Persistent viral vector-mediated transgene expression in the airways requires delivery to cells with progenitor capacity and avoidance of immune responses. Previously, we observed that GP64-pseudotyped feline immunodeficiency virus (FIV)-mediated gene transfer was more efficient in the nasal airways than the large airways of the murine lung. We hypothesized that in vivo gene transfer was limited by immunological and physiological barriers in the murine intrapulmonary airways. Here, we systematically investigate multiple potential barriers to lentiviral gene transfer in the airways of mice. We show that GP64-FIV vector transduced primary cultures of well-differentiated murine nasal epithelia with greater efficiency than primary cultures of murine tracheal epithelia. We further demonstrate that neutrophils, type I interferon (IFN) responses, as well as T and B lymphocytes are not the major factors limiting the transduction of murine conducting airways. In addition, we observed better transduction of GP64-pseudotyped vesicular stomatitis virus (VSV) in the nasal epithelia compared with the intrapulmonary airways in mice. VSVG glycoprotein pseudotyped VSV transduced intrapulmonary epithelia with similar efficiency as nasal epithelia. Our results suggest that the differential transduction efficiency of nasal versus intrapulmonary airways by FIV vector is not a result of immunological barriers or surface area, but rather differential expression of cellular factors specific for FIV vector transduction.

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Subject Category: Methods section

Introduction

The aim of the gene therapy vector development for life-long genetic diseases such as cystic fibrosis is to create a vehicle with the ability to efficiently, safely, and persistently express a transgene in the appropriate cell types. Current lentiviral vector technology has made considerable progress towards this goal. Several features of lentiviral vectors make them attractive for technology has made considerable progress towards this goal. Several features of lentiviral vectors make them attractive vehicles for delivering therapeutic genes, including their large coding capacity, efficient gene transfer capabilities, persistent expression, the capacity to transduce mitotically quiescent cells, and lack of virus-encoded proteins that could elicit undesirable immune responses. Towards this end, we developed gene transfer vectors derived from a nonprimate lentivirus, feline immunodeficiency virus (FIV), for our studies.

To develop efficient viral vectors, several barriers that may inhibit gene transfer must be considered. Due to constant environmental exposure, the airway and alveolar surfaces evolved multiple effective barriers to prevent foreign particles and pathogens from penetrating the epithelial lining of the lung. The airways are lined with a pseudo-stratified columnar epithelial cells, joined by cell-to-cell tight junctions. The mucus layer over-laying the airway surface provides a physical barrier that traps and clears inhaled particles. Immune responses are additional barriers that may prevent efficient transduction of target tissues. Innate immunity is activated, in part, by recognition of viral antigens or gene transfer vectors. Pattern recognition receptors are expressed in macrophages, dendritic cells, and airway epithelial cells. Recognition by receptors triggers the secretion of inflammatory cytokines and maturation of antigen presenting cells. Adaptive immune responses may also limit the persistence of transgene expression in the target cells. Vector antigens, vector-encoded proteins, or the transgene products may induce CD8+ T-cell responses as well as production of neutralizing antibody.

To date, we have screened many envelope glycoproteins to determine those with optimal tropism for the airways. Pseudovirions made with the envelope glycoprotein GP64, from baculovirus Autographa californica multinucleocapsid nucleopolyhedrosis virus, yield high titer FIV preparations (~10⁸–10⁹ transducing units (TU)/ml). GP64 confers apical entry into the polarized air-liquid primary cultures of human airway epithelia. Using a luciferase reporter gene driven by the RSV promoter and bioluminescence imaging, we observed persistent gene expression from in vivo gene transfer in the murine nose with GP64-pseudotyped FIV (GP64-FIV). Longitudinal bioluminescence analysis documented expression in the murine nasal epithelia for >1 year without significant decline. By histological analysis using a LacZ reporter gene, we found that olfactory and respiratory
epithelial cells were transduced. These data suggest that GP64-FIV efficiently transduces and persists in the nasal epithelium of mice and that the cellular tight junction integrity. Additional studies show that GP64-FIV can be repeatedly applied to the nasal epithelium to increase the gene transfer efficiency without generating immune responses that block transduction.

In previous studies, we observed that in vivo GP64-FIV mediated gene transfer was greater than tenfold more efficient in the murine nasal airways than the large airways of the lung. We hypothesized that the difference between nasal and intrapulmonary airway transduction efficiencies could be attributed to the combinations of immunological and physical barriers. To test this hypothesis, we examined GP64-FIV transduction of respiratory epithelia of the nasal and intrapulmonary airways in multiple mouse models and in well-differentiated primary airway cultures.

