Pseudotyping Lentiviral Vectors with Lymphocytic Choriomeningitis Virus Glycoproteins for Transduction of Dendritic Cells and *In Vivo* Immunization

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Abstract

Lentiviral vectors (LVs) are promising delivery systems for gene therapy, and they can be further engineered to increase their potential for effectively delivering transgenes to desired cell populations. Here, we have engineered LVs pseudotyped with envelope glycoproteins derived from lymphocytic choriomeningitis virus (LCMV) for antigen delivery to elicit vaccine-directed immune responses. Two variants, LCMV-WE and LCMV-Arm53b, were evaluated for their ability to mediate LV-based cellular transduction *in vitro*. LCMV-WE with a leucine residue at position 260 (260L) is known for its high-affinity binding with a cellular receptor, α -dystroglycan (α -DG), whereas LCMV-Arm53b has low-affinity binding resulting from a phenylalanine residue at the same position. In contrast to LCMV-Arm53b, we found that LVs pseudotyped with LCMV-WE could transduce 293T cells and murine dendritic cells much more efficiently based, at least in part, on their favorable interaction with α -DG. In mice, LCMV-WE-bearing LVs encoding a model antigen, invariant chain ovalbumin, could elicit substantial antigen-specific CD8⁺ T cell immune response. The response could be further enhanced by a homologous boosting immunization with the same vector. These findings offer evidence to support the potential utilization of LCMV-WE-bearing LVs for vectored vaccines against cancer and infectious diseases.

Introduction

LENTIVIRUS-BASED VIRAL VECTORS, commonly termed lentiviral vectors (LVs), such as human immunodeficiency virus type 1 (HIV-1), have been utilized widely as a consequence of their highly efficient transduction, their ability to transduce both dividing and nondividing cells (Naldini *et al.*, 1996), their ability to integrate transgenes into host cell genome (Kootstra and Verma, 2003), and their low immunogenicity (Abordo-Adesida *et al.*, 2005). However, the natural tropism of HIV-1 envelope (Env) glycoprotein to CD4⁺ T cells and macrophages has restricted the host range.

To address this complication, one strategy modifies LVs by replacing the original Env glycoprotein with glycoproteins from other enveloped viruses. These phenotypically mixed particles, or pseudotypes, are constructed by incorporating heterologous glycoproteins onto the LV surface through the budding process in the producing cells (Cronin *et al.*, 2005). The most commonly used Env glycoprotein of

pseudotyped LVs is derived from vesicular stomatitis virus (VSV-G), which mediates viral entry through the binding of membrane phospholipids (Froelich *et al.*, 2010; Giacca and Zacchigna, 2012), and a recent study showed that LDL receptor serves as the cellular receptors for VSV-G-pseudotyped LVs (Finkelshtein *et al.*, 2013). Although VSV-G-pseudotyped LVs have broadened the range of infected cell types and possess good stability (Froelich *et al.*, 2010), they also have some disadvantages, including toxicity of constitutive expression in producer cell lines and the susceptibility of inactivation by human complement (Ory *et al.*, 1996; DePolo *et al.*, 2000). Therefore, glycoproteins of other enveloped viruses have been introduced to LVs with the aim of improving safety and/or efficiency.

Lymphocytic choriomeningitis virus (LCMV) is a rodent-borne, enveloped, and bisegmented single-stranded RNA virus belonging to *Arenaviridae* family of viruses. The entry of LCMV into cells is mediated by a fusion glycoprotein (GP) located in the outer lipid envelope of the virus. This

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protein, encoded by the RNA fragment, is first translated into a precursor protein and then cleaved into two subunits, GP1 and GP2 (Reignier *et al.*, 2006). GP1 is a peripheral membrane protein that contains the binding site for the cellular receptor (Parekh and Buchmeier, 1986). GP2 is an integral membrane protein and anchors the complex in the viral and host cell membrane (Burns and Buchmeier, 1991).

Several LCMV strains and other arenaviruses have been found to enter cells via interaction with a cellular receptor termed α -dystroglycan (α -DG) (Cao *et al.*, 1998). α -DG is the peripheral moiety of DG, which is highly conserved and ubiquitously expressed on the cell surface as the receptor for extracellular matrix proteins (Henry and Campbell, 1996; Cao *et al.*, 1998; Durbeej *et al.*, 1998). DG is initially encoded as a single polypeptide and then cleaved into extracellular α -DG and membrane-anchored β -DG (Holt *et al.*, 2000; Barresi and Campbell, 2006). The binding affinity of LCMV to α -DG determines viral tropism and infection outcome in mice (Cao *et al.*, 1998; Zajac *et al.*, 1998; Smelt *et al.*, 2001). By evaluating the binding interaction between α -DG and several LCMV strains and variants, these viruses could be divided into two groups with respect to the affinity of α -DG binding (Smelt *et al.*, 2001).

