

Human Immunodeficiency Viral Vector Pseudotyped with the Spike Envelope of Severe Acute Respiratory Syndrome Coronavirus Transduces Human Airway Epithelial Cells and Dendritic Cells

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ABSTRACT

The human severe acute respiratory syndrome coronavirus (SARS-CoV) is a highly infectious virus that causes severe respiratory infections in humans. The spike envelope glycoprotein of SARS-CoV, the main determinant of SARS-CoV tropism, was isolated and used to pseudotype a human immunodeficiency virus (HIV)-based vector. Spike-pseudotyped HIV vector was generated and evaluated *in vitro* on well-differentiated human airway epithelial cells and bronchial explants and *in vivo* in murine airways. The spike envelope was less efficient at promoting HIV vector transduction of murine airway epithelium than an optimized deletion mutant of the Zaire ebolavirus envelope glycoprotein (NTD6L), which was used as a benchmark. However, spike-pseudotyped HIV vector was substantially more efficient than NTD6L-pseudotyped vector on human airway epithelium as demonstrated by *lacZ* gene transfer in primary cultures of epithelial cells and bronchial explants. In addition, this study shows that spike-pseudotyped HIV-based vector can efficiently transduce human dendritic cells and epithelial cells of the esophagus, which may have implications in investigating mechanisms of SARS-CoV pathogenesis. Spike-pseudotyped HIV-based vector is a novel lung-directed gene transfer vehicle that holds promise for the treatment of genetic lung diseases such as cystic fibrosis or α_1 -antitrypsin deficiency.

INTRODUCTION

LENTIVIRAL VECTORS can be efficiently pseudotyped with envelope proteins from many viruses including the glycoprotein from vesicular stomatitis virus (VSV-G) (Naldini *et al.*, 1996). Although VSV-G-pseudotyped lentiviral vectors can transduce many cell types with good efficacy (Blomer *et al.*, 1997; Miyoshi *et al.*, 1997; Li *et al.*, 1998), *in vivo* transduction of airway cells was detected only after disruption of epithelial integrity (Johnson *et al.*, 2000; Limberis *et al.*, 2002). To achieve good transduction of lung airway epithelia *in vivo*, envelope glycoproteins derived from other viruses have been evaluated in the context of pseudotyped human immunodeficiency virus (HIV)-based vectors. These studies revealed that

the envelope glycoprotein of the Zaire ebolavirus can successfully pseudotype HIV-based vectors and promote efficient gene transfer to airway epithelia and submucosal glands *in vivo* (Kobinger *et al.*, 2001; Medina *et al.*, 2003). Additional development of the Zaire ebolavirus led to the generation of two optimized deletion mutants (deletions of the mucin domain; NTD4L and NTD6L) that further enhanced gene transfer to the airways of mice (Medina *et al.*, 2003) and nonhuman primates (G.P. Kobinger, M.P. Limberis, S. Somanathan, G. Schumer, P. Bell, and J.M. Wilson, unpublished data).

The severe acute respiratory syndrome coronavirus (SARS-CoV) that emerged in Asia at the end of 2002 had an apparent tropism for the lung, based on the fact that infected patients developed severe pneumonia (Drosten *et al.*, 2003). Several stud-

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ies indicated that SARS-CoV acutely replicated in pulmonary epithelia, initiating the cascade of events leading to respiratory distress syndrome and death in approximately 10% of cases (Drosten *et al.*, 2003; Lee *et al.*, 2003). The spike envelope glycoprotein of SARS-CoV is the main determinant for virus tropism and mediates binding to its cellular receptor, angiotensin-converting enzyme 2 (ACE2 receptor) (Li *et al.*, 2003; Hofmann and Pohlmann, 2004). Spike glycoprotein was also shown to pseudotype HIV-based vector with reasonable efficiency and to mediate transduction of various cell lines *in vitro* (Hofmann *et al.*, 2004; Moore *et al.*, 2004; Yang *et al.*, 2004).

The present study evaluates the transduction efficiency of spike-pseudotyped HIV-based vector in primary cultures of human airway epithelial and dendritic cells, tracheal explants, and *in vivo* in murine airways after *in vivo* administration with the goal of delineating SARS pathogenesis and creating better vectors for lung gene transfer.

MATERIALS AND METHODS

DNA constructs and vector production

The helper packaging construct pCMV Δ R8.2 encoding the HIV helper function, the transfer vector pHx'LacZWP encoding β -galactosidase (β -Gal) (Watson *et al.*, 2002), and plasmids encoding envelope proteins were used for vector production. Plasmid pcDNA-NTD6L or pShCAG2-nSpike encoding, respectively, the NTD6L or spike viral envelope was used to generate pseudotyped HIV vector. The generation of plasmid pcDNA-NTD6L, encoding a deletion mutant of the Zaire ebolavirus envelope glycoprotein, has been described previously (Medina *et al.*, 2003). Two variants of the spike envelope gene of SARS-CoV were generated and evaluated with the cytomegalovirus (CMV) or CMV immediate-early enhancer/chicken β -actin/rabbit β -globin hybrid (CAG) promoter by Western blot and vector production. The spike envelope glycoprotein complementary DNA (cDNA) was first isolated from a Canadian isolate of SARS-CoV (strain Tor2) by reverse transcriptase-polymerase chain reaction (RT-PCR) (generating the spike gene). The PCR fragment was cloned with a TOPO TA Cloning kit (Invitrogen, Carlsbad, CA) and characterized by sequencing (performed by SeqWright, Houston, TX), and was found to be 100% identical to the published sequence (Marra *et al.*, 2003). The spike gene was also optimized for codon usage in human cells to generate n-spike. The n-spike gene was generated by PCR with overlapping oligonucleotides introduced to optimize human codon usage. The resulting overlapping PCR fragments were fused and a full-length codon-optimized spike cDNA was created (Zhi *et al.*, 2005). The spike or n-spike gene was cloned in pShuttle (Clontech, Palo Alto, CA) 3' of the CMV promoter to generate pShCMV-Spike and pShCMV-nSpike, respectively. The CMV promoter was then replaced with the CAG promoter isolated from pCAGGS (Niwa *et al.*, 1991) in both pShCMV-Spike and pShCMV-nSpike to generate pShCAG2-Spike and pShCAG2-nSpike, respectively.

