hr. At this temperature, viral particle internalization is reduced. As shown in Fig. 4D, GFP-marked virions were detected on the surface of 293T/αCD20 cells, whereas no detectable virus was seen on the surface of 293T cells. As a control, incubation of either 293T or 293T/αCD20 cells with viral supernatants harvested from producing cells transfected with plasmids devoid of the plasmid encoding Gag/Pol resulted in no bound particles. Addition of soluble anti-CD20 antibody was observed to reduce the number of virions bound to 293T/CD20 cells (data not shown). Thus, CD20 incorporated into the lentiviral particles can retain specific binding to αCD20-expressing cells.

Targeted transduction of cell lines in vitro

To test the ability of the engineered recombinant lentivectors to transduce only cells expressing the selected surface immunoglobulin, the supernatants from cells transfected to produce FUGW/CD20+SINmu, FUGW/CD20, FUGW/SINmu, or FUGW/VSVG were harvested and incubated with stable cell lines expressing the target immunoglobulin (293T/αCD20 and Jurkat/αCD20). The parental cell lines
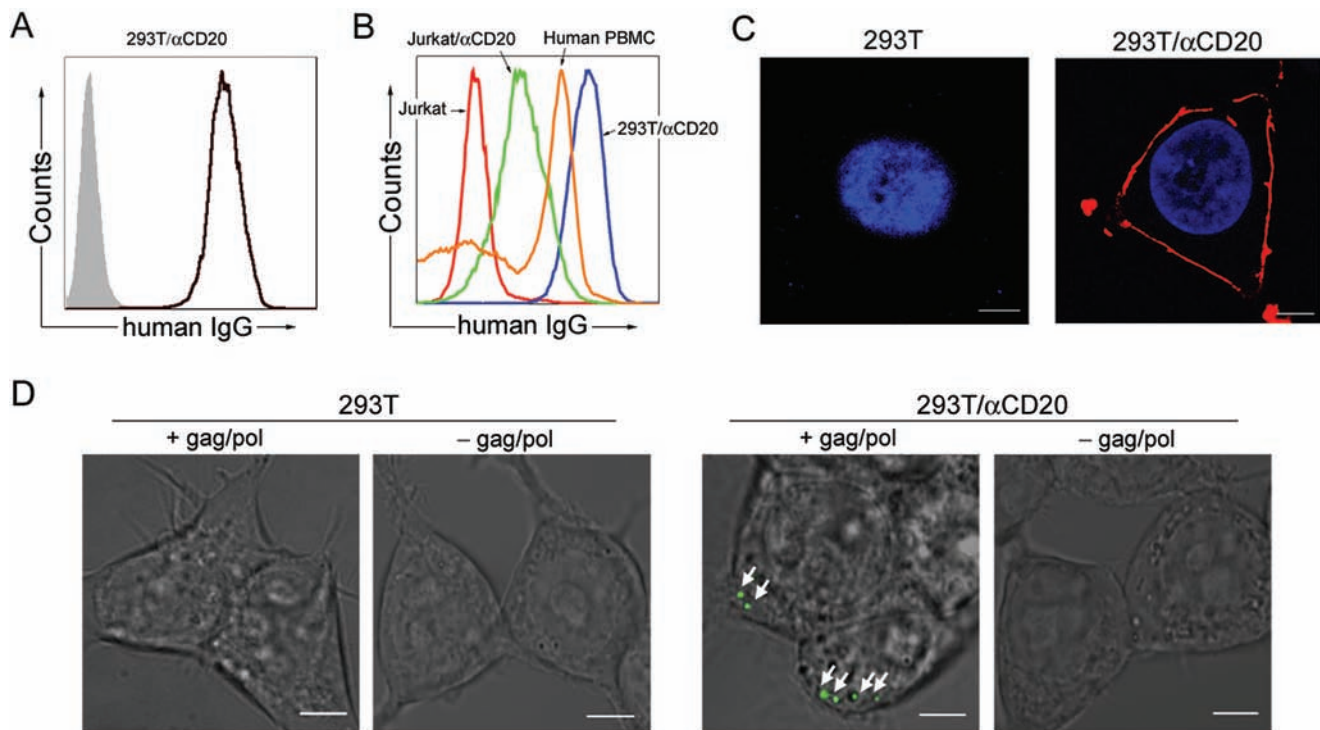