Viruses exhibiting high-affinity binding to α -DG, such as the Cl13 variant, were blocked by soluble α -DG from infecting 3T6 mouse fibroblasts and could infiltrate the spleen white pulp area, causing ablation of cytotoxic T-lymphocyte (CTL) response postinfection in mice (Smelt *et al.*, 2001). This immunosuppressive variant showed a preferential replication mainly in CD11c⁺ and DEC-205⁺ dendritic cells (DCs) in spleen (Sevilla *et al.*, 2000). Other viruses, such as Arm53b, with low-affinity binding to α -DG were only partially inhibited and were found to be restricted to the red pulp area of spleen (Smelt *et al.*, 2001). A point mutation in the LCMV glycoprotein was required for receptor α -DG binding and long-term persistence (Salvato *et al.*, 1991; Sullivan *et al.*, 2011). Cl13 with the mutation (L at GP1 260) possesses exceptionally strong binding affinity to α -DG, which is expressed mostly on DCs, and this is essential for mediating long-term persistence in Cl13 infection (Sevilla *et al.*, 2003; Sullivan *et al.*, 2011). With natural tropism for DCs, LCMV has already been evaluated as a vaccine carrier. One study showed that engineered LCMV vaccine vectors elicited efficient CTL responses. This mechanistic study further verified that the vectors target and activate DCs *in vivo* (Flatz *et al.*, 2010).

The glycoprotein of LCMV also represents the only target for LCMV neutralizing antibodies (nAbs) (Flatz *et al.*, 2010). Previous findings indicated that the kinetics of nAbs was solely determined by the viral glycoprotein and not by the virus backbone (Pinschewer *et al.*, 2004). The nAb response to LCMV is weak, and convalescent serum fails to prevent reinfection (Pinschewer *et al.*, 2004; Eschli *et al.*, 2007; Flatz *et al.*, 2010). Although viral vectors failed to elicit vector-specific antibody immunity in one study of LCMV-based vaccine delivery, CTL response was boosted after readministration of the same vectors (Flatz *et al.*, 2010). More recently, the glycoprotein of LCMV WE strain was used for pseudotyped vesicular stomatitis virus as vaccine vectors. Since these vaccine vectors did not induce nAbs to the vector itself, it was possible to boost with the same vectors (Tober *et al.*, 2014).

In the present study, the envelope glycoprotein from two LCMV strains, WE and Arm53b, was used to pseudotype LVs. WE is originated from one of the immunosuppressive LCMV strains that adopts the F260L mutation. We found that LVs bearing glycoproteins from the WE strain could efficiently transduce cells through interaction with the cellular receptor α -DG, while Arm53b-pseudotyped LVs obtained lower titer by 1–2 logs in the 293T cell line and could barely transduce bone marrow-derived DCs (BMDCs). Direct administration of the recombinant LVs pseudotyped with WE glycoprotein encoding a model ovalbumin (OVA) antigen induced a strong antigen-specific T-cell immune response *in vivo*, and the response could be enhanced via homologous boost injection. These results indicate that the LCMV-WE glycoprotein could be a promising candidate to pseudotype LVs for vaccine delivery.

Materials and Methods

Mice

C57BL/6 mice were purchased from the Jackson Laboratory. All mice were held under specific pathogen-reduced conditions in the animal facility of the University of Southern California. All experiments were performed in accordance with the guidelines set forth by the National Institutes of Health and the University of Southern California on the Care and Use of Animals.

Plasmid construction

The HIV-1-based vector FUGW expressing green fluorescence protein (GFP) driven by human ubiquitin-C promoter was described previously (Lois *et al.*, 2002). FUGW-Ii-OVA was generated by replacing GFP with the C-terminal end of invariant chain (Ii) fused with the amino acid sequences spanning positions 242–353 in truncated chicken OVA (Diebold *et al.*, 2001). The two plasmids of LCMV envelope glycoproteins are phCMV-LCMV-WE (GenBank ID: AF186080) and phCMV-LCMV-Arm53b (GenBank ID: AY847350). Plasmid phCMV-LCMV-WEmu, which carries an L260F mutation, was generated from the parent phCMV-LCMV-WE using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies).

Production of pseudotyped viral particles

Recombinant LVs were generated in the producer 293T cell line by transient transfection through the standard calcium phosphate precipitation protocol (Pear *et al.*, 1993). The 293T cells were cultured in 6 cm tissue culture plates (BD Biosciences) and D10 medium (Dulbecco's modified Eagle's medium; Invitrogen) supplemented with 10% fetal bovine serum (Sigma-Aldrich), 2 mM L-glutamine, 100 U/ml of penicillin, and 100 mg/ml of streptomycin. Cells were cotransfected with the lentiviral backbone plasmid (FUGW or FUGW-Ii-OVA) (5 μ g), packaging plasmids pMDLg/pRRE and pRSV-REV (2.5 μ g each), and the envelope plasmids phCMV-LCMV-WE, phCMV-LCMV-Arm53b, or phCMV-LCMV-WEmu (2.5 μ g). The viral supernatants were harvested 48 hr posttransfection and filtered through a 0.45 μ m filter (Nalgene). To prepare the concentrated virus, the viral supernatants were submitted to ultracentrifuge (Optima L-80 XP Preparative Ultracentrifuge; Beckman Coulter) at

50,000 *g* for 90 min. The viral pellets were then resuspended in an appropriate volume of cold phosphate buffered saline (PBS) buffer. The number of viral particles was quantified by quantitative reverse-transcription (RT) PCR. Briefly, the viral RNA was purified from viral supernatants using the QIAamp Viral RNA mini kit (Qiagen) and quantified using the Lenti-X qRT-PCR Titration Kit (Clontech). The number of viral RNA copies was calculated based on the standard curve.