Pseudotyped HIV-based vector was produced by triple transfection of 293T cells, using the CaPO₄ precipitation method as previously described (Kobinger *et al.*, 2001). Briefly, cells were transfected with the appropriate envelope expression vector, the

HIV packaging plasmid pCMV Δ R8.2, and the transfer vector pHxLacZWP and washed twice with serum-free Dulbecco's modified Eagle's medium (DMEM) 16 hr later. Pseudotyped virus-like particles were concentrated from cell-free supernatant by ultracentrifugation 60 hr posttransfection. Vector was resuspended in complete DMEM and stored at -80°C . Transducing units per milliliter (TU/ml) was determined for each vector stock by counting β -Gal-positive cells by limiting dilution on 293 T cells. Vector stocks with titers of $1-6 \times 10^8$ TU/ml for NTD6L-HIV vector or $1-8 \times 10^5$ TU/ml for spike-HIV vector were used for experimentation. All experiments involving the production and functional analysis of replication-incompetent pseudotyped HIV-based vectors were performed under biosafety level 2+ containment, as approved by the Wistar Institute (Philadelphia, PA) and the University of Pennsylvania (Philadelphia, PA) Institutional Biosafety Committees.

Airway cultures

Human airway cells were isolated from the trachea and main stem bronchi of donor lungs provided by the National Disease Research Exchange (NDRE, Philadelphia, PA). After enzymatic dispersion the cells were seeded on collagen-coated semipermeable membrane supports (Transwell-COL, 12-mm diameter and 0.4- μm pore size; Corning Life Sciences, Acton, MA), as previously described (Kobinger *et al.*, 2001). Once cells reached confluence, the apical medium was removed and the cells were maintained at an air-liquid interface (ALI) to allow differentiation of the epithelial cell subpopulations. Typically, the ALI cultures were used 4 to 6 weeks after introduction to air, at which time transepithelial resistance was on the order of $\geq 500 \Omega \cdot \text{cm}^2$. To evaluate pseudotyped HIV-based vectors, ALI cultures were transduced with 100 μl of vector stock ($1-6 \times 10^7$ LacZ-293T-TU/ml for NTD6L-HIV vector or $4-8 \times 10^4$ 293T-TU/ml for spike-HIV vector) applied to the apical side of the culture. To exclude damage of epithelial integrity the transepithelial resistance was measured 24 hr after transduction, at which time it remained at $>500 \Omega \cdot \text{cm}^2$ (data not shown), indicating that the epithelial integrity was not compromised. The cultures were stained with 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) to reveal β -Gal expression 4 days after transduction. β -Gal-expressing cells were counted by examining 20 fields at $\times 100$ magnification and extrapolating for the surface area (1 cm^2) of the membrane support.

Bronchial explants

Small portions (0.5 cm^2) of bronchi were excised from normal human airways and placed on collagen-coated permeable supports (Transwell-COL, 6.5-mm diameter and 0.4- μm pore size; Corning Life Sciences). The tissue was fed from the basolateral reservoir. Tissues were transduced with 50-100 μl of vector stock (approximately 1×10^7 LacZ-293T-TU/ml for NTD6L-HIV vector and 1×10^4 293T-TU/ml for spike-HIV vector) from the apical side and incubated for 2-4 hr at 37°C . Fresh medium was then added so as to submerge the tissue. Medium was replaced every 12 hr for 48 hr. For β -Gal expression, the tissue was fixed in 0.5% glutaraldehyde in phosphate-buffered saline (PBS) for 10 min at room temperature and stained with X-Gal at 37°C for 3-12 hr.

Isolation and transduction of human monocyte-derived dendritic cells

Peripheral blood mononuclear cells, isolated from healthy donors, were obtained from the Center for AIDS Research (CFAR) facility at the University of Pennsylvania. Monocytes adhered to a plastic surface by incubation for 2 hr at 37°C in AIM-V medium (Invitrogen). Subsequently the nonadherent cells were removed and the plastic-adherent monocytes were differentiated by application of a combination of granulocyte-macrophage colony-stimulating factor (GM-CSF, 800 U/ml; Berlex, Montville, NJ) and interleukin (IL)-4 (500 U/ml; R&D Systems, Minneapolis, MN) for 7 days. Immature dendritic cells were harvested on day 7 and had the following immunophenotype: CD14⁺HLA-DR⁺CD11c⁺CD80⁺CD86⁺CD83⁻. Cells were transduced with either human adenovirus 5 expressing LacZ (AdHu5LacZ) or spike-pseudotyped HIV-LacZ vector at a multiplicity of infection (MOI) of 1000 and 0.2, respectively. Seventy-two hours later cells were stained for β -Gal expression and imaged with an inverted Nikon TE300 microscope (Nikon Instruments, Melville, NY).

Animal model

C57BL/6 mice (6 to 8 weeks of age) were anesthetized by an intraperitoneal injection of ketamine-xylazine. Using standard techniques, the trachea was exposed through a midline incision, 100 μ l of vector preparation containing 1–6 \times 10⁷ LacZ-293T-TU/ml (NTD6L-HIV vector) or 1–8 \times 10⁴ 293T-TU/ml (spike-HIV vector) was instilled with a syringe, and the subcutaneous tissues were sutured closed. At necropsy, the lungs were inflated with O.C.T. compound (Sakura Finetek USA, Torrance, CA)–PBS (1:1) and processed for cryofixation. Cryosections (10 μ m) were prepared and stained with X-Gal overnight. Transduction efficiency was estimated by examining 20–25 high-power fields from 16 cryosections spaced throughout the tissue block (at 400- μ m intervals). Five animals were treated with each pseudotyped vector, and the experiment was repeated once. All animal procedures were approved by the Wistar Institute and the University of Pennsylvania Institutional Animal Care and Use Committees.