FIG. 4. Confocal imaging of the engineered lentivector bound to target cells. (**A**) Expression of αCD20 on the 293T/αCD20 cell line (solid line) compared with the parental 293T cells (shaded area) analyzed by flow cytometric staining with an anti-human IgG antibody. (**B**) Expression of IgG on the Jurkat/αCD20 cell line (green) compared with the indicated cells (Jurkat [red], 293T/αCD20 [blue], or human peripheral blood mononuclear cells [PBMCs] [orange]). (**C**) 293T/αCD20 cell line expressing αCD20. The nuclei of fixed 293T and 293T/αCD20 cells were labeled with DAPI. Cells were subsequently stained with Alexa Fluor 594-conjugated goat anti-human IgG antibody. Scale bars: 5 μm. (**D**) Binding of virus to target cell. 293T/αCD20 and 293T cells were incubated at 4°C with virus harvested from producing cells transfected with the plasmids necessary to produce FUW-GFPvpr/CD20+SINmu (labeled as "+ gag/pol"). Cells incubated with viral supernatants harvested from producing cells transfected with all the plasmids expect for gag/pol (labeled as "– gag/pol") were included as a control. GFP-positive viral particles are shown in green. Scale bars: 5 μm.

(293T and Jurkat), negative for expression of the target antibody, were used as controls. Four days posttransduction, GFP expression, which constituted a sign of positive transduction, was analyzed by flow cytometry. Figure 5 shows the result of targeted transduction *in vitro*. A major peak shift, an indication of GFP expression, was observed for all cells exposed to the positive control lentivector (FUGW/VSVG). In contrast, transduction of targeting lentivector FUGW/CD20+SINmu resulted in significant GFP expression only in αCD20-expressing cells (Fig. 5); approximately 79.5% of 293T/αCD20 cells and 10.2% of Jurkat/αCD20 cells were GFP[+]. As controls for the role of the fusogen and binding protein in transduction, lentivectors displaying only the binding protein (CD20) showed no transduction for all cell lines tested, and lentivectors displaying only the fusogen (SINmu) showed low background transduction. αCD20-negative cells transduced with FUGW/CD20+SINmu yielded only background GFP expression, confirming that the targeted transduction was αCD20 dependent. Titering on 293T/αCD20 cells showed that the specific titer of fresh, unconcentrated lentivector FUGW/CD20+SINmu was approximately 5×10^6 transduction units (TU)/ml. We observed that the specific transduction of Jurkat/αCD20 cells was significantly lower than that of 293T/αCD20 cells. This could be due to the lower expression of αCD20 on the surface of the Jurkat/αCD20 cell line. Primary peripheral blood B cell IgG expression was found to be higher than the level of IgG expression on Jurkat/αCD20 cells but less than the expression of IgG on 293T/αCD20 cells (Fig. 4B). It is also possible that different intracellular trafficking, sorting, and endosome properties between these two cell lines on specific binding of FUGW/CD20+SINmu could contribute to the different levels of transduction efficiency.

