Specific Targeting of Human Interleukin (IL)-13 Receptor α 2-Positive Cells with Lentiviral Vectors Displaying IL-13

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Abstract

The ability to selectively and efficiently target transgene delivery to specific cell types *in vitro* and *in vivo* remains one of the formidable challenges in gene therapy. Lentiviral vectors have several advantages that make them attractive as gene delivery vehicles and their tropism can be altered through pseudotyping, allowing transgene delivery to specific populations of cells. The human interleukin-13 receptor α 2 (IL-13R α 2) is uniquely over-expressed in many different human tumors, making it an attractive target for cancer therapy. In this study, we examined whether IL-13R α 2-positive tumor cells can be specifically targeted with lentiviral vector pseudotypes containing a truncated fusion (F) protein derived from measles virus (MV) and a tail-truncated and receptor-blind MV hemagglutinin (H) protein bearing IL-13 at the C terminus. The retargeted lentiviral vector efficiently transduced cells that express high levels of IL-13R α 2, but not cells expressing low levels of IL-13R α 2 *in vitro*. *In vivo*, it specifically targeted IL-13R α 2-positive glioma cell xenografts in immunodeficient mice in the context of subcutaneous and intracranial glioma models. Similar lentiviral vectors may be developed for targeting other tumors expressing specific cell surface receptors.

Introduction

RETROVIRAL VECTORS based on gammaretroviruses and lentiviruses provide powerful tools for gene delivery *in vitro*, *ex vivo*, and *in vivo* (D'Costa *et al.*, 2009; Matrai *et al.*, 2010; Dropulic, 2011). To exploit the full therapeutic potential of retroviral vectors, they must be capable of transducing target cells while avoiding impacting nontarget cells. A major technical challenge in facilitating the efficient transduction of the desired target cells by viral vectors is that the native tropism of the virus often does not meet the therapeutic need (Waehler *et al.*, 2007). To bypass this issue, the host range of retroviral vectors including that of lentiviral vectors can be altered by a process known as pseudotyping. Pseudotyped retroviral vectors consist of vector particles bearing envelope (Env) glycoproteins derived from other enveloped viruses. Such particles possess the tropism of the virus from which the glycoprotein was originally derived (Cronin *et al.*, 2005).

The development of lentiviral vectors that display a reduced tropism for the natural receptor and an increased specificity for a chosen receptor to allow targeted transduction

of specific cell types *in vitro* and *in vivo* has been challenging, due to a number of issues such as targeting specificity and vector titers (Frecha *et al.*, 2008). A novel strategy for lentiviral vector targeting based on engineered measles virus (MV) H and F glycoproteins has emerged (Funke *et al.*, 2008). This strategy is promising because of its flexibility and because background transduction in nontarget cells is low (Funke *et al.*, 2008; Anliker *et al.*, 2010; Munch *et al.*, 2011). The native H protein mediates attachment to the viral receptor CD46, signaling lymphocytic activation molecule (SLAM; Yanagi *et al.*, 2006), or Nectin-4 (Muhlebach *et al.*, 2011) on the cell surface, and signals to the F protein to trigger cell fusion. The steps required to retarget this cell fusion reaction are ablation of H protein-mediated CD46 and SLAM receptor recognition and introduction of a new binding specificity in the H glycoprotein (Nakamura *et al.*, 2004). Various ligands such as epidermal growth factor (EGF) (Funke *et al.*, 2008), single-chain antibodies (Anliker *et al.*, 2010), and designed ankyrin repeat proteins (DARPs) (Munch *et al.*, 2011) have been successfully displayed on MV H, allowing retargeted lentiviral vector delivery to the corresponding target cells.

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The flexibility of the H glycoprotein to accommodate cell-targeting ligands was also demonstrated in the context of other viruses. For example, Allen and colleagues (2008) successfully made a retargeted recombinant MV displaying interleukin (IL)-13, fused to the C terminus of the H protein, and demonstrated specific targeting to human IL-13 receptor $\alpha 2$ (IL-13R $\alpha 2$)-positive cells both *in vitro* and *in vivo*. IL-13R $\alpha 2$ is an appealing target because it is overexpressed in various types of tumors, including but not limited to glioblastoma, ovarian cancer, pancreatic cancer, prostate cancer, renal cell carcinoma, head and neck cancer, AIDS-Kaposi's sarcoma, and oral squamous cell carcinoma (Joshi and Puri, 2009).

The purpose of our study was to develop lentiviral vectors that bear MV glycoproteins and human IL-13 to test whether such vectors can specifically target IL-13R $\alpha 2$ -positive tumor cells *in vitro* and *in vivo*.