Immunocytochemistry and electron microscopy

ALI cultures were infected with 1 \times 10⁷ plaque-forming units (PFU) of SARS-CoV (strain Tor2) per well. Ten days later, cells were fixed and analyzed by immunohistochemical staining or processed for electron microscopy analysis. For immunohistochemical staining, cells were fixed with 10% formalin and embedded in paraffin. Sections (5 μ m thick) were deparaffinized; boiled in a microwave for 6 min in 10 mM citrate buffer (pH 6.0); treated sequentially with 2% H₂O₂, avidin-biotin blocking reagents (Vector Laboratories, Burlingame, CA), and protein blocking agent (Fisher Scientific, Hampton, NH); followed by incubation with primary and biotinylated secondary antibodies (Vector Laboratories). Primary antibodies were directed against the N terminus of the nucleocapsid (N) protein (rabbit serum from Abgent, San Diego, CA; diluted 1:50). Bound secondary antibodies were visualized with a VECTA-STAIN Elite ABC kit (Vector Laboratories) and 3,3'-diaminobenzidine (DAB) as substrate. In all cases, incubation was

followed by extensive washing with PBS. Negative controls consisted of preincubation with PBS, omission of the primary antibody, and substitution of the primary antibody by an isotype-matched nonimmune control antibody. For electron microscopy, cells were fixed in 2.5% glutaraldehyde in PBS and enclosed in 1% low melting point agarose. After chilling on ice, the cells in small agarose blocks were refixed overnight in 2.5% glutaraldehyde–2% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.4), washed in cacodylate buffer, and incubated in 2% OsO₄ for 2 hr. The samples were then stained for 1 hr with 1% uranyl acetate in 150 mM maleate buffer (pH 6.0), washed, dehydrated with ethanol, and finally embedded in resin (LX-112; Ladd Research Industries, Williston, VT). Ultrathin sections (80 nm thick) were stained with uranyl acetate and lead citrate according to standard protocols, and samples were examined under a Philips CM-100 transmission electron microscope (FEI, Hillsboro, OR).

Western blot

293T cells were transfected with each plasmid encoding spike protein, using the CaPO₄ precipitation method (described previously). Forty-eight hours later, cells were harvested, resuspended in lysis buffer, and frozen at –20°C. The total protein content of all samples, determined by Bradford assay (Bio-Rad, Hercules, CA), was normalized to the lysate with the lowest total protein by diluting with 4 \times sodium dodecyl sulfate (SDS) sample buffer (Invitrogen) including 5% 2-mercaptoethanol. Diluted samples were heated to 94°C for 4 min and 20 μ g of each sample was loaded onto an SDS–polyacrylamide gel. After electrophoresis, proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane. Blots were blocked with 8% milk diluted in PBS–Tween 20 (PBS/T) for 45 min, washed three times (10 min each) with PBS/T, and probed with primary antibody at room temperature for 1 hr. Primary antibody consisted of whole serum isolated from rabbits inoculated with purified spike protein diluted in 5% milk–PBS/T to a final concentration of 1:500. The blots were then washed three times with PBS/T before being incubated with secondary antibody for 45 min at room temperature and washed again three times with PBS/T. Secondary antibody was anti-rabbit horseradish peroxidase (HRP) diluted in 5% milk–PBS/T to a final concentration of 1:2000 (Santa Cruz Biotechnology, Santa Cruz, CA). Protein bands were developed with SuperSignal West Pico chemiluminescent substrate (Pierce Biotechnology, Rockford, IL) and exposed on Kodak BioMax film (Eastman Kodak, Rochester, NY).

RESULTS

Generation of HIV-based vector pseudotyped with the spike envelope protein of SARS-CoV

The gene encoding the spike envelope glycoprotein was generated by RT-PCR from viral RNA isolated from strain Tor2 of SARS-CoV. The sequence of the spike gene was identical to the previously published sequence for the SARS-CoV Tor2 isolate (Marra *et al.*, 2003). The spike gene was cloned under the control of a CMV promoter in the pShuttle vector, which

was used to produce pseudotyped HIV-based vector by triple transfection in 293T cells. Initial yields of concentrated spike-pseudotyped HIV-based vector were on the order of $2\text{--}3 \times 10^3$ TU/ml, which is 5–6 logs lower than preparations of HIV-based vector pseudotyped with VSV-G or Zaire ebolavirus glycoprotein. The production of spike-HIV vector preparations with low titer was possibly due to weak intracellular expression of the spike glycoprotein or suboptimal packaging of the envelope into virus-like particles. Codon optimization was previously used to improve expression of spike and presumably vector titers from pseudotyped retroviral vector preparations (Babcock *et al.*, 2004; Moore *et al.*, 2004; Yang *et al.*, 2004). Here, intracellular expression of spike was enhanced both by codon-optimizing the sequence for translation in mammalian cells (n-spike) and by using the hybrid CAG promoter. Western blot analysis indicated that the expression of spike was significantly increased by both codon optimization and the CAG promoter (Fig. 1A, compare lanes 2 and 3, lanes 4 and 5, lanes 2 and 4, and lanes 3 and 5, respectively), which resulted in a 2- to 3-log increase in the production of spike-pseudotyped HIV transducing units per milliliter (Fig. 1B). Subsequently, pShCAG2-nSpike plasmid expressing the codon-optimized spike under the control of the AG promoter was used to generate all working stocks of spike-pseudotyped HIV-based vector employed in this study. A similar approach was used to optimize the expression of spike in adenovirus-based vaccines (Zhi *et al.*, 2005).