LV transduction

293T cells were plated in 96- or 24-well culture plates and spin-infected with viral supernatant (100 μ l per well or 1 ml per well) at 2500 rpm and 25°C for 90 min, using a Sorvall RT Legend centrifuge. Subsequently, the supernatant was replaced with fresh culture medium and incubated at 37°C with 5% CO₂ for 3–5 days. For the FUGW-based vector, GFP expression measured by flow cytometry was used to determine the vector titer. The transduction titer was determined by the dilution range that exhibited a linear response. For the FUW-Ii-OVA-based vector, the titer was determined by measuring OVA expression in transduced cells via qPCR. Briefly, genomic DNA of transduced cells was purified using DNeasy Blood & Tissue Kit (Qiagen). The number of OVA transgene copies was quantitated by qPCR using an OVA-specific primer pair (sense primer: 5'-TCA AAG TGT ACT TAC CTC GCA TGA A-3'; antisense primer: 5'-TGC CCA TAG CCA TTA AGA CAG A-3') and calculated based on the standard curve.

Transduction of DCs in vitro

Murine bone marrow cells were isolated from the femur of C57BL/6 mice, and BMDCs were generated as previously described (Yang and Baltimore, 2005). BMDCs were plated in 24-well culture dishes and spin-infected with viral supernatants (2 ml per well) at 2500 rpm and 25°C for 90 min, using the same Sorvall RT Legend centrifuge. The supernatant was then replaced with fresh RPMI medium with 10% FBS and GM-CSF (from J558L cells, 1:20 conditioned medium). The cells were cultured 5 days before performing flow cytometric analysis of GFP expression.

Flow cytometric analysis of cell-surface protein expression

293T cells were stained with anti- α -DG monoclonal antibody (mAb) IIH6C4 (Millipore) for 10 min under 4°C, washed, and then stained with Alexa488-conjugated goat anti-mouse antibody (Invitrogen) at 4°C for 10 min. To analyze BMDCs, IIH6C4 was first labeled with NHS-fluorescein and purified using the FITC and Fluorescein Dyes and Labeling Kits (Thermo Scientific). BMDCs were blocked with anti-mouse CD16/CD32 (BD Bioscience) and stained with the labeled antibody for 10 min at 4°C. The stained cells were then analyzed by flow cytometry.

Assays to examine receptor-mediated transduction

293T cells (2×10^4) or BMDCs (1×10^5) were incubated for 45 min with anti- α -DG mAb IIH6C4 (200 μ g/ml), with isotype-matched mouse mAb IgG_{2b, κ} (BD Biosciences) (200 μ g/ml), or without reagent. The cells were then infected

with the LCMV-WE-enveloped or VSV-G-enveloped FUGW vector (MOI <1) overnight in the presence of blocking reagents. The supernatants were then replaced by fresh medium. GFP expression was assayed by flow cytometry 3 days posttransduction for 293T cells and 5 days for BMDCs.

Confocal imaging

Fluorescence images were acquired on a Yokogawa spinning-disk confocal scanner system (Solamere Technology Group) using a Nikon eclipse Ti-E microscope (Nikon) equipped with an X60/1.49 Apo TIRF oil objective and a Cascade II: 512 EMCCD camera (Photometrics). Transduced BMDCs in a 96-well plate were washed with cold PBS and fixed with 4% formaldehyde, and then the cells were permeabilized with 0.1% Triton X-100 and stained with DAPI (4',6-diamidino-2-phenylindole). The images were analyzed by the Nikon NIS Element software.

In vivo immunization and analysis of immune responses

The concentrated viral vectors (FUW-Ii-OVA) were injected into C57BL/6 mice through footpad (f.p.), intravenous (i.v.), intraperitoneal (i.p.), or intradermal (i.d., at the base of tail) routes. The immunized mice were analyzed for immune responses 2 weeks after injection. For the analysis of immune responses, mouse splenocytes were collected and washed with PBS. Splenocytes were stimulated for 6 hr with OVA (257SIINFEKL264) peptide (1 μ g/ml), with GolgiPlug (BD Biosciences) to inhibit IFN- γ secretion. The cells were stained with anti-mouse CD16/32 to block Fc receptors and stained with anti-mouse CD8-FITC and anti-mouse CD4-PE/Cy5. Cells were then permeabilized and stained with anti-mouse IFN- γ -PE. Stained cells were analyzed by flow cytometry. All monoclonal antibodies for staining were from BD Biosciences or BioLegend.