Materials and Methods

Plasmid constructs

The pSLIK-Neo/TRE Pitt-IL-13R $\alpha 2$ lentiviral vector plasmid was constructed as follows: The TRE Pitt promoter fragment (Pluta *et al.*, 2005) derived from pLitTREPitt (Addgene plasmid #18661) was subcloned into pENTR2B (Invitrogen, Carlsbad, CA). A cDNA fragment encoding human IL-13R $\alpha 2$, derived from pSPORT6/hIL-13R $\alpha 2$ (Open Biosystems, Huntsville, AL), was inserted downstream of the TRE Pitt promoter, resulting in pENTR2B/TRE Pitt/IL-13R $\alpha 2$. The final pSLIK-Neo/TRE Pitt-IL-13R $\alpha 2$ plasmid was generated by Gateway recombination (Invitrogen) between the pENTR2B/TRE Pitt/IL-13R $\alpha 2$ entry plasmid and the pSLIK-Neo destination vector (Addgene plasmid #25735) (Shin *et al.*, 2006), using LR Clonase II (Invitrogen). The pNL(CMV)EGFP/CMV/WPRE Δ U3 plasmid was derived from the pNL-EGFP/CMV/WPRE Δ U3 plasmid (Ricks *et al.*, 2008). It bears a hybrid 5' long terminal repeat (LTR) that was derived from pHIV dl κ B SP1 CMV IE(a) (Chang *et al.*, 1993). The pNL(CMV)Fluc/CMV/WPRE Δ U3 plasmid was derived from pNL(CMV)EGFP/CMV/WPRE Δ U3 by replacing the enhanced green fluorescent protein (EGFP) coding region with a PCR fragment encoding firefly luciferase (Fluc); the PCR fragment was generated with the pGL4.20-Luc2/Puro plasmid (Promega, Madison, WI) as a template. The pNL(CMV)GLuc/CMV/WPRE Δ U3 lentiviral vector plasmid encoding membrane-bound *Gaussia* luciferase (GLuc) was generated by Gateway recombination between the pENTR2B/CD8-SP-GLuc-TM entry plasmid and the pNL(CMV)DEST/CMV/WPRE Δ U3 destination vector. The CD8-SP-TM sequence encoding the CD8 leader peptide and the CD8 transmembrane domain (Santos *et al.*, 2009) was synthesized by GenScript USA (Piscataway, NJ). A fragment encoding humanized GLuc lacking its native signal peptide (NanoLight Technology, Pinetop, AZ) was inserted in-frame between the CD8 leader peptide and the CD8 transmembrane domain.

The pCD/NL-BH Δ 1 packaging plasmid was derived from pCD/NL-BH* $\Delta\Delta\Delta$ (Zhang *et al.*, 2004). It contains the Gag- and Pol-coding regions but lacks functional Tat-, Rev-, and accessory protein-encoding sequences.

The pCG-F Δ 30 plasmid encoding the MV Edmonston strain F glycoprotein, bearing a 30-amino acid truncation at the cytoplasmic tail, was described previously (Moll *et al.*, 2002). Plasmid pCG-H Δ 20-AA-IL-13 encodes MV virus Ed-

monston strain-derived hemagglutinin (H) fused to IL-13 (Allen *et al.*, 2008). Amino acids Y481 and R533 of the H protein were mutated to alanine to ablate the ability of the protein to bind to the native MV receptors CD46 and SLAM (Nakamura *et al.*, 2004). The pCG-H Δ 20-AA-IL-13 plasmid was constructed by subcloning a 1774-bp *NsiI-SpeI* fragment derived from pCG-H Δ AA-IL-13-H6 (Allen *et al.*, 2008) into pCG-H Δ 20 (Moll *et al.*, 2002). Plasmid pCG-H Δ 18-AA-IL-13 was derived from the pCG-H Δ 20-AA-IL-13 plasmid by further truncation of the cytoplasmic tail-encoding sequence, using a synthetic DNA fragment prepared by GenScript USA. The pCG-H Δ 18-AA plasmid lacks the IL-13-coding region. It was derived from pCG-H Δ 18-AA-IL-13 by deletion of the IL-13-encoding *XmaI-SpeI* fragment and substituting for it a synthetic DNA fragment encoding a hexahistidine (His $_6$) tail. The pCEF-VSV-G plasmid contains a hybrid CEF promoter (human cytomegalovirus enhancer combined with human EF-1 α promoter) (Kuroda *et al.*, 2011) and bears a vesicular stomatitis virus G protein (VSV-G)-coding region, derived from pLTR-G (Reiser *et al.*, 1996).

Cell lines

Human embryonic kidney 293T (CRL-11268; American Type Culture Collection [ATCC], Manassas, VA) cells and human osteosarcoma (HOS) (CRL-1543; ATCC) cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Lonza, Walkersville, MD) containing 10% heat-inactivated fetal bovine serum (FBS), 2.5 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 μ g/ml) (all from Invitrogen, Grand Island, NY). HOS-IL-13R $\alpha 2$ cells were generated by transduction of HOS cells with the pSLIK-Neo/TRE Pitt-IL-13R $\alpha 2$ lentiviral vector and selection in medium containing G418 (500 μ g/ml). Pooled cell clones were induced with doxycycline (Dox, 1 μ g/ml), stained with a phycoerythrin (PE)-conjugated mouse anti-human IL-13R $\alpha 2$ antibody (Diacclone, Besançon, France), and sorted for cells expressing high levels of IL-13R $\alpha 2$.

Human U251 glioblastoma cells (National Cancer Institute [NCI], Frederick, MD) were cultured in RPMI 1640 medium. U87 glioma cells (U87; ATCC) and T98G glioblastoma multiforme cells (CRL-1690; ATCC) were cultured in Eagle's minimal essential medium (EMEM). Both RPMI 1640 and EMEM were supplemented with 10% FBS, 25 mM HEPES, 0.1 mM nonessential amino acids (NEAAs), 2 mM glutamine, 1 mM sodium pyruvate, penicillin (100 U/ml), and streptomycin (100 μ g/ml) (Invitrogen). DU145 human prostate carcinoma cells (HTB-81; ATCC) were cultured in EMEM supplemented with 10% heat-inactivated FBS, 2 mM glutamine, penicillin (100 U/ml), and streptomycin (100 μ g/ml) (Invitrogen).

Cytotoxicity assay

Cytotoxicity mediated by chimeric fusion immunotoxin consisting of IL-13 linked to *Pseudomonas aeruginosa* exotoxin (IL-13-PE) was determined as described previously (Joshi *et al.*, 2002; Joshi and Puri, 2005).