Susceptibility of human airway epithelium to SARS-CoV and spike-pseudotyped HIV-based vector

Wild-type SARS-CoV was applied to the apical surface of human airway ALI cultures to address tropism of the virus for

human conducting airway epithelia. Infected ALI cultures were analyzed 10 days later for expression of SARS-CoV antigen by immunohistochemical staining with a nucleocapsid (N)-specific antibody. Epithelial cells positive for N were detected in infected cultures (Fig. 2B) but not in cultures exposed only to vehicle (Fig. 2A). Analysis of SARS-CoV-infected ALI cultures by electron microscopy revealed the presence of progeny virions in the nucleus of epithelial cells (Fig. 2C and D). Spike-pseudotyped HIV-based vector was evaluated next to assess whether relevant levels of transduction are possible with concentrated vector preparations. Concentrated stock (100 μ l per well containing $4\text{--}8 \times 10^4$ TU) of spike-pseudotyped HIV vector expressing the *lacZ* marker gene was added to the apical side of ALI cultures and β -Gal expression was analyzed 4 days later. Controls included vector-free DMEM and NTD6L-pseudotyped HIV vector ($1\text{--}6 \times 10^7$ TU/well) expressing LacZ, previously shown to transduce ALI cultures. Representative photomicrographs of cultures transduced with LacZ-expressing vectors are shown in Fig. 3. Spike-pseudotyped HIV-based vector demonstrated transduction of, on average, 70–75% (up to 95%) of the surface epithelium (Fig. 3C and F), which was superior to the 40–45% transduction efficiency obtained with NTD6L-pseudotyped HIV vector (Fig. 3B and E); no β -Gal expression was detected in ALI cultures exposed to vehicle (Fig. 3A and D). To control for pseudo-transduction, experiments were carried out in the presence of zidovudine (AZT; 5 μ M) to inhibit RT and ablate vector-encoded transgene. Like uninfected cultures, AZT-treated cultures demonstrated no β -Gal expression, indicating that the β -Gal activity is indeed the result of transduction rather than protein transfer (data not shown). These results indicate that primary cultures of human airway epithelial cells are susceptible to SARS-CoV and spike-pseudotyped HIV vector.

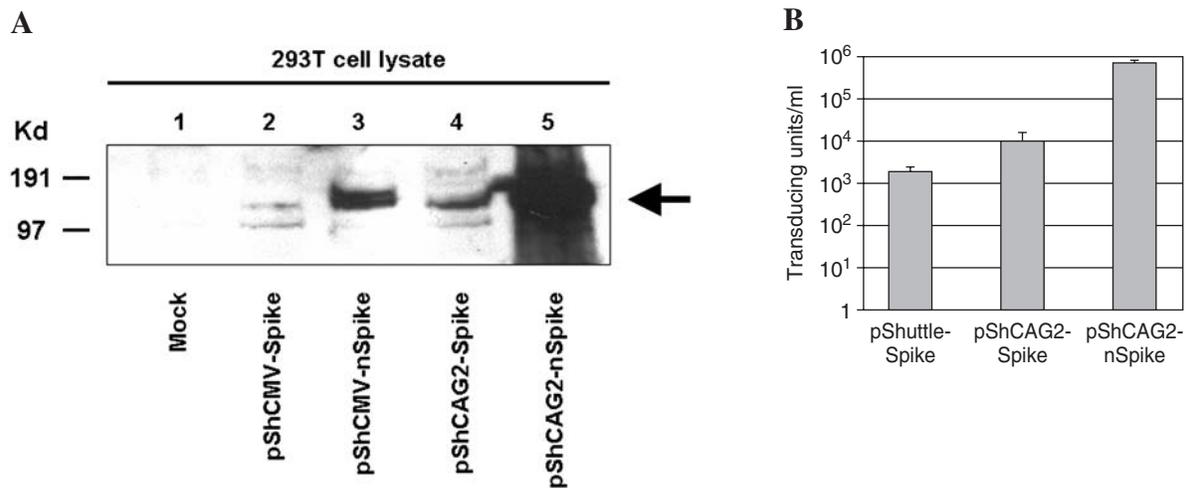


FIG. 1. Optimization of spike expression and pseudotyped-HIV-based vector production. (A) Expression of SARS-CoV spike protein from three expression plasmids transfected into 293T cells. See Materials and Methods for information regarding the plasmids. Cell lysates were separated by SDS-PAGE and expression of spike was detected by Western blotting, using whole serum collected from rabbits inoculated with purified spike protein. Mock-transfected cell lysate served as a negative control. Molecular weight markers are shown on the left. The expected spike protein band is indicated (arrow). (B) Titers of LacZ-expressing vector stock produced with various expression cassettes of spike. Vector titers were determined by limiting dilution on 293T cells and are represented as transducing units per milliliter (TU/ml). The column for each condition represents the mean of the four best titrating experiments among six to eight productions, and each bar shows the standard deviation of the mean.