Neutralizing antibody assay

Vector-specific nAb responses were assessed by a GFP-based virus neutralization assay. The 293T cells were plated at the density of 2×10^4 cells per well in a 96-well plate. FUGW lentiviral particles pseudotyped with either LCMV-WE or VSV-G were then added at a MOI=0.2 with 2-fold serial dilutions of serum from mice immunized with either LCMV-WE- or VSV-G-enveloped FUW-Ii-OVA. Spin infection was conducted at 2500 rpm and 25°C for 90 min, and the supernatants were replaced with fresh culture media. After culturing for an additional 3 days, the GFP expression was evaluated by flow cytometry. The neutralization titer was defined as the maximum serum dilution that neutralized 50% of GFP expression.

Results

Generation of LCMV-pseudotyped LVs

Previously, glycoproteins from the *Arenaviridae* family, including LCMV, were shown to be capable of incorporating into retroviral and LVs, generating functional surrogates for the study of arenavirus entry (Beyer *et al.*, 2002; Dylla *et al.*, 2011). In the present study, HIV-1-derived LVs bearing LCMV glycoproteins were generated by transient

cotransfection with the lentiviral backbone FUGW (Fig. 1A) and packaging plasmids encoding *gag*, *pol*, *rev*, and the respective envelope GP gene. Two constructs encoding the two strains of LCMV GPs, WE and Arm53b, were evaluated for their ability to pseudotype LVs (Fig. 1A). The infectious titers of pseudotyped LVs were measured on the 293T cell line, which is considered generally permissive to infection and is frequently chosen for titration (Cronin *et al.*, 2005). LVs pseudotyped with VSV-G (FUGW/VSV-G) could transduce 293T cells with a titer of more than $\sim 10 \times 10^6$ transduction unit (TU)/ml. When the LCMV glycoprotein WE was incorporated into LV particles (FUGW/LCMV-WE), the transduction in 293T cells was $\sim 3 \times 10^6$ TU/ml,

FIG. 1. Generation of lentiviral vectors pseudotyped by LCMV glycoprotein. (A) Schematic diagrams of constructs encoding the lentiviral backbone FUGW and the envelope glycoprotein. Ubi, human ubiquitin-C promoter; GFP, enhanced green fluorescence protein; WRE, woodchuck hepatitis virus posttranscriptional regulatory element to increase the level of transcription; ΔU3, deleted U3 region, resulting in the transcriptional activation of the integrated viral LTR promoter; pA, polyadenylation signal; LCMV-WE and LCMV-Arm53b, LCMV-WE- and LCMV-Arm53b-expressing plasmids containing the rabbit β-globin intron and polyadenylation signal. (B) Transduction titer of LVs was quantified by serially diluting fresh viral supernatants of FUGW/LCMV-WE, LCMV-Arm53b, and FUGW/VSV-G, followed by transduction of 2×10^4 293T cells. Three days later, the transduction efficiency was measured by analyzing GFP expression using flow cytometry, and the corresponding viral titer was calculated. LCMV, lymphocytic choriomeningitis virus; LVs, lentiviral vectors.

TABLE 1. TRANSDUCTION TITERS OF LCMV-WE, LCMV-ARM53B, AND VSV-G-PSEUDOTYPED LENTIVIRAL VECTORS IN 293T CELLS

Envelope glycoprotein	Transduction titer (TU/ml)
LCMV-WE	$(3.16 \pm 0.21) \times 10^6$
LCMV-Arm53b	$(1.24 \pm 0.58) \times 10^5$
VSV-G	$(2.80 \pm 0.89) \times 10^7$

LCMV, lymphocytic choriomeningitis virus.

no more than ~ 4 -fold lower than the VSV-G pseudotype. However, the titer of Arm53b-pseudotyped LVs (FUGW/LCMV-Arm53b) was about 10 times lower than that of FUGW/LCMV-WE (Fig. 1B and Table 1).

The different vector–receptor interactions offered by these two glycoproteins could explain the difference in titer that resulted. Considering that LCMV-WE with the F260L mutation has a high binding affinity to the cellular receptor α-DG, a reverse mutation was made on LCMV-WE to (1) restore phenylalanine (F) at position 260 (designated as LCMV-WE_{mu}, Fig. 2A) and (2) determine whether this amino acid was crucial for the transduction of 293T cells. Of the three GPs, FUGW/LCMV-WE yielded the highest percentage of GFP-positive cells, while LVs bearing either LCMV-WE_{mu} or LCMV-Arm53b still showed low transduction efficiency (Fig. 2B).

We next produced LVs with the three LCMV-GPs encoding a transgene of an invariant chain OVA (Ii-OVA) protein (Fig. 2B). Quantitative PCR was employed to measure the yield of the physical LV particles and transduction titer. The LV yield based on genomic RNA copy of LCMV-WE-pseudotyped LVs (FUGW-Ii-OVA/LCMV-WE) was $(6.4 \pm 0.7) \times 10^8$ copies/ml, while FUGW-Ii-OVA pseudotyped with LCMV-WE_{mu} and Arm53b possessed an even higher copy number [$(1.4 \pm 0.1) \times 10^{10}$ copies/ml and $(1.0 \pm 0.1) \times 10^{10}$ copies/ml, respectively] (Fig. 2C). However, in sharp contrast to the genome titer, the transduction capability of FUGW-Ii-OVA/LCMV-WE at a titer of $(2.7 \pm 0.6) \times 10^6$ TU/ml was better than the other two pseudotyped LVs in that LVs bearing LCMV-WE_{mu} and Arm53b yielded approximately one log lower titer in 293T cells (Fig. 2C and Table 2).