Results
GP64-FIV mediated gene transfer in the airways
We delivered seven doses (one dose per day, 7 consecutive days) of GP64-FIV (Figure 1a,b) or VSVG-FIV (Figure 1c) expressing firefly luciferase to the nasal airways via direct nasal instillation or the pulmonary airways via tracheal intubation. GP64-FIV is formulated with 1% methylcellulose, which restricts gene transfer to the conducting airways and limits viral vector transduction of the alveoli. Consistent with previous observations, nasal expression was significantly (14-fold) higher than pulmonary expression (Figure 1a). In addition, we routinely observed higher nasal expression in mice that received vector delivery via tracheal intubation. The mechanism for this observation is unknown; however, mucociliary clearance can rapidly clear particles from the airways into the nasopharynx. These data suggested that FIV transduces nasal epithelia with greater efficacy than pulmonary epithelia. The difference was more pronounced for GP64-FIV than VSVG-FIV. We observed the same pattern of transduction efficiencies of nasal and intrapulmonary airways using GP64-pseudotyped HIV in mice (data not shown). This differential transduction efficiency could be the result of anatomical, immunological, or cellular differences between the two anatomical regions. Because the expression levels were higher with GP64-FIV, and the difference in transduction efficiencies was more pronounced, we elected to move forward using GP64 as our pseudotyping envelope.

Phagocytes as barriers to FIV-mediated gene transfer in the airways
Adenoviral vectors elicit inflammation that can reduce the effectiveness of pulmonary gene transfer. To investigate the pulmonary inflammation resulting from GP64-FIV, we administered one dose of vector (approximately $1 \times 10^7$ TU) to mice via tracheal intubation. Bronchoalveolar lavage (BAL) fluid was collected at 4 hours and 24 hours after vector administration to assess inflammatory cell recruitment. BAL total and differential cell counts were used to quantify the severity of inflammation. We compared GP64-FIV with a vehicle (filter-sterilized α-lactose buffer and 1% methylcellulose) negative control as well as matched titer of Ad5 and 25 µg of lipopolysaccharide (LPS) (derived from 055:B5 Escherichia coli; Sigma-Aldrich, St Louis, MO) positive control. An additional sham negative control was included in which mice were anesthetized and the teflon catheter was inserted into the trachea and removed, but no material was delivered. Delivery of LPS increased neutrophil counts at 4 hours after delivery and neutrophils continued to predominate at 24 hours after delivery as expected (Figures 2a,b). In contrast, no neutrophil infiltration was observed 4 hours following vector delivery (Figure 2a). Neutrophils were observed 24 hours after delivery in mice receiving GP64-FIV and Ad5 (Figure 2b). These trends were also seen in mice that received vehicle. At 24 hours following transduction, a matched volume of sterile vehicle also recruited neutrophils to the lung. Vehicle alone was sufficient to induce low-level inflammation. These data encouraged us to further examine the possible role of the host immune response in preventing transduction of pulmonary airways by GP64-FIV.

Figure 1 Transduction efficiency of FIV in nasal and intrapulmonary epithelia in vivo. (a) Titer-matched GP64-FIV formulated with 1% methylcellulose was administered to mice via intranasal instillation or tracheal intubation. (b) Bioluminescent imaging was conducted 7 days following vector delivery. For nasal imaging, mice were laid in the prone position and regions of interest were drawn around the muzzle. For lung imaging, mice were laid in the supine position and photon emission was quantified from regions of interest (red ovals) drawn around the chest. Titer-matched VSVG-FIV formulated with 1% methylcellulose was administered to mice via intranasal instillation or tracheal intubation and bioluminescent imaging was conducted 7 days following vector delivery (c). $n = 5$ mice per group; *** $P < 0.001$. FIV, feline immunodeficiency virus; VSVG, vesicular stomatitis virus-G.
Neutrophils are one of the first host immune defenses against infection.\textsuperscript{19} We observed that neutrophils were recruited in the airways after viral vector delivery (Figure 2b). This observation prompted us to examine the effect of neutrophils on gene transfer to the airways. We transiently depleted neutrophils using 100 µg of mouse RB6-8C5 antibody (RB6). This antibody was chosen because it also depletes macrophages, dendritic cells, and lymphocytes.\textsuperscript{20} Mice received RB6 or IgG isotype control 24 hours before and 24 hours and 72 hours after the first dose of vector. Cell counts confirmed neutrophil depletion (Supplementary Figure S1). Four doses over 4 days (one dose per day) of GP64-FIV expressing luciferase were administered via nasal instillation or tracheal intubation. Luciferase expression was quantified at the indicated time points by bioluminescent imaging. \( n = 5 \) mice per group. Data are representative of three individual experiments. BAL, bronchoalveolar lavage; FIV, feline immunodeficiency virus; LPS, lipopolysaccharide; PMN, polymorphonuclear neutrophil; TU, transducing units. *** \( P < 0.001 \).