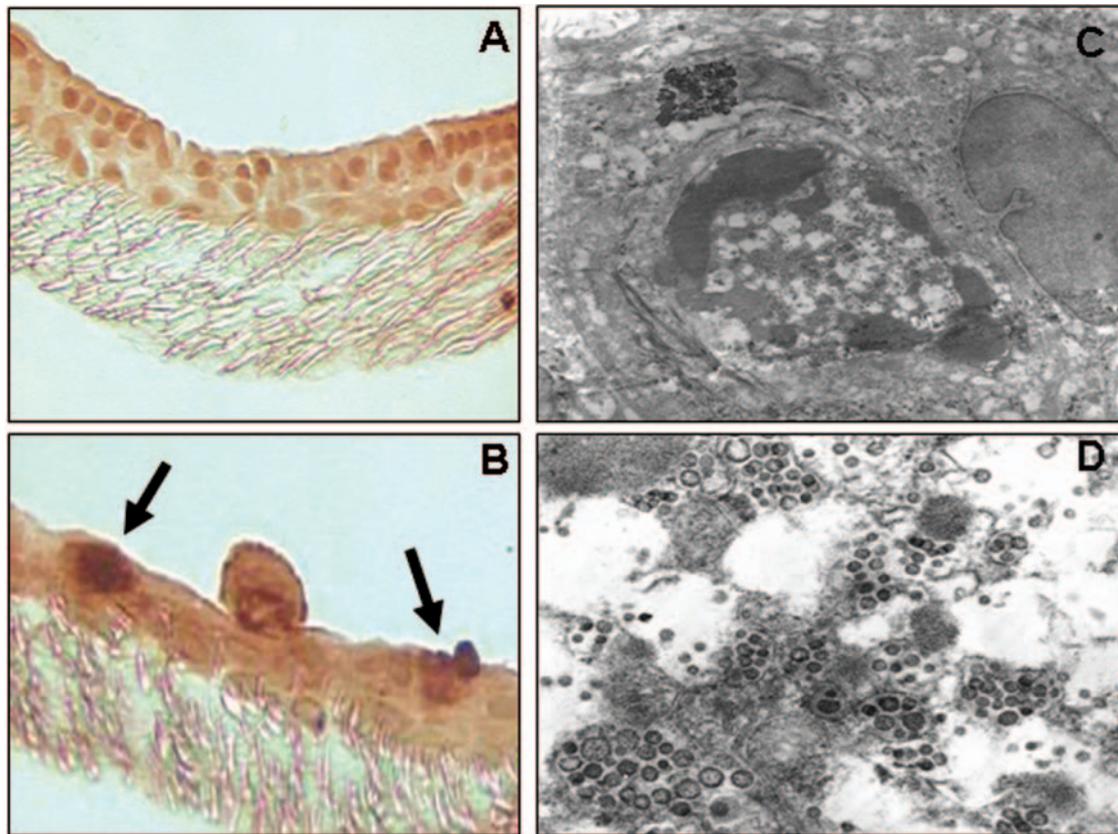


FIG. 2. Susceptibility of ALI cultures of human airway epithelial cells to SARS-CoV. ALI cultures were infected or not with SARS-CoV, fixed, and subjected to immunostaining with an antibody specific to the nucleocapsid protein [(A) vehicle control; (B) SARS-CoV] or analyzed by electron microscopy [low and high magnifications, (C) and (D), respectively]. Arrows indicate cells positive for N in (B). In (C), that part of the lumen indicative of the apical side is at the top left corner. Infections of ALI cultures (triplicate wells) with wild-type SARS-CoV were performed in two independent experiments with airway epithelial cells isolated from two donors. Pictures are from the second experiment, where immunocytochemistry generated the highest number of positive cells for the nucleocapsid protein.

Transduction of human bronchial explants and murine lung airways by spike-pseudotyped HIV-based vector

Excised sections of human bronchi were incubated for 2–4 hr with concentrated spike- or NTD6L-pseudotyped HIV vector encoding β -Gal to further characterize and compare the transduction efficiency of both vectors in intact airway epithelium. Tissue was fixed and stained with X-Gal 16 hr after application of the vector. Spike-pseudotyped vector resulted in robust expression of β -Gal in the surface epithelium, stronger than with NTD6L-pseudotyped vector (Fig. 4, compare panels C and F with panels B and E). Histological photomicrographs of these tissues demonstrated no specific expression in the vehicle control, a low level of expression in NTD6L-pseudotyped vector-treated tissue, and strong expression of β -Gal in the tissues transduced with spike-pseudotyped HIV-based vector (Fig. 4A–F).

NTD6L- and spike-pseudotyped HIV vectors encoding β -Gal were also administered to immune-competent mice by intratracheal instillation. Mouse tracheas were stained and analyzed for β -Gal expression 28 days postadministration of vector. In the murine model, airway epithelial cells expressing β -Gal were frequently observed in different regions of the tra-

chea after treatment with NTD6L-pseudotyped HIV-based vector (Fig. 5A). However, only a few β -Gal-positive cells were observed throughout the trachea and no positive cells were seen in the distal airways or alveolar regions in mice treated with spike-pseudotyped vector, although submucosal glands were found positive in many areas (Fig. 5B). β -Gal was undetectable in the airway of control mice administered DMEM, using the same staining conditions (data not shown). Interestingly, high levels of β -Gal expression were noted in epithelial cells of the esophagus of mice administered spike-pseudotyped HIV vector (Fig. 5D). We had already observed this phenomenon, although with less efficiency, with other pseudotyped HIV vectors. This occurs after the intratracheal administration of a large volume (100 μ l) of vector preparation, which can overspill in the oral cavity and be swallowed back by the treated mouse. No positive staining was observed in the esophagus of NTD6L-pseudotyped vector-treated mice (Fig. 5C).

Human dendritic cells are susceptible to transduction by spike-pseudotyped HIV-based vector

Transduction of human dendritic cells by spike-pseudotyped HIV vector was evaluated. Monocyte-derived immature human