α-DG-mediated transduction by LCMV-WE-enveloped LVs

LCMV-WE glycoprotein with a leucine on amino acid 260 (L260) had high binding affinity to the cellular receptor α-DG, but the mutation of L260F caused a loss in 293T transduction titer. Therefore, to investigate whether α-DG expression correlated with transduction, 293T cells were stained by an anti-α-DG monoclonal antibody, IIH6C4, followed by flow cytometry. IIH6C4 is an anti-α-DG antibody that recognizes the laminin binding site of α-DG. More than 40% of 293T cells were found with positive signals of α-DG expression (Fig. 3A). This could explain the observation that LCMV-WE-pseudotyped LVs transduced 293T cells more efficiently because LVs bearing LCMV-WE could bind to the 293T cell surface through the interaction with the α-DG receptor and then enter the cells. To further examine this receptor-mediated transduction, we performed

FIG. 2. Impact of F260L mutation in LCMV GP1 based on its ability to mediate LV transduction. **(A)** GP1 of LCMV-WE was mutated at amino acid position 260 to produce a low-affinity α -DG binding envelope (LCMV-WE_{mu}) similar to the wild-type envelope of LCMV-Arm53b. **(B)** Transduction in 293T cells by LVs pseudotyped with LCMV-WE, LCMV-WE_{mu}, and LCMV-Arm53b. **(C)** *Upper:* Schematic diagram of the construct FUW-li-OVA encoding invariant chain OVA; *lower:* comparison of viral RNA copy number and transduction titer of FUW-li-OVA enveloped with LCMV-WE, LCMV-WE_{mu}, or LCMV-Arm53b.

an inhibition assay, again using IIH6C4. In the presence of increasing concentrations of IIH6C4, we found that transduction was progressively inhibited.

With IIH6C4 treatment at a concentration of 200 μ g/ml, the transduction of FUGW/LCMV-WE was inhibited by more than 60%. In contrast, FUGW/LCMV-WE-mediated transduction resulted in a moderate reduction upon the treatment of isotype-matched control antibody at the same concentration (Fig. 3B and C). VSV-G-pseudotyped LVs, which has broad tropism, were also used as a control in the inhibition assay. 293T cells were incubated with FUGW/LCMV-WE and FUGW/VSVG after the IIH6C4 treatment. The inhibition was only found in FUGW/LCMV-WE transduced cells (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/hgtb). The LCMV viral binding site on α -DG was mapped between

amino acids 169 and 408, which overlaps, at least partially, with the binding site of laminin-1 (Imperiali *et al.*, 2005). These results indicated that the transduction of LCMV-WE-pseudotyped LVs depended, at least partially, on receptor α -DG expression on the target cell surface.

DC transduction by LCMV-pseudotyped LVs

Wild-type LCMV exhibits tropism for DCs. LCMV strains and variants with high binding affinity to α -DG, such as C113, are associated with preferential replication in a majority of CD11c⁺ and DEC-205⁺ cells, leading to immunosuppression (Borrow *et al.*, 1995; Sevilla *et al.*, 2000). CD11c⁺ cells represent a major DC population in the spleen expressing α -DG. Therefore, we further evaluated α -DG expression on DCs and its role in DC transduction. To

TABLE 2. VIRAL PARTICLE TITER AND TRANSDUCTION TITERS OF PSEUDOTYPED LENTIVIRAL VECTORS IN 293T CELLS

Lentiviral vector	Viral particles (genome copies/ml)	Transduction titer (TU/ml)
FUW-IiOVA/LCMV-WE	$(6.4 \pm 0.7) \times 10^8$	$(2.7 \pm 0.6) \times 10^6$
FUW-IiOVA/ LCMV-WE _{mu}	$(1.4 \pm 0.1) \times 10^{10}$	$(1.4 \pm 0.2) \times 10^5$
FUW-IiOVA/ LCMV-Arm53b	$(1.0 \pm 0.1) \times 10^{10}$	$(0.8 \pm 0.1) \times 10^5$

accomplish this, BMDCs were prepared by isolating bone marrow from C57BL/6 mice and culturing with the cytokine GM-CSF. Anti- α -DG antibody IIH6C4 conjugated with fluorescein was used to probe α -DG expression on the cultured BMDCs. From the flow cytometric result shown in Fig. 4A, it was apparent that CD11c⁺ cells expressed α -DG, while no significant difference was observed between the isotype-matched control and the anti- α -DG antibody for CD11c⁺ cells.