Binding molecule necessary for targeted transduction

To further test whether the specific interaction between CD20 on the lentiviral surface and αCD20 on the surface of the target cells mediated the observed targeted transduction of FUGW/CD20+SINmu, soluble αCD20 antibody was added at various concentrations to the virus–cell mixture during transduction. The soluble antibody could compete with the binding of the viral vector to the target cells, 293T/αCD20, therefore blocking the viral transduction. An isotype control antibody was also used as a negative control. As expected, Fig. 5B shows that αCD20 antibody concentration negatively correlated with targeted transduction, whereas no correlation was observed between the amount
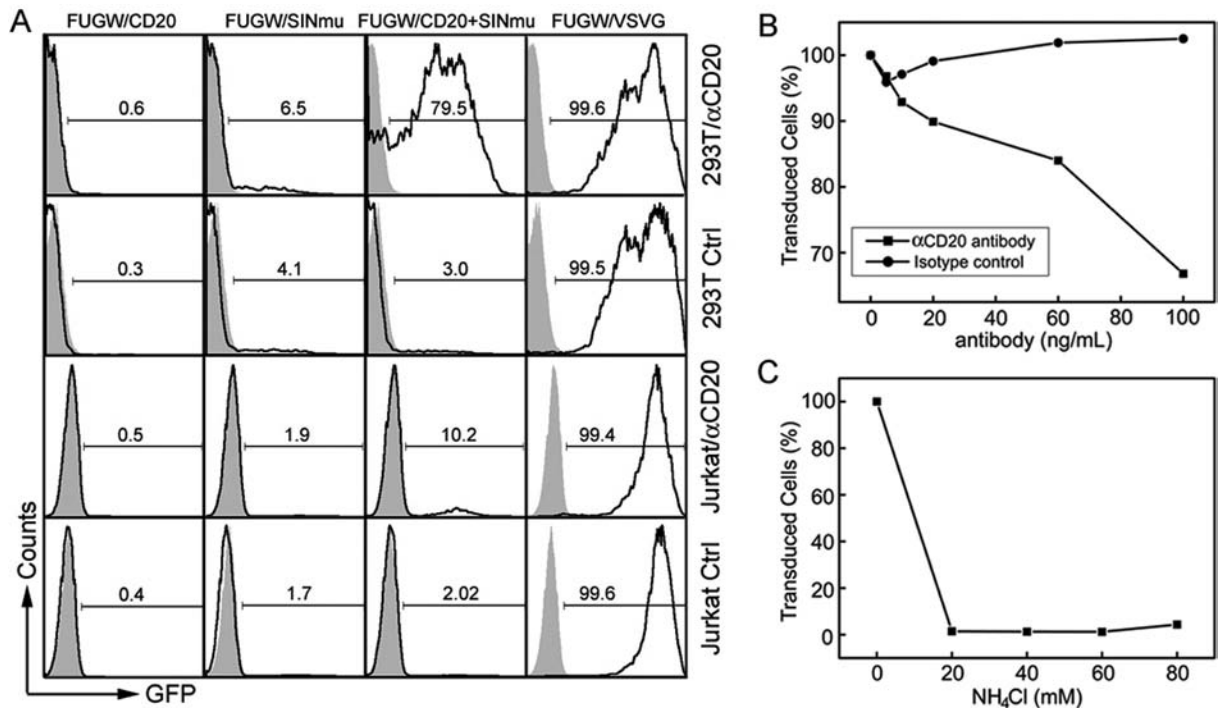

FIG. 5. *In vitro*-targeted transduction of αCD20-expressing cells. (**A**) Cell lines expressing αCD20 (293T/αCD20 and Jurkat/αCD20; 2×10^5) were transduced with 2 ml of fresh, unconcentrated FUGW/CD20+SINmu, FUGW/SINmu, FUGW/CD20, FUGW/VSVG, or no virus (as a negative control). Parental cell lines lacking expression of αCD20 were included as controls. GFP expression was analyzed by flow cytometry. The shaded area represents untransduced cells (without exposure to the viral vector) and the solid line represents transduced cells. (**B** and **C**) 293T were seeded at 0.1×10^6 cells per well in a 24-well dish. FUGW/CD20+SINmu (1 ml) was added to each well. (**B**) Effect of addition of soluble αCD20 antibody on targeted transduction. Either αCD20 antibody or an isotype control was added to the wells during transduction at the listed concentrations. Eight hours later, the medium was replaced with fresh medium and the cells were allowed to grow for 4 days. Cells were then analyzed for GFP expression by flow cytometry. (**C**) Effect of neutralizing agent, NH_4Cl, on transduction. The indicated concentration of NH_4Cl was added to the wells during transduction. Eight hours later, the medium was replaced with fresh medium and the cells were allowed to grow for 4 days. Cells were analyzed for GFP expression by flow cytometry.

of isotype control antibody and transduction efficiency. This verified that binding of viral CD20 to cell surface αCD20 is necessary for specific transduction.

pH change necessary for targeted transduction

Envelope glycoprotein derived from Sindbis virus is activated by the acidic pH within the lumen of endosomes to allow fusion of the viral particle to the host cell's endosomal membrane (Glomb-Reinmund and Kielian, 1998). This fusion allows the viral particle to release its contents into the cell's cytosol. To test whether the drop in pH was necessary to trigger SINmu to mediate fusion, we added ammonium chloride (NH$_4$Cl) at various concentrations into an incubation mixture of 293T/αCD20 cells and lentivector FUGW/CD20+SINmu; NH$_4$Cl could raise the pH to neutralize the acidic endosomal compartments. Figure 5C shows a significant decrease in transduction with the addition of NH$_4$Cl, suggesting that the lentiviral vector displaying pH-sensitive fusogen SINmu relies on the low pH within the endosome to mediate fusion to achieve targeted transduction.