Lentiviral vector production

Lentiviral vector production was carried out with 6-well plates, 15-cm dishes, or HYPERFlask cell culture vessels

(Corning, Lowell, MA), as described previously (Kuroda *et al.*, 2009, 2011; Kutner *et al.*, 2009a,b). For transfection using 6-well plates (4×10^5 HEK 293T cells per well), 0.53 μ g of the pNL(CMV)EGFP/CMV/WPRE Δ U3, pNL(CMV)Fluc/CMV/WPRE Δ U3, or pNL(CMV)GLuc/CMV/WPRE Δ U3 vector plasmid, 0.32 μ g of the pCD/NL-BH Δ 1 packaging plasmid, 0.76 μ g of the pCMV-rev plasmid (Lewis *et al.*, 1990) (obtained from M.-L. Hammarskjöld and D. Rekosh through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH), 0.066 μ g of the pCG-F Δ 30 plasmid, and 0.033 μ g of the pCG-H Δ 18-AA-IL-13 plasmid were used per well. For experiments involving VSV-G pseudotypes, the pCEF-VSV-G plasmid (0.18 μ g/well) was used. For 15-cm dishes and HYPERFlask vessels, the number of cells and the quantities of the various plasmids were proportionally increased according to the cell culture surface area. The cell culture media were changed the day after transfection and the vector-containing supernatants were harvested on the third day (about 60 hr posttransfection), filtered through a 0.45- μ m pore size filter (Millipore, Billerica, MA), and concentrated by ultracentrifugation through a 20% sucrose cushion. The vector pellets were resuspended in phosphate-buffered saline (PBS) supplemented with Polybrene (3 μ g/ml), and stored at -80°C . Vector stocks were titrated by qPCR using genomic DNA from U251 cells transduced using woodchuck hepatitis virus posttranscriptional regulatory element (WPRE)-specific primers (Kutner *et al.*, 2009b). Vector titers are expressed as transducing units (TU)/ml. Relative vector particle numbers were determined with a p24 ELISA kit (Kutner *et al.*, 2009b).

Transduction in vitro and vector neutralization

HOS-IL-13R α 2, U251, or DU145 cells were seeded in 12-well plates at a density of 1×10^5 cells per well the day before transduction. When needed, Dox was added to the cell culture medium at a final concentration of 1 μ g/ml. The next day, cell culture medium was removed from each well and a 0.5-ml aliquot of unconcentrated lentiviral vector sample and 0.5 ml of cell culture medium supplemented with Polybrene (16 μ g/ml) were added. After overnight incubation in a CO₂ incubator, the vector-Polybrene mixture was replaced with 1 ml of fresh medium.

For vector neutralization, polyclonal goat anti-human IL-13R α 2 antibodies (R&D Systems, Minneapolis, MN) were used. Cells were preincubated with the antibody for 1 hr at room temperature. The antibody was also present during the 12-hr transduction step. Afterward, the vector-antibody mixture was replaced with 1 ml of fresh medium.

For vector neutralization using the recombinant IL-13R α 2 extracellular domain (R&D Systems), vectors were first preincubated with the recombinant protein at 37°C in a humidified 5% CO₂ incubator for 1 hr. Twelve hours later, the vector-containing medium was removed, and the cells were washed once with PBS and replenished with fresh complete medium. Three days posttransduction, cells were harvested with 0.25% trypsin-EDTA (Invitrogen), fixed for 10 min with an equal volume of PBS supplemented with 2% paraformaldehyde (Sigma-Aldrich, St. Louis, MO), and washed once with PBS. The percentage of EGFP-positive cells was determined by fluorescence-activated cell sorting (FACS)

with a BD FACSCalibur or FACSCanto II system (BD Biosciences, San Jose, CA). GLuc activity was measured with the Dual Luciferase assay system from Promega, and Fluc activity was measured with the regular luciferase assay system from Promega.

Determination of IL-13R α 2 levels by FACS

U251, U87, T98G and HOS-IL-13R α 2 cells were washed twice with plain PBS (Ca²⁺ and Mg²⁺ free), detached with PBS supplemented with 5 mM EDTA and 2.5% FBS, centrifuged at $500 \times g$ for 5 min, and resuspended in staining solution (CO₂-independent medium [Invitrogen], supplemented with 2.5% FBS and 2.5 mM EDTA) at a concentration of 5 million cells/ml. For each sample, 100- μ l aliquots of the cells were used. The cells were stained with a biotin-labeled goat anti-IL-13R α 2 antibody (10 μ g/ml) or biotin-labeled normal goat IgG (R&D Systems), for 1 hr at 4°C . The cells were washed once with plain PBS, resuspended in staining solution containing Alexa 647-labeled streptavidin (10 μ g/ml; Invitrogen), incubated for 1 hr at 4°C , washed twice with plain PBS, and resuspended in 0.5 ml of staining solution containing YO-PRO-1 viability dye (1 μ l/ml; Invitrogen). YO-PRO-1-negative (live) cells were analyzed with the FACSCalibur system (BD Biosciences).