We then evaluated the transduction of BMDCs by FUGW/LCMV-WE, FUGW/LCMV-WE_{mu}, and FUGW/LCMV-Arm53b with equal viral particle amount quantified by viral genome RT-PCR. As shown in Fig. 4B, the percentage of GFP⁺CD11c⁺ cells in FUGW/LCMV-WE transduction is around 9.48%, yet FUGW/LCMV-WE_{mu} and FUGW/LCMV-Arm53b can barely transduce DCs. In order to examine the transduction specificity of FUGW/

LCMV-WE, FUGW/VSV-G was used as a transduction control. Flow cytometry showed that LVs bearing LCMV-WE had a greater specificity in CD11c⁺ cells compared to that of VSV-G (Fig. 4B). We also investigated the capability of IIH6C4 to block the transduction of FUGW/LCMV-WE on BMDCs. Microscopic imaging revealed that GFP expression was downregulated after incubation with IIH6C4, whereas no significant change of GFP signal occurred after incubation with the isotype-matched control antibody (Fig. 4C). These results suggested that the interaction with receptor α -DG also mediated the LCMV-WE transduction in BMDCs.

Antigen-specific CD8⁺ T cell responses by LCMV-pseudotyped LVs

DCs are specialized antigen-presenting cells that can present the transgene antigen to other cells of the immune system like T cells. Since we observed that LCMV-WE-pseudotyped LVs could effectively transduce BMDCs *in vitro*, the potential of this vector to be used for vectored vaccines was evaluated. Naive C57BL/6 mice were immunized with a single injection of FUW-Ii-OVA/LCMV-WE ($\sim 1 \times 10^7$ TU) by the f.p., i.v., i.p., and i.d. (at the base of tail) routes. OVA-specific CD8⁺ T cells were monitored by intracellular cytokine staining (ICCS) of harvested splenocytes stimulated with an OVA-specific peptide 2 weeks postimmunization. By comparison, immunization through the f.p. route resulted in the highest percentage of OVA-specific CD8⁺ T cells ($\sim 15.2\%$, Fig. 5A). Intradermal injection elicited around 7.65% OVA-specific CD8⁺ T cell responses, while the other two routes yielded much lower

FIG. 3. Role of α -DG in mediating transduction of LVs pseudotyped with LCMV glycoprotein. (A) Surface staining of α -DG expression on 293T cells using anti- α -DG mAb IIH6C4 (solid fill: without anti- α -DG mAb; open fill: with anti- α -DG mAb). (B) 293T cells (2×10^4) were incubated for 45 min with anti- α -DG mAb (200 μ g/ml), with isotype-matched mAb (200 μ g/ml), or without reagent. The cells were then infected with FUGW/LCMV-WE (MOI < 1) overnight in the presence of blocking reagents. GFP expression was assayed by flow cytometry 3 days posttransduction where one representative result is shown with indicated values. (C) The transduction value was given as a mean of duplicates $\pm$ SD.

FIG. 4. Receptor-mediated transduction of BMDCs by LCMV-WE-pseudotyped LVs. **(A)** BMDCs were stained with NHS-fluorescein-labeled anti- α -DG mAb IIH6C4 and analyzed by flow cytometry. **(B)** BMDCs were transduced by FUGW/LCMV-WE, FUGW/LCMV-WE mu, and FUGW/LCMV-Arm53b with the equal amount of viral particles quantified by viral genome RT-PCR. Five days after transduction, BMDCs were stained by PE/cy5-anti-CD11c, and GFP expression was analyzed by flow cytometry. **(C)** BMDCs were transduced by FUGW/LCMV-WE or the control FUGW/VSVG-G with the same transduction units. GFP expression was analyzed with the same method in **(B)**. **(D)** Fluorescence images of BMDC inhibition assay. BMDCs (1×10^5) were incubated for 1 hr with anti- α -DG (200 μ g/ml), isotype-matched (200 μ g/ml) antibody, or without reagent. The cells were then transduced with FUGW/LCMV-WE (MOI < 1) overnight in the presence of blocking reagents. Cells expressing GFP were enumerated 5 days posttransduction, and the value was given as a mean of triplicates $\pm$ SD. BMDCs, bone marrow-derived dendritic cells. Color images available online at www.liebertpub.com/hgtb

FIG. 5. Induction of CD8⁺ T cell responses by LCMV-pseudotyped LVs. **(A)** C57BL/6 mice were immunized with 1×10^7 TU of FUGW-li-OVA/LCMV-WE via footpad (f.p.), intravenous (i.v.), intraperitoneal (i.p.), and intradermal (i.d. at the base of tail) routes. Two weeks postimmunization, spleen cells were restimulated *in vitro* with the OVA peptide, and the IFN- γ response was evaluated by ICCS. **(B)** To characterize the dose-dependent immunization, various indicated doses of FUGW-li-OVA/LCMV-WE were injected into C57BL/6 mice via the f.p. route, and the CD8⁺ T cell frequencies specific for OVA were assessed 2-week postimmunization by ICCS. ICCS, intracellular cytokine staining.

responses. LCMV-Arm53b-pseudotyped LVs were also administered in naive C57BL/6 mice through the same injection routes, and it was found that injection through the f.p. route resulted in the highest percentage of OVA-specific CD8⁺ T cells (~6.1%, Supplementary Fig. S2), which is significantly lower than the immune response generated by LCMV-WE-enveloped LVs. This was consistent with the lower transduction of BMDCs *in vitro* by the LCMV-Arm53b vector (Fig. 4B).