Targeted transduction in vivo

We used NOD/SCID immunocompromised mice to examine the ability of the engineered lentivector to specifically transduce target cells *in vivo*. In addition, we also compared the efficiency of the targeting lentivector against a nontargeting VSVG-pseudotyped lentivector to transduce tumors *in vivo*. On the right flank of each mouse, 10×10^6 tumor cells (Jurkat/αCD20) were injected subcutaneously as a target for the viral vector. The dorsal left side of the mice received no tumor cells, which was used as a control. A non-

invasive bioluminescence imaging assay that employed a firefly luciferase-bearing lentiviral vector, FUWLuc (Fig. 6A), was used to monitor targeted transduction. We injected FUWLuc bearing CD20 and SINmu subcutaneously 8 hr after tumor cell inoculation. Three mice received 100×10^6 TU (MOI of 10) of the targeting virus (FUWLuc/CD20+SINmu) in both the right side, in the same area where the target cells were injected, and in the left side, bearing no tumor (Fig. 6B). To compare the targeting virus with a ubiquitously infectious virus, mice were injected at MOIs of 10, 20, or 50 with VSVG-pseudotyped lentivector. Six days after injection of the viral vector, the substrate for firefly luciferase (D-luciferin) was injected intraperitoneally into the mice and images of light emission from anesthetized mice were obtained with the IVIS 200 system. For the mice that received the targeting vector FUWLuc/CD20+SINmu, we observed luciferase activity predominantly in the area implanted with tumor cells (right side), representing the success of targeted viral vector transduction of tumor cells expressing αCD20 *in vivo* (Fig. 6C, far right). The mice that received targeting virus at an MOI of 10 had a luminescence level on the tumor side that was comparable to that of the mice receiving nontargeting FUWLuc/VSVG at an MOI of 50 (Fig. 6C). As compared with FUWLuc/VSVG, the background luminescence on the left side, bearing no tumor cells, was significantly less for targeting vector FUWLuc/CD20+SINmu, indicating the significant specificity of our engineered lentivector.

Targeted suicide gene therapy in vivo

To test the potentially therapeutic role of our targeting system, we used targeting vector to deliver a suicide gene, en-
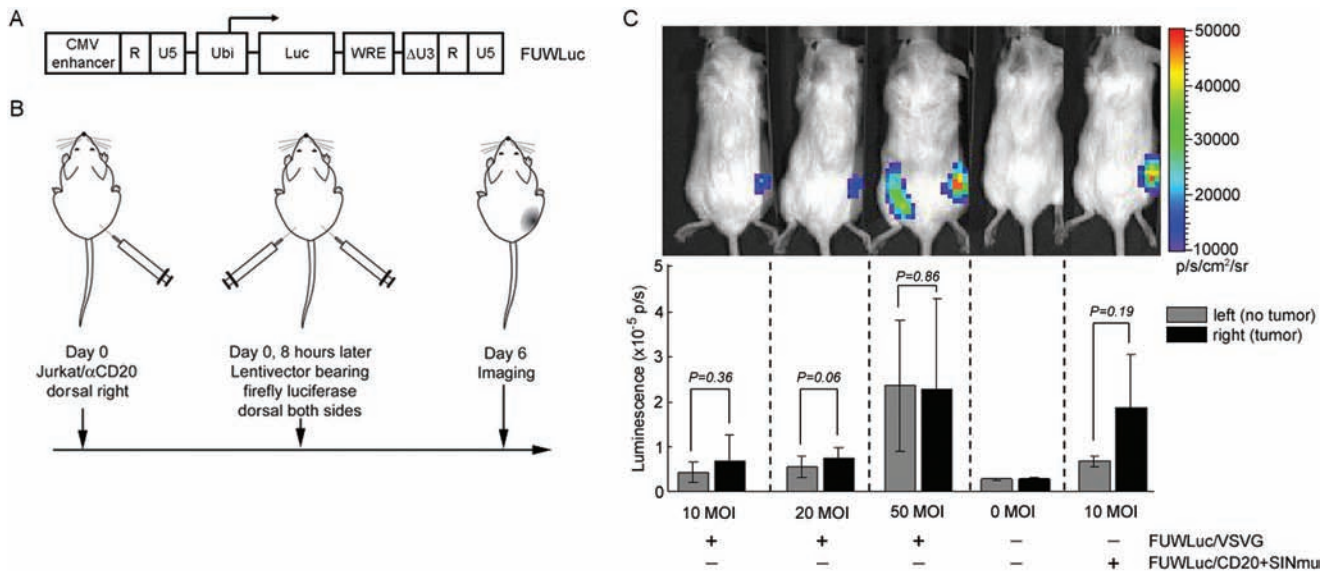