Assessment of vector targeting in vivo with a subcutaneous model

The animal protocol and procedures were approved by Institutional Animal Care and Use Committee at the Center for Biologics Evaluation and Research (Bethesda, MD) (protocol 2011-02). The animal facilities are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. All experiments were performed according to institutional guidelines. Five million U251 glioma cells or DU145 prostate carcinoma cells in 100 μ l of PBS were grafted subcutaneously into 6- to 8-week-old male athymic *nu/nu* mice (NCI). One to 3 weeks later, when tumor volumes had reached about 100 mm³, 30- μ l aliquots (3×10^6 TU) of GLuc-encoding lentiviral vectors (MV H Δ 18-AA-IL-13/F Δ 30) were injected into the tumors. Three days after vector injection, mice were imaged with the IVIS system (Caliper Life Sciences, Hopkinton, MA) immediately after 100 μ l of the coelenterazine substrate (Nanolight Technology) was injected into each tumor.

Assessment of vector targeting in vivo using an intracranial model

The animal protocol and procedures were approved by the Institutional Animal Care and Use Committees at the Center for Biologics Evaluation and Research (protocol 2003-13). One million U251 glioma cells in 10 μ l of PBS were grafted intracranially by a bolus injection (Kawakami *et al.*, 2004) into 6- to 8-week-old female athymic *nu/nu* mice (NCI), using a model 900 small animal stereotaxic instrument from David Kopf Instruments (Tujunga, CA). Five days later, 10- μ l aliquots (7×10^5 TU) of Fluc-encoding lentiviral vector (MV H Δ 18-AA-IL-13/F Δ 30) were injected at the same location where the tumor cells were originally injected. At various times after vector injection, mice were imaged with the IVIS system (Caliper Life Sciences) 15 min after intraperitoneal

injection of the D-luciferin substrate (150 mg/kg; Caliper Life Sciences).

Results

Design of lentiviral vectors for targeted transgene delivery into IL-13R α 2-positive cells

For targeted transgene delivery into IL-13R α 2-positive cells *in vitro* and *in vivo*, safety-improved lentiviral vectors, based on a previously described second-generation lentiviral vector packaging system (Zhang *et al.*, 2004; Ricks *et al.*, 2008), were developed (Fig. 1). The improved packaging system is Tat independent and includes the pCD/NL-BH Δ 1 helper plasmid encoding the HIV-1 Gag and Pol functions; it also includes the pCMV-rev plasmid (Lewis *et al.*, 1990) that encodes Rev (Fig. 1B). The vector plasmids used encode either *Gaussia* luciferase (GLuc), firefly luciferase (Fluc), or EGFP (Fig. 1A) and bear a hybrid 5' LTR (Chang *et al.*, 1993) containing a CMV-IE promoter sequence. The Env glycoprotein constructs used include the MV-based pCG-H Δ 18-AA-IL-13, pCG-H Δ 18-AA, and pCG-F Δ 30 plasmids, as well as the pCEF-VSV-G plasmid encoding the vesicular stomatitis virus G glycoprotein (Fig. 1C).

Targeted transduction of IL-13R α 2-positive cells *in vitro*

To test the ability of lentiviral vectors pseudotyped with the MV H Δ 18-AA-IL-13 and F Δ 30 glycoproteins to selectively transduce target cells *in vitro*, an engineered human osteosarcoma (HOS) cell line (HOS-IL-13R α 2) that expresses human IL-13R α 2 in response to Dox addition was developed. Consistent with results reported previously (Saito *et al.*, 2005), unmodified HOS cells and HOS-IL-13R α 2 cells in the absence of Dox did not reveal any IL-13R α 2-positive cells above background by FACS (Fig. 2A); however, in the presence of Dox, a substantial fraction of the cells were positive after staining with an anti-IL-13R α 2 antibody. This indicates that IL-13R α 2 overexpression in HOS-IL-13R α 2 cells is strictly dependent on Dox addition.

To test whether IL-13R α 2 receptors expressed by HOS-IL-13R α 2 cells in the presence of Dox are functional, we treated the cells with various concentrations of a chimeric fusion immunotoxin consisting of IL-13 linked to *Pseudomonas aeruginosa* exotoxin (IL-13-PE). Uptake of IL-13-PE and inhibition of protein synthesis have been shown previously to correlate with IL-13R α 2 levels (Obiri *et al.*, 1996;

FIG. 1. Representation of the lentiviral vector system. **(A)** Vector constructs. pNL(CMV)GLuc/CMV/WPRE Δ U3, pNL(CMV)Fluc/CMV/WPRE Δ U3, and pNL(CMV)EGFP/CMV/WPRE Δ U3 bear *Gaussia* luciferase (GLuc), firefly luciferase (Fluc), or enhanced green fluorescent protein (EGFP) transgene sequences, respectively. CMV, human cytomegalovirus immediate-early promoter; RRE, Rev response element; LTR, long terminal repeat; PPT, central polypurine tract; WPRE, woodchuck hepatitis virus posttranscriptional element. **(B)** Helper (packaging) constructs. pCD/NL-BH Δ 1 encodes the Gag and Pol proteins and pCMV-rev (Lewis *et al.*, 1990) encodes Rev. **(C)** Env glycoprotein constructs. The pCG-H Δ 18-AA-IL-13 plasmid encodes a fusion protein consisting of a receptor-blind variant of the measles virus (MV) H protein that lacks 18 residues of the cytoplasmic tail fused to interleukin (IL)-13. The pCG-H Δ 18-AA plasmid lacks the IL-13 coding sequence. Plasmid pCG-F Δ 30 encodes the MV F-protein lacking 30 amino acids of the cytoplasmic tail. Plasmid pCEF-VSV-G expresses the vesicular stomatitis virus G glycoprotein.