![Figure 2](image)

**Figure 2 Macrophages and neutrophils**. A single dose of GP64-FIV (approximately 1 × 10\(^7\) TU), titer-matched Ad5 vector, or 25 ng of LPS was administered to Balb/c mice via tracheal intubation. Vehicle (1% methylcellulose in \( \alpha \)-lactose buffer), sham, and naïve mice served as negative controls. BAL fluid was collected and differential cell counts were determined at (a) 4 hours and (b) 24 hours. Black bar indicates macrophages, white bar indicates neutrophils, and gray bar indicates other cell types. \( n = 4 \) mice per group; For neutrophil depletion studies, mice were pretreated with 100 µg of mouse RB6-8C5 antibody (RB6) or rat IgG2b isotype control (IgG) 24 hours before and 24 hours and 72 hours after the first dose (approximately 1 × 10\(^7\) TU) of GP64-FIV. Four doses (one dose per day) of GP64-FIV vector were administered via (c) nasal instillation or (d) tracheal intubation. Improvement in gene transfer was observed in either nasal instillation or tracheal intubation. No improvement in gene transfer was observed in either nasal instillation or tracheal intubation. No improvement in gene transfer was observed in intrapulmonary airways in mice. 

Type I interferon responses and FIV-mediated gene transfer in the airways

Type I interferon (IFN) responses are triggered by the activation of Toll-like receptors and other host pattern recognition receptors in response to viral nucleic acids or proteins. IFN\( \alpha \) and IFN\( \beta \) are crucial factors that induce the expression of proteins involved in innate defenses against viral infection. To determine if FIV-mediated lung gene transfer efficiency is improved in the absence of type I IFN responses, we utilized type I IFN receptor-deficient (IFN\( \alpha \beta R^{-/-} \)) mice.\textsuperscript{23} We delivered three doses over 3 days (one dose per day) of GP64-FIV expressing luciferase or Ad5 expressing luciferase to IFN\( \alpha \beta R^{-/-} \) mice or matched wild-type controls via nasal instillation or tracheal intubation. No improvement was observed in FIV-mediated gene transfer to either the nasal (Figure 3a) or intrapulmonary airways (Figure 3b) of IFN\( \alpha \beta R^{-/-} \) mice as compared with the wild-type controls. In contrast, improved long-term transgene expression in the lung was observed for 8 weeks in IFN\( \alpha \beta R^{-/-} \) mice compared with the matched wild-type controls when Ad5 was delivered to the lung (Figure 3b); however, no improvement in Ad5-mediated luciferase persistence was observed in the nasal airways of IFN\( \alpha \beta R^{-/-} \) mice. These results suggest that type-I-mediated transduction of intrapulmonary airways and cannot account for the observed differences in transduction efficiency.
IFN responses are not a major barrier to FIV gene transfer in the intrapulmonary airways.

**B and T lymphocytes and FIV-mediated gene transfer in the airways**

We next examined whether the efficiency of FIV gene transfer to the intrapulmonary airways is improved in the absence of B and T cells by utilizing RAG-1-deficient mice. RAG-1-deficient mice are unable to perform V(D)J recombination and consequently do not develop mature B and T lymphocytes. We delivered three doses over 3 days (one dose per day) of GP64-FIV expressing luciferase or Ad5 expressing luciferase to RAG-1-deficient mice or matched wild-type controls via nasal or tracheal instillation. We observed no significant improvement in FIV-mediated gene transfer of RAG-1-deficient mice as compared with the matched controls (Figures 3c,d). In contrast, Ad5-mediated expression improved and persisted 19 weeks in both nasal (Figure 3c) and intrapulmonary airways (Figure 3d). We repeated this study in SCID mice that have similar immunodeficient characteristics as RAG-1-deficient mice except SCID mice are on a Balb/c background. Similar to RAG-1 mice, we observed no increase in FIV transduction in the nasal or intrapulmonary airways of SCID mice as compared with the matched controls (data not shown). These data suggest that inhibiting the adaptive immunity mediated by T and B cells does not significantly improve GP64-FIV mediated gene transfer. These data further support our previous observations that GP64-FIV can be repeatedly administered to the airways without stimulating a host immune response.14