FIG. 6. Targeted transduction of Jurkat/αCD20 cells *in vivo*. (**A**) Schematic representation of lentivector FUWLuc expressing firefly luciferase. Luc, firefly luciferase; Ubi, WRE, and ΔU3 are as described in Fig. 2A. (**B**) Jurkat/αCD20 cells (10×10^6) were injected subcutaneously into the dorsal right side of NOD/SCID-HLA mice. Eight hours later, concentrated FUWLuc/CD20+SINmu, FUWLuc/VSVG, or no virus (as a control) was injected subcutaneously into both the right and left sides of the mice. Six days later, mice were injected with 3 mg of D-luciferin and imaged with an IVIS 200 system. (**C**) *Top*: Image of whole mice. p/s/cm^2/sr, photons/sec/cm^2/steradian. *Bottom*: Quantification of luminescence signal after gating on the injection site. Gray columns, signal from the left side of the mice; solid columns, signal from the right side of the mice. p/s, photons/sec. The average signal is shown for each group, consisting of three mice. Error bars represent the standard deviation. *p* Values were calculated by *t* test.

coding herpes simplex virus type 1 thymidine kinase (HSV1-TK), to xenografted tumors in an immunocompromised mouse model. The cells transduced to express HSV1-TK can be deleted by treatment with the prodrug GCV, which is transformed into a toxic metabolite form by HSV1-TK (Blumenthal *et al.*, 2007). Suicide gene therapy based on HSV1-TK has been evaluated clinically by *in situ* injection of gammaretroviral vectors into solid tumors (Ram *et al.*, 1997; Satoh *et al.*, 2005). Targeted delivery of HSV1-TK could potentially improve the safety and efficacy of the therapy. We constructed a lentivector encoding a mutant form of HSV1-TK, termed SR39tk, which has been shown to be more reactive toward the GCV substrate and therefore has enhanced ability to kill tumor cells (Black *et al.*, 2001). The resulting vector was designated FUWSR39tk (Fig. 7A). Tumor cells bearing surface αCD20 (Jurkat/αCD20) were first marked *in vitro* by transduction with the lentivector FUWLuc to express firefly luciferase (designated as Jurkat/αCD20Luc cells). Figure 7B shows a schematic of the experiment. First, Jurkat/αCD20Luc cells (5×10^6) were injected subcutaneously into the right flank of each mouse. Because the xenografted tumor stably expressed firefly luciferase, the relative size and kinetic growth of the tumor could be visualized by imaging the living animals. Eight hours after injecting Jurkat/αCD20Luc cells, 10 MOI of FUWSR39tk/CD20+SINmu or FUWSR39tk/VSVG was injected subcutaneously into the

same area as the initial tumor inoculation; no viral vector injection was included as a control. From day 7 to day 12, selected mice were treated daily with an intraperitoneal injection of the prodrug GCV at 50 mg/kg. Mice were imaged throughout the process to chart the effect of each step on tumor growth (Fig. 7C and D). Growth of the tumor before GCV treatment appeared to be unaltered by transduction of tumor cells with the SR39tk-expressing lentivector, as similar tumor growth was observed between mice injected with FUWSR39tk/CD20+SINmu and mice that received no viral vector (Fig. 7D). The GCV treatment alone initially slowed the growth of the tumor, but normal growth rate was retained after the completion of the 5-day GCV treatment (Fig. 7C and D). The largest effect could be seen on the mice that were injected with the targeting lentivector FUWSR39tk/CD20+SINmu or the nontargeted FUWSR39tk/VSVG and the prodrug GCV. In these mice, tumor growth appeared to be efficiently suppressed for more than 3 weeks, as can be seen in Fig. 7C and D.