FIG. 2. Regulated overexpression of IL-13R α 2 in human osteosarcoma (HOS) cells. **(A)** FACS analysis of IL-13R α 2 overexpression in HOS cells. *Left:* HOS-IL-13R α 2 cells with or without doxycycline (Dox). *Right:* Unmodified HOS cells with or without Dox. Blue line, cells exposed to Dox (1 μ g/ml); red line, cells without Dox. **(B)** Protein synthesis inhibition in HOS-IL-13R α 2 cells, using IL-13 linked to *Pseudomonas aeruginosa* exotoxin (IL-13-PE). HOS-IL-13R α 2 cells induced with Dox or uninduced cells were treated with IL-13-PE; the concentrations of IL-13-PE used varied from 0.01 to 1000 ng/ml; renal carcinoma cells (PM-RCCs), which are positive for IL-13R α 2, served as a positive control. **(C)** HOS cells and HOS-IL-13R α 2 cells treated with or without Dox were transduced with an EGFP-encoding lentiviral vector pseudotyped with the MV H_{CA18-AA}-IL-13/F_{CA30} glycoproteins and analyzed for EGFP expression 3 days later with a FACSCalibur. Color images available online at www.liebertpub.com/hgtb

Joshi *et al.*, 2002; Joshi and Puri, 2005). As shown in Fig. 2B, treatment with IL-13-PE inhibited protein synthesis in HOS-IL-13R α 2 cells exposed to Dox; renal cell carcinoma cells (PM-RCCs; Obiri *et al.*, 1996) were used as a positive control. In contrast, protein synthesis was not affected in HOS-IL-13R α 2 cells lacking Dox (uninduced cells) when using IL-13-PE up to 100 ng/ml. These findings are consistent with the results shown in Fig. 2A; they indicate that the levels of functional IL-13 receptors in uninduced HOS-IL-13R α 2 cells are low and that the production of functional IL-13R α 2 receptors in HOS-IL-13R α 2 cells can be induced by Dox addition.

The capacity of EGFP-encoding lentiviral vectors pseudotyped with the MV H_{CA18-AA}-IL-13 and F_{CA30} glycoproteins to selectively transduce Dox-induced HOS-IL-13R α 2 cells was investigated next. As shown in Fig. 2C, EGFP-positive cells emerged among cells that were treated with Dox before and during transduction but not among parental HOS cells or HOS-IL-13R α 2 cells that lacked Dox, indicating that transduction was IL-13R α 2 dependent.

Three additional control experiments were performed to further confirm that efficient transduction of HOS-IL-13R α 2 cells was dependent on IL-13 and IL-13R α 2 (Fig. 3). In the first control experiment, IL-13R α 2-expressing target cells were transduced with an otherwise identical lentiviral vector except that the chimeric MV H protein did not contain IL-13. The results presented in Fig. 3A show that there was no

transduction of HOS-IL-13R α 2 cells in the absence of the IL-13 ligand. In the second control experiment, IL-13R α 2-expressing target cells were treated with a polyclonal goat anti-IL-13R α 2 antibody 1 hr before and during transduction. Figure 3B shows that transduction was blocked in a dose-dependent manner for the vector pseudotyped with MV chimeric H glycoprotein, but not for the vector pseudotyped with VSV-G. The third control experiment involved treatment of the vector with the soluble extracellular domain of IL-13R α 2 (rIL-13R α 2) 1 hr before and during transduction. Transduction using the MV chimeric H glycoprotein-containing vector was blocked in a dose-dependent manner, whereas the VSV-G-containing vector was unaffected (Fig. 3C). Taken together, these experiments indicate that transduction of HOS-IL-13R α 2 cells was specifically mediated by IL-13 and IL-13R α 2.

We next tested whether pseudotyped lentiviral vectors containing the MV H_{CA18-AA}-IL-13 and F_{CA30} glycoproteins could transduce tumor cells that naturally express IL-13R α 2. To that end, we transduced the IL-13R α 2-positive U251 and U87 glioma cell lines; HOS-IL-13R α 2 cells treated with Dox and T98G glioblastoma multiforme cells were used as positive and negative controls, respectively. Figure 4B shows that all cell lines could be transduced successfully except for T98G cells, consistent with the finding that IL-13R α 2 expression in T98G cells was close to background levels, but positive for the other cell lines (Fig. 4A).

FIG. 3. Specificity of *in vitro* transduction. **(A)** IL-13R α 2-expressing HOS cells were transduced with EGFP-encoding lentiviral vectors pseudotyped either with H_{cΔ18-AA}-IL-13/F_{cΔ30} glycoprotein (right) or F_{cΔ30}/H_{cΔ18-AA} glycoprotein (left). **(B)** IL-13R α 2-expressing HOS cells were treated with a goat anti-IL-13R α 2 polyclonal antibody before and during transduction; MV, MV H_{cΔ18-AA}-IL-13/F_{cΔ30}-pseudotyped lentiviral vector; VSV-G, VSV-G-pseudotyped lentiviral vector. **(C)** The pseudotyped lentiviral vectors were treated with recombinant rIL-13R α 2, the soluble extracellular domain of IL-13R α 2, before and during transduction; MV, MV H_{cΔ18-AA}-IL-13/F_{cΔ30}-pseudotyped lentiviral vector; VSV-G, VSV-G-pseudotyped lentiviral vector.