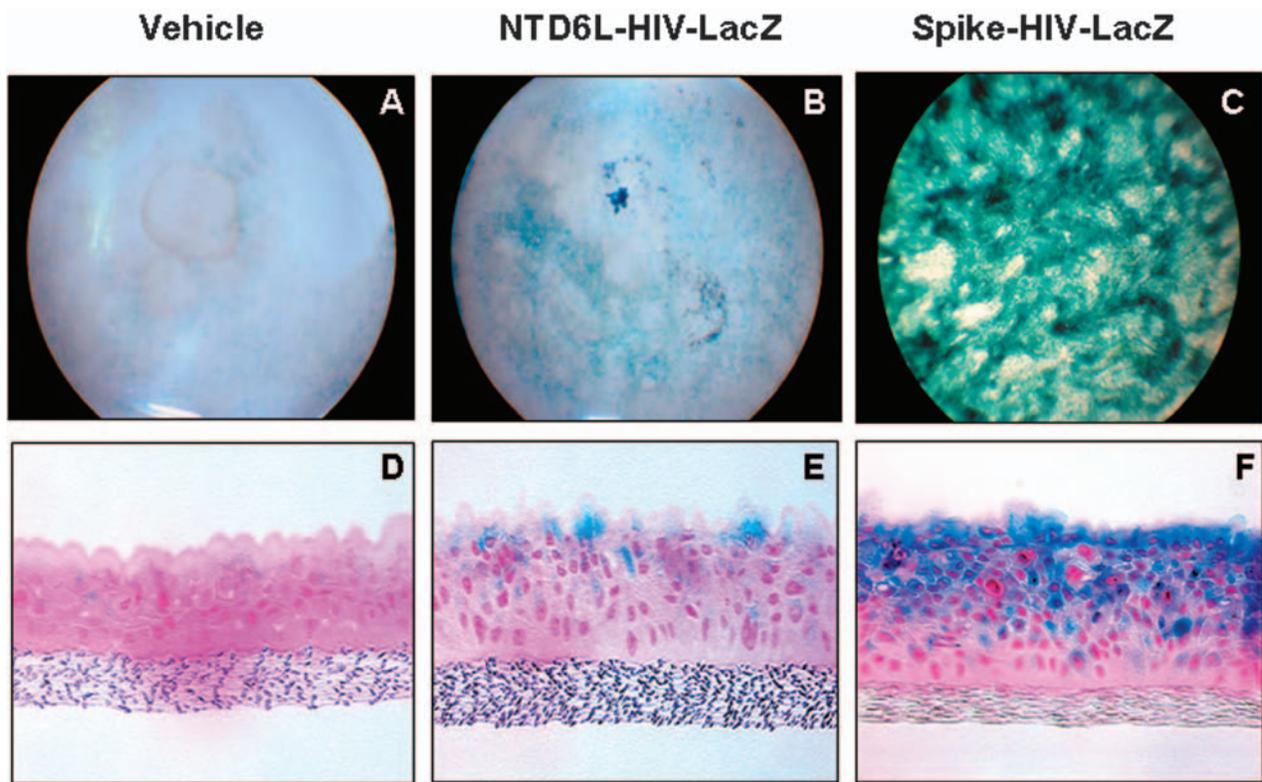


FIG. 3. β -Gal expression in HIV-based vector-transduced ALI cultures of human airway epithelial cells. (A–C) Low-magnification microphotographs showing β -Gal expression by control or pseudotyped-HIV-based vectors 4 days after apical application of 100 μ l of concentrated vector stock. (D–F) High-magnification microphotographs of transverse sections prepared from the corresponding sample [see (A–C)]. Transduction experiments were performed with two different vector preparations on ALI cultures generated from three different normal human donor lungs. Pictures are representative of samples from the same experiment in which human airway ALI cultures were well differentiated and more than 20% ciliated.

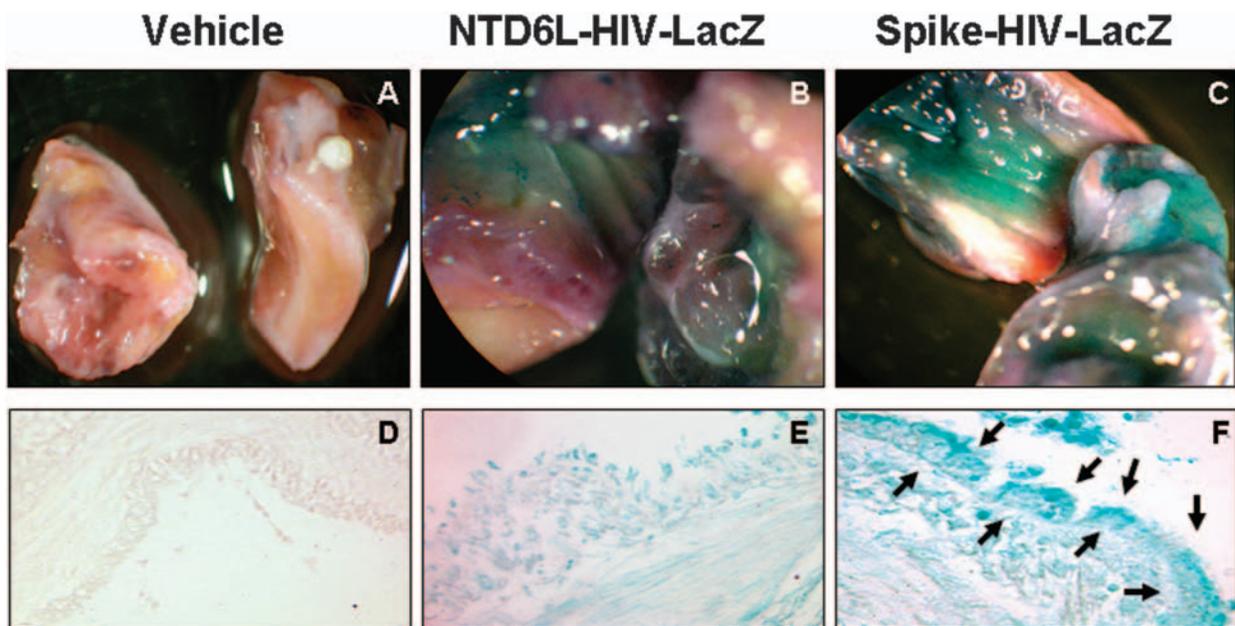


FIG. 4. *Ex vivo* gene transfer to excised human bronchi. (A–C) Images of bronchi isolated from a normal lung after vector treatment for 16 hr and subsequently stained with X-Gal. (D–F) Microphotographs of paraffin sections made from corresponding tissue [see (A–C)] after X-Gal staining. Under these conditions, no expression is seen in the vehicle-treated tissue. Specific staining is seen in the NTD6L-pseudotyped vector and, more importantly, in the spike-pseudotyped HIV vector-treated cells (arrows). Transduction experiments were performed in triplicate with two different vector preparations and human bronchi were isolated from two normal human lung donors. Pictures are representative of samples from the same experiment showing the best preservation of tissue integrity.