Discussion

Our results demonstrate that recombinant lentivector can be engineered to target cells expressing an antigen-specific immunoglobulin *in vitro* and *in vivo*. The general targeting strategy is to display an antigen protein and a fusogenic pro-
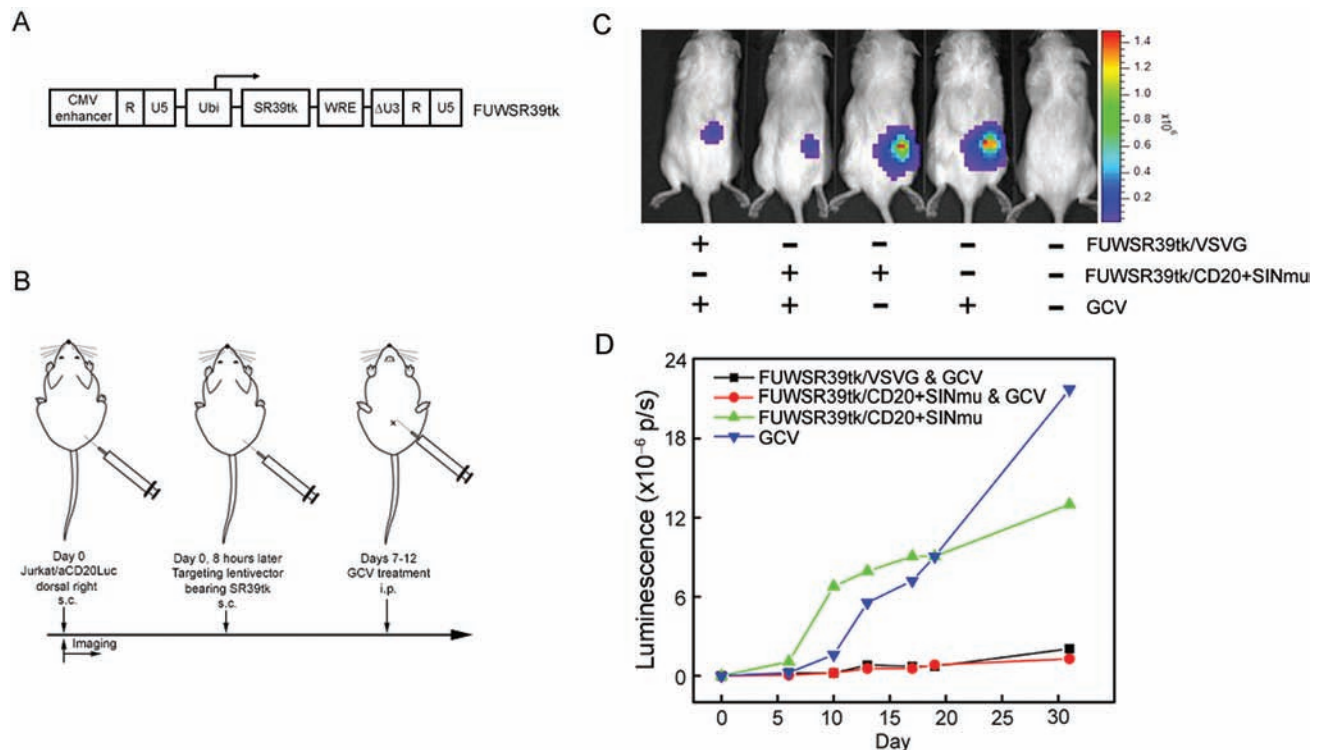

FIG. 7. Targeted suicide gene therapy to Jurkat/αCD20 *in vivo*. (**A**) Schematic representation of transfer lentiviral vector FUWSR39tk expressing SR39tk: a mutant form of the herpes simplex virus type 1 thymidine kinase. Ubi, WRE, and ΔU3 are described in Fig. 2A. (**B–D**) Jurkat/αCD20Luc cells (5×10^6) were injected subcutaneously into the right flank of NODSCID-HLA mice; one mouse did not receive the treatment as a control. Eight hours later, the lentivector (FUWTK/CD20+SINmu or FUWSR39tk/VSVG) was injected at the area implanted with tumor cells. During days 7 to 12, selected mice received intraperitoneal injection of GCV prodrug. (**B**) Experimental scheme. (**C**) Bioluminescence image 1 week after the completion of prodrug treatment. p/s/cm^2/sr, photons/sec/cm^2/steradian. (**D**) Time course of tumor growth as indicated by luminescence signal measured by gating on the right flank of tumor area. p/s, photons/sec.