Optimized production of lentiviral vectors displaying IL-13

To facilitate the investigation of the performance of lentiviral vectors pseudotyped with MV H_{cΔ18-AA}-IL-13 and F_{cΔ30} glycoproteins *in vivo*, vector production was optimized. Ratios of the F_{cΔ30} and H_{cΔ18-AA}-IL-13 glycoprotein-encoding plasmids used during vector production were optimized first. As shown in Fig. 5A, the optimal ratio was between 3:1 and 1.67:1. A ratio of 2:1 was repeatedly found to be the best (data not shown) and was used for the rest of the study. In addition, the ratio of the vector and helper plasmids in relation to the sum of the F_{cΔ30} and H_{cΔ18-AA}-IL-13 glycoprotein plasmids was optimized. As shown in Fig. 5B, the optimal ratio of the vector and helper to Env glycoprotein plasmids (pCG-H_{cΔ18-AA}-IL-13 and

pCG-F_{cΔ30}) was 27:1. The final conditions used for transfecting HEK 293T cells in 6-well plates were as follows: 0.53 μ g of pNL(CMV)EGFP/CMV/WPREΔU3, pNL(CMV)GLuc/CMV/WPREΔU3, or pNL(CMV)Fluc/CMV/WPREΔU3 plasmid, 0.32 μ g of pCD/NL-BHA1 plasmid, 0.76 μ g of pCMV-rev plasmid, and 0.066 μ g of pCG-F_{cΔ30} plasmid and 0.033 μ g of pCG-H_{cΔ18-AA}-IL-13 plasmid per well. For large-scale production of lentiviral vectors pseudotyped with MV H_{cΔ18-AA}-IL-13 and F_{cΔ30} glycoproteins, the conditions used for the 6-well plate format were scaled up proportionally.

Targeted transduction in vivo

The capacity of lentiviral vectors pseudotyped with the MV H_{cΔ18-AA}-IL-13 and F_{cΔ30} glycoproteins to selectively

FIG. 4. Targeted transduction of IL-13R α 2-positive human tumor cell lines *in vitro*. **(A)** Surface expression of IL-13R α 2 on human glioma cells (T98G, U87, and U251) and on IL-13R α 2-expressing HOS cells. Cells were stained with a polyclonal goat-anti IL-13R α 2 antibody (black lines) or a control goat antibody (shaded areas) and an Alexa 647-conjugated secondary antibody and were analyzed by FACS, using a FACSCalibur instrument. **(B)** Human glioma cells (T98G, U87, and U251) and IL-13R α 2-expressing HOS cells were transduced with an EGFP-encoding lentiviral vector and analyzed as described for Fig. 2C.

FIG. 5. Optimization of vector production for targeted transduction *in vivo*. **(A)** Optimization of ratios of pCG-H_{ca18-AA}-IL-13 and pCG-F_{ca30} plasmids. Samples containing 0.53 μ g of pNL(CMV)EGFP/CMV/WPRE Δ U3, 0.32 μ g of pCD/NL-BH Δ 1, 0.76 μ g of pCMV-rev, and various amounts of pCG-F_{ca30} and pCG-H_{ca18-AA}-IL-13 plasmid DNAs were prepared to transfect HEK 293T cells in 6-well plates. The combined amounts of pCG-F_{ca30} and pCG-H_{ca18-AA}-IL-13 plasmid DNAs used were 0.18 μ g; this amount was kept the same for each sample. Sixty hours posttransfection, vector-containing supernatants were harvested and used to transduce U251 cells. Transduced cells were analyzed by FACS 48 hr posttransduction. Vector titers were calculated on the basis of the percentage of EGFP-positive cells. **(B)** Optimization of vector/helper to Env glycoprotein plasmid ratios. The ratio of the vector to the helper plasmids (1.5:1) was the same for each sample; the ratio of pCG-F_{ca30} to pCG-H_{ca18-AA}-IL-13 Env glycoprotein plasmid (2:1) was kept the same; the total amount of plasmid DNA in each sample (1.08 μ g) was kept the same but the ratios of the combined amounts of vector and helper plasmids to the Env glycoprotein plasmid varied as indicated.

transduce target cells *in vivo* in an IL-13/IL-13R α 2-dependent manner was investigated next. To do this, we first established subcutaneous tumors in nude mice, using the IL-13R α 2-positive U251 cell line. The DU145 prostate carcinoma cell line, which lacks IL-13R α 2 expression (Kawakami *et al.*, 2002), was used as a negative control. The *in vitro* analysis presented in Fig. 6B confirms that DU145 cells were not transduced above background levels whereas U251 cells were transduced efficiently. To monitor the specificity of vector targeting in the context of subcutaneous tumors, an MV H_{ca18-AA}-IL-13- and F_{ca30}-pseudotyped lentiviral vector encoding GLuc (Tannous *et al.*, 2005) was used. Figure 6A documents the production of GLuc activity in transduced U251 cells *in vitro*. As shown in Fig. 6C (top), subcutaneous U251 glioma tumors injected with 3×10^6 transducing units (TU) of the vector and the coelenterazine substrate were positive, using the IVIS imaging system, whereas tumors

that did not receive any vector were negative. DU145 tumors treated in the same way did not produce a detectable signal (Fig. 6C, bottom). Taken together, these results indicate that the targeting specificity *in situ* of IL-13-containing lentiviral vectors was maintained in the context of the subcutaneous tumor model.