To further characterize the immunogenicity of LCMV-pseudotyped LVs, a dose-dependent assay was conducted. Several doses of FUGW-Ii-OVA/LCMV-WE were injected into C57BL/6 mice by the f.p. route, and after 2 weeks, the OVA-specific CD8⁺ T cell frequencies were assessed (Fig. 5B). At the lowest viral vector dose of 2×10^5 TU, almost no significant CD8⁺ T cell response was noted, and the immune response remained at a low level between 2% and 3%, as the dose was increased to 2×10^6 TU, whereas at the higher dose of 2×10^7 TU, the CD8⁺ T cell frequency rose sharply to 12–13%. At the highest dose in the range we tested (6×10^7 TU), an impressive response level of approximately 20% was observed. This dosage study revealed that the effective range of LCMV-WE-bearing LVs for immunization was $>10^6$ TU per mouse.

Enhanced OVA-specific immunity by homologous prime-boost regimen

The efficacy of LCMV-pseudotyped LVs was then characterized in homologous prime/boost vaccination. Two groups of C57BL/6 mice received LVs of FUGW-Ii-OVA/LCMV-WE with the dose of $\sim 8 \times 10^6$ TU on day 0. Two weeks later, IFN- γ -producing CD8⁺ T cells were quantified by flow cytometry for the prime immunization (Fig. 6A). On day 30, C57BL/6 mice were boosted with the same vector at the same dose. On day 40, the boosting immune response was examined by IFN- γ -producing CD8⁺ T cells. The result showed that the prime/boost regimen attained 2–3-fold higher frequency of OVA-specific CD8⁺ T cells.

It has been demonstrated that LCMV virions trigger a slow and poor nAb response (Pinschewer *et al.*, 2004) and that antibodies to LCMV have only a limited capacity to prevent reinfection (Fitz *et al.*, 2010). Moreover, studies have shown that the nAb response is primarily determined by the viral glycoprotein (Pinschewer *et al.*, 2004). Such results prompted us to investigate the antivector immunogenicity of LCMV-bearing LVs by measuring the vector-specific nAb titer in immunized mice. To do this, FUGW/LCMV-WE (MOI <0.2) was incubated with the serum

FIG. 6. OVA-specific immunity by homologous prime-boost regimen and induction of nAb against the vector. **(A)** C57BL/6 mice were injected with PBS as a control or FUGW-Ii-OVA/LCMV-WE ($\sim 10^7$ TU). Two weeks later, IFN- γ -secreting CD8 T cells within the freshly harvested splenocytes were assessed for immune responses in the prime phase. At day 30, FUGW-Ii-OVA/LCMV-WE ($\sim 10^7$ TU) was injected as homologous boost immunization. At day 40, splenocytes were restimulated *in vitro* with the OVA peptide, and the IFN- γ response was evaluated by ICCS. **(B)** Antivector immune responses were assessed 2 weeks postimmunization by a neutralization assay. Naive C57BL/6 mice were immunized with FUGW-Ii-OVA/LCMV-WE and FUGW-Ii-OVA/VS-G at $\sim 10^7$ TU. Student's *t*-test (two-tailed) was used for the statistical analysis. The experiment was duplicated, and error bars indicate mean $\pm$ SD.

from mice immunized with FUW-Ii-OVA/LCMV-WE, followed by transduction of 293T cells. The nAb titer was calculated from the dilution that caused 50% decrease of vector transduction in 293T cells. From the data in Fig. 6B, the titer elicited by LCMV-WE was around 10, which was 7–8-fold lower than that generated by VSV-G-pseudotyped LVs. This lower titer was consistent with the result of homologous boosting immunization by LCMV-WE-enveloped LVs, which requires that the antivector antibody response in the prime phase be so low that it is incapable of significantly blocking vectors administered in the boosting phase.

Discussion

In this work, we demonstrated that LCMV GP could successfully pseudotype HIV-1-derived LVs and that LVs carrying LCMV GP could induce potent antigen-specific CD8⁺ T cell immunity *in vivo*. LCMV belongs to the *Arenaviridae* family, and its glycoprotein mediates receptor binding and cell entry (Borrow and Oldstone, 1992; Cao *et al.*, 1998; Reignier *et al.*, 2006). Envelope proteins from two LCMV strains, LCMV-WE and LCMV-Arm53b, were utilized to pseudotype HIV-1-derived LVs. Based on the distinct binding affinity to cell receptor α -DG, two functional groups have been established among LCMV strains and variants (Smelt *et al.*, 2001). LCMV-WE and LCMV-Arm53b, coming from the two groups, differ in the genome sequence causing mutation F260L on GP1, which is associated with the binding to the cellular receptor α -DG. LCMV-Arm53b was previously reported in pseudotyping HIV-1-based LVs, and the titer was around two logs lower than that of VSV-G pseudotype (Cronin *et al.*, 2005). Interestingly, LVs with LCMV-WE yielded a titer similar to that of amphotropic MLV and VSV-G (Cronin *et al.*, 2005). In the present study, incorporation of the glycoprotein WE and Arm53b onto LVs resulted in significantly different 293T transduction in that the titer of LVs bearing WE was consistently about 10-fold higher than that of Arm53b.