tein on the lentiviral surface. In the model system reported here, the antigen protein is CD20, which is the specific antigen for the surface immunoglobulin αCD20, and the fusogenic protein is SINmu, which is a binding-deficient and fusion-competent envelope protein derived from the Sindbis virus. To generate targeting viral particles, the virus-producing cells are transfected to express both the binding and fusion molecules. The natural budding mechanism of the lentivirus incorporates the surface-expressed binding and fusogenic molecules on the surface of the resultant virions, which is confirmed by fluorescence imaging of GFP–Vpr-labeled viral particles.

The mechanism for viral entry of the targeting virus is believed to begin with binding of CD20 to the targeted immunoglobulin αCD20. Using confocal imaging, we could only binding of the virus see to cells expressing αCD20. When soluble αCD20 antibody was added to the cell culture system to compete with the targeting lentivector for binding to CD20-expressing cells, viral transduction was greatly inhibited. This shows that binding through the αCD20 immunoglobulin is necessary for the targeting viral vector to enter the cell. Binding of the immunoglobulin could induce endocytosis. In the endosomal compartment, the drop in pH triggers the fusion function of pH-sensitive fusogen SINmu, as shown in an endosomal neutralization assay in which we observed a sharp decrease in transduction with the addition of NH_4Cl.

Targeted transduction *in vitro* shows that the targeting lentivector preferentially transduces cells expressing the αCD20 immunoglobulin. Whereas the viral vector carrying only CD20 or SINmu resulted in no transduction, the lentivector bearing both CD20 and SINmu can efficiently transduce 293T/αCD20 cells (79.5%). We further showed *in vivo* targeted transduction of a xenografted Jurkat/αCD20 tumor. Jurkat/αCD20 cells were injected subcutaneously into the right flank of immunocompromised mice, followed by the injection of viral vector delivering the firefly luciferase gene into both the right and left flanks of the mice. One week postdelivery, various concentrations of VSVG-pseudotyped viral vectors were compared with 10 MOI of the engineered lentivector FUWLuc/CD20+SINmu by measuring bioluminescence in live animals. Fewer infectious particles were needed for the targeting vector to achieve the same level of expression seen in the tumors transduced with the nontargeting VSVG-pseudotyped viral vector. We speculate that the nonspecific binding of VSVG to multiple types of animal tissue cells results in less viral vector reaching the target cells as compared with the targeting vector, although more direct evidence needs to be obtained experimentally in order to support this argument. Nevertheless, this result highlights the advantage of developing targeted delivery vehicles for *in vivo* gene therapy.

Previously we reported a method to engineer lentivectors to target B cells by incorporating a membrane-bound antibody (αCD20) and a fusogen (SINmu) into the lentiviral surface (Yang *et al.*, 2006). The resulting vectors could efficiently transduce B cells expressing the cognate CD20 antigen. In this study, we demonstrated that it is feasible to design a reciprocal targeting strategy to direct lentivectors to deliver therapeutic genes to cells expressing a particular immunoglobulin. This also constitutes the first report of efficient targeting of lentivector to monospecific immunoglob-

ulin-expressing cells. Our method of incorporating a specific antigen on the lentiviral surface is a feasible approach to targeting clonal and autoreactive B cells for certain autoimmune diseases with known antigens such as pemphigus vulgaris and Graves' disease (Davidson and Diamond, 2001). For cases in which the antigen against a specific immunoglobulin is unknown, we can use the previously reported method (Yang *et al.*, 2006) to incorporate a secondary, anti-idiotypic antibody onto the lentiviral surface. Thus, these two methods could complement each other to provide novel ways to achieve gene delivery to defined populations of B cells.