To test the targeting specificity of MV H_{ca18-AA}-IL-13 glycoprotein-pseudotyped lentiviral vectors in the context of an orthotopic glioblastoma model, U251 cells were stereotactically implanted into the brains of nude mice as described previously (Kawakami *et al.*, 2004; Allen *et al.*, 2008). A lentiviral vector pseudotyped with the MV H_{ca18-AA} glycoprotein lacking IL-13 was used as a negative control. The vector amount was normalized on the basis of p24 present in the vector samples. A transgene encoding Fluc, the substrate of which, D-luciferin, can cross the blood-brain barrier (Deroose *et al.*, 2006), was used. The results presented in Fig. 7A show that no Fluc activity above background levels was detected in animals that were injected with the control vector bearing the MV H_{ca18-AA} glycoprotein lacking IL-13. However, in animals injected with vector pseudotyped with the MV H_{ca18-AA}-IL-13 glycoprotein, Fluc activity was detected in five of six animals injected, 2 days after vector injection (Fig. 7B). These results are consistent with the view that transduction of U251 tumor cells *in vivo* in the context of intracranial glioblastomas was mediated by IL-13/IL-13R α 2.

Discussion

Efficient transgene delivery *in vivo* using retargeted lentiviral vectors has been challenging because of a number of issues including low titers. A number of approaches aimed at improving the safety and efficiency of transgene delivery *in vivo*, involving retargeted lentiviral vectors, have been described. One such approach uses pseudotyped lentiviral vectors involving engineered versions of the Sindbis virus E2 glycoprotein, allowing ligand-mediated cell targeting in the presence of the Sindbis virus E1 fusion protein (Morizono *et al.*, 2001; Yang *et al.*, 2006). *In vivo* applications of this approach have involved the use of lentiviral vectors to target P glycoprotein-expressing melanoma cells in the lungs of a mouse model of metastatic melanoma (Morizono *et al.*, 2005). A drawback of this approach is that background transduction levels in the absence of a targeting ligand or with cells lacking the corresponding receptors can be substantial. Also, cell entry of retargeted Sindbis virus is pH dependent (Morizono and Chen, 2011); this may limit this approach to receptors that are endocytosed.

Zhang and colleagues (2010) described an alternative targeting strategy involving a cell-specific ligand that is incorporated into lentiviral vector particles containing a truncated version of the VSV-G glycoprotein (VSV-GS) that promotes cell fusion (Jeetendra *et al.*, 2002). In one study, Goyvaerts and colleagues (2012) used this approach to target antigen-presenting cells (APCs) both *in vitro* and *in vivo*. To do this, an APC-specific camelid-derived heavy chain antibody and the VSV-GS fusion protein were displayed on lentiviral vector particles. Antibody-dependent transduction of mouse dendritic cells and macrophages was demonstrated both *in vitro* and *in situ*. An attractive feature of this particular approach is that vectors can be concentrated to high titers (Zhang *et al.*, 2010; Goyvaerts *et al.*, 2012).

FIG. 6. Targeting IL-13R α 2-positive tumor cells *in vivo*, using a subcutaneous tumor model. **(A)** U251 cells were transduced with various amounts of the GLuc-encoding lentiviral vector pseudotyped with MV H_{CA18-AA}-IL-13 and F_{CA30} glycoproteins and assayed for luciferase activity 3 days after transduction. **(B)** U251 cells and DU145 cells were transduced with an EGFP-encoding lentiviral vector pseudotyped with MV H_{CA18-AA}-IL-13/F_{CA30} glycoproteins and analyzed as described for Fig. 2C. **(C)** Mice bearing subcutaneous U251 glioma cell-induced tumors or DU145 prostate carcinoma cell-induced tumors were injected with GLuc-encoding lentiviral vectors pseudotyped with the MV H_{CA18-AA}-IL-13/F_{CA30} glycoproteins; vectors were administered directly into the tumors. Control injections consisted of PBS. Three days later, the mice were imaged with the IVIS system after 100 μ l (2.5 mg/ml) of the coelenterazine substrate was injected into the tumors. Color images available online at www.liebertpub.com/hgtb

The targeting strategy for lentiviral vectors involving engineered MV H and F glycoproteins is promising because of its flexibility and because background transduction levels are low (Funke *et al.*, 2008; Anliker *et al.*, 2010; Munch *et al.*, 2011). Also, because MV enters cells through direct fusion at the cell membrane, it allows for vector entry in a pH-independent

manner. To test the strategy *in vivo*, Munch and colleagues used lentiviral vectors bearing chimeric H proteins containing DARPins to target HER2/neu-positive tumors in SCID mice (Munch *et al.*, 2011), and Anliker and colleagues used a single-chain antibody to target GluA-expressing neurons in mice (Anliker *et al.*, 2010).

FIG. 7. Targeting of IL-13R α 2-positive tumors in an intracranial model. One million U251 cells in 10 μ l of PBS were injected into the brains of 6- to 8-week-old athymic *nu/nu* mice. Five days later, 10- μ l aliquots of Fluc-encoding lentiviral vectors pseudotyped with **(A)** MV H_{CA18-AA}/F_{CA30} or **(B)** MV H_{CA18-AA}-IL-13/F_{CA30} were injected through the same burr hole. The amount of p24 injected for the animals shown in **(A)** was 264 ng, and for the animals shown in **(B)** it was 248 ng. Two days after vector injection, mice were imaged with the IVIS imaging system 15 min after intraperitoneal injection of the luciferin substrate (150 mg/kg body weight). Each group contained 6 mice and 3 mice were imaged together. Color images available online at www.liebertpub.com/hgtb