To investigate whether the F260L mutation could alter viral entry, a mutant construct LCMV-WE_{mu} was generated, carrying the same sequence of LCMV-WE except for the replacement of L with F at amino acid 260. By comparing the genomic viral RNA of WE, WE_{mu}, and Arm53b, it was found that WE_{mu} and Arm53b possessed around 1 log higher viral RNA copies than WE with the same amount of viral supernatant. However, in line with analysis of GFP expression, the DNA copies of the OVA transgene in transduced 293T were at least 10-fold higher in the WE strain than the other two. Studying LCMV receptor function via α -DG O-mannosylation (Imperiali *et al.*, 2005) used the antibody clone VIA4-1 to detect the expression of α -DG in a 293T cell line. In addition to VIA4-1, we herein used another anti- α -DG mAb, IIH6C4, to stain the 293T cell surface. More than 40% cells were positive for α -DG expression, which could explain why LCMV-WE-pseudotyped LVs could transduce the 293T cell line much more effectively even with fewer viral particles in the viral supernatant.

To further validate that LCMV-WE could enter 293T cells via interaction with α -DG, the anti- α -DG clone IIH6C4 was used to block transduction on 293T cells. Transduction of FUGW/LCMV-WE was inhibited up to 67.3% in the presence of IIH6C4, while the isotype-matched control an-

tibody showed no significant inhibition. IIH6C4 is not the only available monoclonal antibody to α -DG, but it acts against the laminin-binding site of glycosylated epitope of α -DG (Hiroi *et al.*, 2011). By performing a molecular analysis of the interaction between LCMV and α -DG, it was revealed that the binding sites for LCMV and laminin-1 on α -DG are overlapped (Kunz *et al.*, 2001). Soluble α -DG purified from tissues or the produced fusion protein Fc-DG has been used to inhibit wild-type LCMV infection as well (Imperiali *et al.*, 2005). Our results agree with those studies indicating that LVs enveloped with LCMV-WE GP can transduce cells in a receptor-dependent manner. A prior study of natural tropism of LCMV showed that CD11c⁺ and DEC-205⁺ splenic DCs, but not B220⁺ B cells or CD4⁺ and CD8⁺ T cells, express α -DG. The preferential transduction of LCMV in DCs leads to immunosuppression and persistent infection (Sevilla *et al.*, 2000). As expected, we observed that LVs transduce DCs in a similar α -DG-mediated manner.

The present study reveals that the DC-targeted transduction property of LCMV-WE-pseudotyped LVs confers its promising application in immunizing mice. The f.p. and i.d. injections of concentrated FUW-IiOVA/LCMV-WE resulted in the strongest OVA-specific immune response. This is consistent with these routes being the best to target skin-derived DCs (Dai *et al.*, 2009). One advantage of LVs is low antivector immunity since LVs do not induce vector-specific immunity, but only weak host immunity against the pseudotyping envelope (Beignon *et al.*, 2009). Pinschewer *et al.* (2004) studied the mechanisms underlying vector-specific nAb responses and found that the impact of the viral glycoprotein, but not the viral backbone, is critical in determining nAb kinetics. The wild-type LCMV was found to be persistent and noncytolytic in the natural host mouse. Compared to the glycoprotein envelope of VSV, LCMV-GP triggered nAb responses slowly and poorly (Pinschewer *et al.*, 2004).

In another study of antiviral antibody responses, LCMV and VSV infections were discussed as two extreme experimental model systems. It was found that LCMV could bypass nAb responses to prolong its lifespan within the host, whereas nAb response was induced rapidly for the survival of the host in the infection of acute and cytopathic VSV (Hangartner *et al.*, 2006). Consistent with those reports, the present studies confirmed that nAb responses to LCMV glycoprotein in immunized mice were poorly induced. A lower antivector immunity offers the potential of a homologous prime-boost regimen using the same vaccine vector. Indeed, as compared with the single-prime immunization, we observed enhanced OVA-specific CD8⁺ T cell immunity upon homologous prime-boost vaccination by LCMV-WE-bearing LVs. This is an important and advantageous feature as a vaccine vector and should be characterized in other antigen settings in order to explore its full potential for vectored vaccine applications.

In conclusion, we evaluated the transduction properties of LVs pseudotyped with glycoproteins from various strains of LCMV. The envelope glycoprotein derived from LCMV-WE was found to pseudotype LVs efficiently and transduce cells through the cellular receptor α -DG. Such an enveloped vector was shown to preferentially transduce DCs, a less-proliferative cell population that is important for initiating,

programming, and maintaining antigen-directed immune response. In mice, LCMV-WE-bearing LVs could induce antigen-specific CD8⁺ T cell responses. Importantly, because of low antivector immunity, such immune responses could be further enhanced by a homologous boosting injection. Given these results, further study should be warranted to determine the application of LCMV in vaccine development for cancers and infectious diseases.

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Author Disclosure Statement

The authors declare no competing financial interests.

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