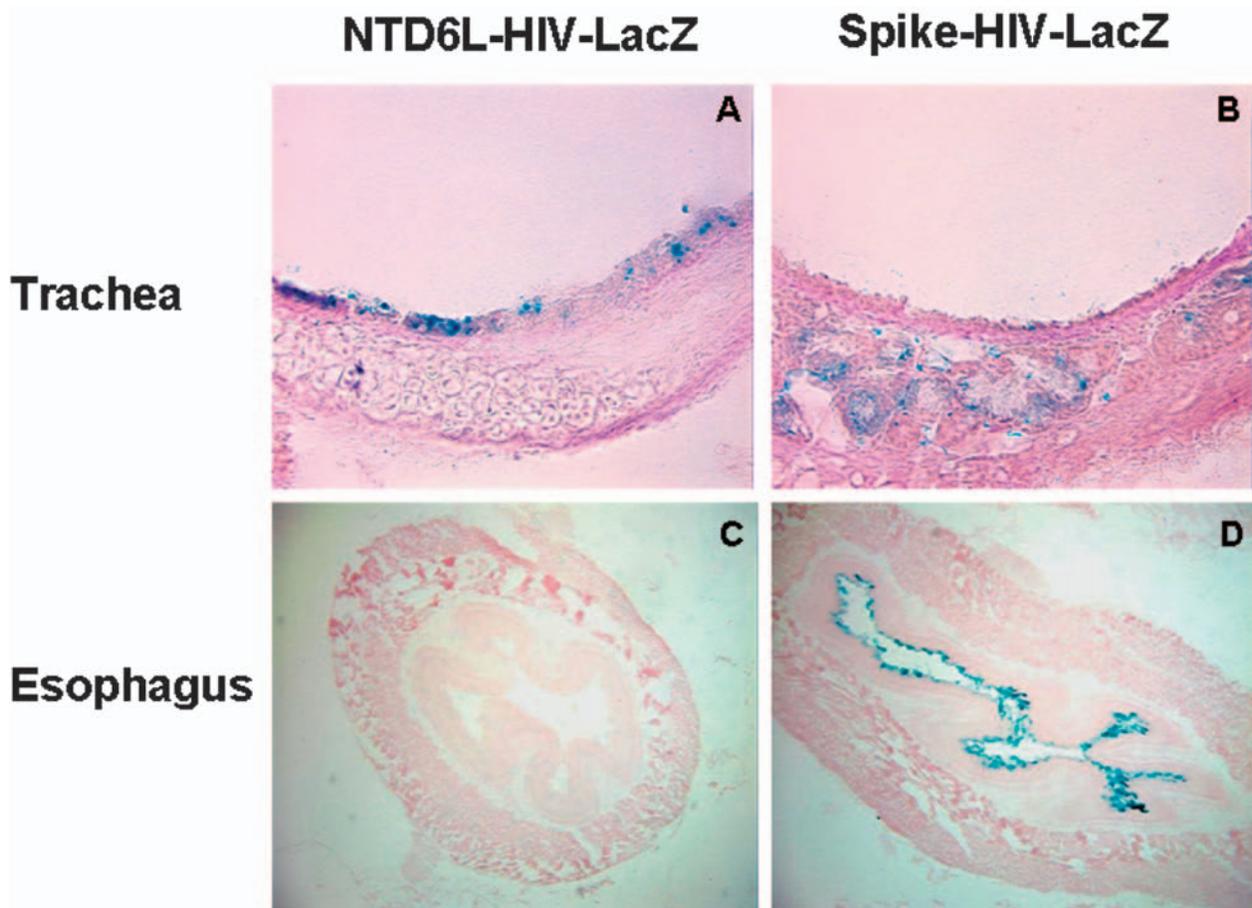


FIG. 5. *In vivo* gene transfer to C57BL/6 mice on day 28. (A and B) Detectable expression is seen in the airway epithelium of animals receiving NTD6L-pseudotyped HIV vector but not in the airway epithelium of mice treated with spike-pseudotyped vector on day 28. (C and D) Strong expression is seen only in the esophagus of animals receiving spike-pseudotyped HIV vector at the same time point. Micrographs of trachea were taken at $\times 300$ magnification and those of esophagus at $\times 200$ magnification. Five mice per group were analyzed twice and pictures represent an average area from one animal of each group showing an average to high number of LacZ-positive airway cells.

dendritic cells (DCs) ($CD14^{-}HLA-DR^{+}CD11c^{+}CD80^{+}CD86^{+}CD83^{-}$) were incubated with spike-pseudotyped HIV-based vector expressing LacZ at a multiplicity of infection (MOI) of 0.2 and stained for β -Gal 72 hr later. No transgene expression was observed in vehicle-transduced cells (Fig. 6A). As a positive control, cultures were infected with an adenovirus expressing LacZ (AdHu5LacZ) at an MOI of 1000, showing transduction of 95% of the cells (Fig. 6B). Interestingly, approximately 45% of β -Gal-positive cells were detected after transduction with spike-HIV vector at an MOI, based on infectious particles, 5000-fold lower than with AdHu5LacZ (Fig. 6C).

DISCUSSION

The initial challenge was to generate sufficient titers of the spike-pseudotyped HIV vector to perform *in vitro* and *in vivo* experiments. A combination of codon optimization for usage in mammalian cells and appropriate promoter resulted in substantial enhancement in the expression of the spike glycoprotein and a 2- to 3-fold increase in the number of transducing

units of spike-pseudotyped HIV vector per milliliter produced from transfected 293T cells. A study previously reported that increasing the envelope glycoprotein expression level by modifying the 5' and 3' untranslated regions, resulted in improved production of infectious feline immunodeficiency virus-based vector by several orders of magnitudes (Sinn *et al.*, 2005). Taken together, these findings support the general concept that production of lentiviral vectors can be optimized by increasing the expression of the envelope glycoprotein selected for pseudotyping. With our improved conditions, the best yields of spike-pseudotyped HIV-based vector were still three orders of magnitude lower than with VSV-G-pseudotyped HIV vector, suggesting that other parameters could be improved for further optimizing the production of lentiviral vector preparation with high infectious titers.