Delivery of modulating genes to antigen-specific B cells could be used to tune B cell function related to a particular antigen. For example, class switching and differentiation of B cells can vary depending on the available cytokine concentration (Tarlinton, 2006). Interleukin (IL)-4 plays important roles in driving the switch to IgG1 and further to IgE (Snapper *et al.*, 1988). Cytokines, such as IL-2, IL-5, and transforming growth factor (TGF)-β, could alter B cells to augment their production of specific IgA (McHeyzer-Williams and McHeyzer-Williams, 2005). Delivery of these genes to chosen B cells can alter the isotype, magnitude, and spectrum of antibodies secreted by antigen-specific B cells. As genes involved in controlling the generation of memory B cells are identified, targeted delivery of such genes to B cells could improve the antigen-specific memory responses (McHeyzer-Williams and McHeyzer-Williams, 2005). The ability to genetically mark antigen-specific B cells *in vivo* could provide a new way to study basic B cell biology (Jacob and Baltimore, 1999; Tarlinton, 2006).

Specific delivery of a suicide gene to eliminate a disease-associated population of B cells in cancer or in autoimmune disease has therapeutic implications. B cell depletion has been successfully used clinically to treat cancer and autoimmune diseases (Goronzy and Weyand, 2003; Edwards and Cambridge, 2005; Martin and Chan, 2006). Most of these depletion therapies target a pan-B cell marker (Martin and Chan, 2006). One of the most successful B cell-depleting methods to date is rituximab, an αCD20 antibody (Edwards and Cambridge, 2005). However, because rituximab recognizes CD20, a widely expressed B cell marker, it is unable to deplete only the specific subset of diseased B cells, leading to undesirable side effects, including hypogammaglobulinemia (Chaiwatanatorn *et al.*, 2003; Imashuku *et al.*, 2004) and increased susceptibility to infection (Wadhwa and Morrison, 2006). Targeting a more specific population of B cells by suicide gene therapy could overcome some of the challenges that rituximab faces. To test whether our system could effectively deliver a suicide gene *in vivo*, we set up a mouse model with a xenografted tumor expressing firefly luciferase. By measuring the bioluminescence from firefly luciferase, we could monitor the kinetic growth of the tumor. We found that a single-dose injection of FUWSR39tk/CD20+SINmu along with the prodrug treatment was able to substantially suppress tumor growth. Although we clearly showed that more cells were targeted by using the targeting vector (Fig. 6C), we did not observe markedly improved suppression of tumor growth when using the targeting vector as opposed to the nontargeting vector (Fig. 7C and D). One possible explanation is the known bystander effect of suicide gene therapy (Freeman *et al.*, 1993). Although only a small fraction of the tumor cells was transduced to express the sui-

cide gene, Freeman and coworkers remained able to detect a significant reduction in tumor size after GCV treatment (Freeman *et al.*, 1993). An increase in the dose and frequency of injection of targeting lentivector bearing the suicide gene could further enhance the efficiency of tumor therapy.

A clinical trial using lentivectors to deliver antisense RNA for anti-HIV therapy has shown no detectable adverse effects (Levine *et al.*, 2006), highlighting the promise of lentivectors for gene therapy (Kohn, 2007). A general concern about the clinical use of lentivectors is insertional mutagenesis, sparked by the X-linked severe combined immunodeficiency (SCID) trial in France, in which some gammaretroviral vector-treated patients developed leukemia as a result of the outgrowth of transduced cells (Hacein-Bey-Abina *et al.*, 2003). Although more studies are needed to fully gauge the oncogenesis of this third-generation lentivector system, Naldini and coworker have shown in a tumor-prone mouse model, using hematopoietic stem cell gene transfer, that lentivectors have lower oncogenic potential (Montini *et al.*, 2006), which could be a major advantage as a gene transfer vector, as opposed to gammaretroviral vectors. A suicide gene therapy could be incorporated into the gene therapy protocol to further increase safety by eliminating, via prodrug treatment, the abnormally proliferative cells that have been marked with a suicide gene (Blumenthal *et al.*, 2007).

Our findings suggest that engineering a gene delivery system based on lentivectors to target antigen-specific immunoglobulin-expressing cells is feasible both *in vitro* and *in vivo*, using direct intratumoral injection. It should be pointed out that such an approach may be appropriate for clinically colocalized disease, such as low-stage non-Hodgkin lymphoma. Future work will be required to validate this method for systemic administration of lentivectors for disseminated disease.

Acknowledgments

Author Disclosure Statement

No competing financial interests exist.

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