Here, our work focuses on IL-13-displaying lentiviral vectors bearing MV H and F glycoproteins to target IL-13R α 2-expressing cells *in vitro* and *in vivo*. IL-13 display to retarget DNA viruses such as herpes simplex virus 1 and replication-defective adenoviral (Ad5) vectors has been described previously (Zhou and Roizman, 2006; Ulasov *et al.*, 2007). To selectively target Ad5 to IL-13R α 2-bearing cells, Ulasov and colleagues (2007) constructed a replication-defective adenoviral vector that possesses an IL-13 ligand presented by a bacteriophage T4-derived fibrin shaft. To assess the ability of the Ad5 vector to effectively transduce glioma cell *in vivo*, a subcutaneous flank xenograft model of human glioma in nude mice was established. In this model, IL-13-displaying vectors showed a 300-fold increase in transgene expression as compared with adenoviral vectors lacking IL-13. IL-13R α 2-directed targeting has also been achieved with RNA-containing viruses, and Allen and colleagues (2008) demonstrated that retargeting of replicating measles virus vectors by IL-13 display is feasible. The titer of the retargeted virus was unchanged relative to that of the unmodified virus, and the *in vivo* antitumor activity was comparable to that of the unmodified MV strain. Attempts to target IL-13R α 2-positive cells with murine leukemia virus-based vectors bearing ecotropic Env glycoproteins with IL-13 inserted at various positions were unsuccessful, due in part to blocks in membrane fusion (Ryu *et al.*, 2008).

The results presented in this paper show that IL-13R α 2-positive tumor cells can be targeted selectively both *in vitro* and *in vivo*, using IL-13-displaying lentiviral vectors. An advantage of lentiviral vectors is their ability to transduce actively dividing cells as well as nondividing cells (Reiser *et al.*, 1996). This is an attractive feature given that tumors typically consist of a heterogeneous mixture of cells including slowly dividing cells as well as nondividing cells. For example, it was estimated that only a fraction (1–38%) of all glioma cells are in mitosis at any given time (Hoshino *et al.*, 1989).

To test the specificity of the system, a Dox-inducible HOS cell line that conditionally overexpresses IL-13R α 2 on Dox addition was constructed. We found that lentiviral vectors pseudotyped with the MV F $_{\Delta 30}$ and H $_{\Delta 18-AA}$ -IL-13 proteins efficiently transduced HOS-IL-13R α 2 cells, but not parental HOS cells, in a Dox-dependent manner. These results indicate that transduction of HOS-IL-13R α 2 cells in the presence of Dox, using vectors pseudotyped with the MV H $_{\Delta 18-AA}$ -IL-13 and F $_{\Delta 30}$ glycoproteins, is mediated by IL-13R α 2. Such vectors also transduced human U251 glioma cells, which naturally express high levels of IL-13R α 2, but not T98G glioma cells, which express low levels of IL-13R α 2. Also, transduction was highly specific as it could be blocked with anti-IL-13R α 2 antibody or a soluble recombinant human IL-13 R α 2 Fc chimera.

Our long-term goal is to establish lentivirus-based tumor receptor targeting strategies *in vivo*. Toward this goal we established an orthotopic glioblastoma model using U251 cells that were implanted in the brain of nude mice (Kawakami *et al.*, 2004; Allen *et al.*, 2008). Lentiviral vectors expressing Fluc were injected 5 days later and a lentiviral vector pseudotyped with the MV H $_{\Delta 18-AA}$ glycoprotein lacking IL-13 was used as a negative control. Consistent with the *in vitro* experiments, mice bearing U251 tumors were positive by luciferase imaging with lentiviral vectors displaying the MV H $_{\Delta 18-AA}$ -IL-13 glycoprotein but not with lentiviral vectors containing the MV H $_{\Delta 18-AA}$ glycoprotein

lacking IL-13. This indicates that that transduction was mediated by IL-13R α 2 present on tumor cells. This view is supported by the observation that IL-13R α 2 levels on non-tumor cells are low (Joshi and Puri, 2009).

Successful tumor targeting involving lentiviral vectors injected systemically will be dependent on a number of factors such as increased vector titers and improvements in receptor affinities including binding thresholds for membrane fusion triggering (Hasegawa *et al.*, 2007). Mutant IL-13 proteins that demonstrate a higher affinity for the IL-13R α 2 receptor have been described previously. For example, substitution of a glutamine acid residue at position 13 with a lysine residue resulted in an IL-13 mutant protein (IL-13 E13K) that bound receptor-expressing tumor cells with 3- to 10-fold higher affinity compared with unmodified IL-13 (Debinski *et al.*, 1998; Oshima *et al.*, 2000). Nevertheless, when the IL-13 E13K mutant protein was fused to *Pseudomonas* exotoxin, the increase in affinity did not translate into superior antitumor efficacy compared with the unmodified IL-13 fusion protein (Kioi *et al.*, 2004). In a different experimental system, an IL-13 mutant protein (IL-13 E13Y) that revealed a 20 times higher affinity for the IL-13R α 2 receptor and a 5 times lower affinity for the IL-13R α 1/IL-4 receptor specifically targeted glioblastoma multiforme cells when displayed on cytotoxic T lymphocytes (Kahlon *et al.*, 2004). The performance of these mutant IL-13 proteins in the context of retargeted lentiviral vectors remains to be determined.

In summary, we have displayed IL-13 on lentiviral vector particles through fusion to the C terminus of the MV H protein, whose binding sites for the native receptors were mutated. The specificity of the *in vitro* transduction process was demonstrated with specific antibodies against IL-13R α 2 and soluble IL-13R α 2. Also, we were able to demonstrate, using luciferase-based imaging, that lentiviral vectors displaying IL-13 specifically targeted IL-13R α 2-positive U251 cells but not IL-13R α 2-negative DU145 cells *in vivo*. We are currently working on improved methods for vector production and concentration to increase vector titers for systemic delivery of retargeted lentiviral vectors in the context of *in vivo* models.

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Author Disclosure Statement

The authors have no competing interests to disclose.

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