The present data indicate that spike-pseudotyped HIV-based vector is substantially more efficient at transducing human airway epithelia in ALI and organ cultures than NTD6L-pseudotyped HIV vector, which was our most efficient lentiviral vector *in vivo* for mouse, monkey, and human lung (Medina *et al.*, 2003; Kobinger *et al.*, unpublished data). However, transduc-

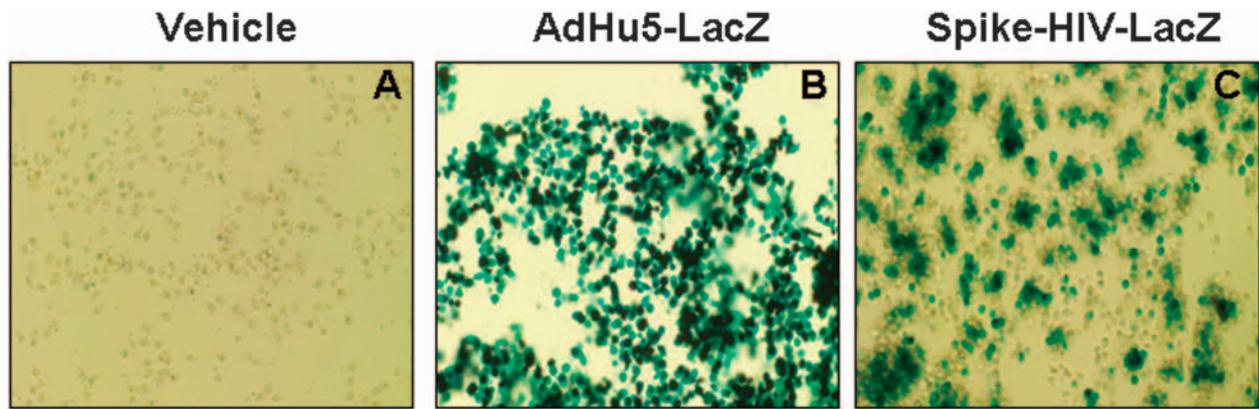


FIG. 6. Transduction of monocyte-derived immature human DCs. Expression of β -Gal-transduced immature human DCs is shown for (A) vehicle-treated DCs (negative control), (B), AdHu5LacZ-treated DCs (positive control), and (C) spike-pseudotyped HIV-based vector-treated DCs. Transduction was performed at an MOI of 10^3 TU/cell (AdHu5LacZ) and an MOI of 0.2 TU/cell (spike-pseudotyped HIV-based vector). Pictures depict the most representative transduction efficiencies from one of three independent experiments performed with DCs isolated from different human donors.

tion of mice airway epithelia by spike-HIV-based vector was relatively low. This could be due to a preferential tropism of the spike envelope for human rather than murine airway epithelia. The distribution and/or affinity of the murine ACE2 receptor may differ from the human ACE2 receptor used by SARS-CoV for viral entry, which could explain the difference between human and murine tissues susceptibility. We did not observe transduction of alveoli in mice, which otherwise have been found to express significant levels of the ACE2 receptor in human lung tissue (Hamming *et al.*, 2004). What has been demonstrated is that mice are less permissive to SARS-CoV replication and do not develop the severe respiratory distress syndrome observed in humans (Subbarao *et al.*, 2004).

The susceptibility of human ALI cultures and bronchial explants to spike-pseudotyped HIV vector is encouraging for its use in lung-directed gene transfer. In addition, this vector system should prove useful for investigating several aspects of SARS-CoV basic virology. The susceptibility of human airway epithelial cells of the conducting airway to SARS-CoV infection has potential implications for models of SARS pathogenesis. On the basis of analysis of pathology specimens of SARS patients with advanced disease, a model of pathogenesis has emerged in which primary infection and inflammation occur in the alveoli (Franks *et al.*, 2003; Nicholls *et al.*, 2006). Our studies of SARS-CoV infection in mice demonstrated early infection in conducting airways and bronchiolitis before involvement of the alveoli (Hogan *et al.*, 2004). The present studies do indeed confirm that human conducting airway could be a site of early disease. Analysis of patient specimens may miss this kind of lesion because they generally were recovered from individuals with advanced disease. The importance of the ACE2 receptor, polarity of viral budding, and increased susceptibility of fully differentiated cultured airway epithelium have been reported, using ALI cultures of human airway epithelial cells (Jia *et al.*, 2005; Sims *et al.*, 2005).

Other cell types were found to be susceptible to spike-pseudotyped HIV-based vector, including epithelial cells of the esophagus and immature human DCs. The hypothesis that in-

fection of immature DCs by SARS-CoV contributes to viral pathogenesis is a tempting idea, with an underlying mechanism that may be more easily investigated with pseudotyped lentiviral vectors. Several reports indicate that SARS-CoV can provoke dysfunctional immune responses such as inadequate chemokines and cytokines production through interactions with DCs (Ziegler *et al.*, 2005; Spiegel *et al.*, 2006; Yen *et al.*, 2006). Transduction of mature human DCs with spike-pseudotyped vector has been reported to be inefficient (Yang *et al.*, 2004). Taken together, these results suggest that maturation of DCs increases their resistance to SARS-CoV infection, a phenomenon that was seen with other viruses such as adenovirus and Dengue virus (Miller *et al.*, 2003; Tassaneeritthep *et al.*, 2003).

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AUTHOR DISCLOSURE STATEMENT

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