**In vitro FIV transduction of murine nasal versus tracheal epithelia**

Physical barriers are known to prevent viral vector-mediated transduction in airways.22 We examined if cellular differences between murine nasal airways and pulmonary conducting airways contribute to GP64-FIV-mediated transduction efficiency difference.14 To discern cellular from anatomical variables between these tissues, we used well-differentiated primary epithelia cultured at the air-liquid interface derived from murine trachea and murine septa from both C57Bl/6 and Balb/c mice. GP64-FIV expressing luciferase was applied to the apical surface of well-differentiated primary cells for 24 hours (multiplicity of infection = 5). Bioluminescence imaging was performed 1 week later (Figure 4a). Importantly, for both strains of mice, the transduction efficiency of the airway cells derived from the septa (black bars) was significantly greater than cells derived from the trachea (gray bars). These in vitro results (Figure 4a) remove variables such as phagocytes, adaptive immunity, surface area, and mucociliary clearance, yet closely recapitulate in vivo observations (Figure 1). These data suggest that a cellular factor(s) that differs between the murine septal and tracheal epithelia is a principal barrier to GP64-FIV transduction in the murine intrapulmonary airways. When we transduced primary murine nasal and tracheal cultures in vitro with GP64-FIV, VSVG-FIV, and Ebola-FIV, all transduced significantly better in nasal airways (Figure 4b). The preferential nasal transduction was independent of the envelope glycoprotein.

We routinely use SEM to confirm that primary cells have fully differentiated. Septal and tracheal derived epithelial cells
were observed to be well-differentiated and exhibited ciliated and nonciliated cell types (Figure 4c,d). The epithelial sheets derived from both anatomical sites were composed of ciliated and nonciliated cells. In addition, cultures were observed with light microscopy following sectioning and staining with toluidine blue (Figure 4e,f). We observed a trend for a greater percentage of ciliated cells in nasal respiratory cell cultures compared with tracheal cell cultures as previously reported; otherwise, there were no striking differences in morphology between the cells cultured from the two regions.

GP64-VSV mediated gene transfer in the airways

Although the cellular receptor for GP64 has not yet been identified, we hypothesized that a receptor for GP64 is differentially distributed between nasal and pulmonary airway epithelial cells in mice. To test this hypothesis, we used a recombinant vesicular stomatitis virus (VSV) that lacks an
envelope glycoprotein and expresses firefly luciferase. The envelope glycoprotein is supplied in trans in the producer cells. We delivered titer-matched VSV pseudotyped with GP64 (GP64-VSV) or VSVG (VSVG-VSV) to Balb/c mice via intranasal instillation or tracheal intubation. Mice were imaged for luciferase expression 24 hours after vector delivery. The transduction efficiency of GP64-VSV was significantly greater in the nasal airways compared with the intrapulmonary airways; whereas, VSVG-VSV transduced both nasal and intrapulmonary airways with similar efficiency (Figure 5a).

To confirm that the transduction levels were not a result of saturated expression, we delivered tenfold dilutions of GP64-VSV to mice via intranasal instillation or tracheal intubation. We observed that luciferase expression decreased proportionately for each route of delivery (Figure 5b). These results suggest that cellular factors such as a GP64 receptor or intracellular restriction factors are the principal barriers to GP64-FIV transduction in the murine intrapulmonary airways.

Discussion

In this study, we examined multiple potential barriers to GP64-FIV gene transfer to the airways. Cellular inflammation was observed in the BAL of mice when one dose of GP64-FIV was administered; however, the vehicle control also triggered neutrophil recruitment. These data suggest that bolus pulmonary delivery of lactose buffer and 1% methylcellulose is sufficient to initiate an inflammatory response. Based on our previous experience, this response is insufficient to prevent expression from re-administered GP64-FIV. Furthermore, these data are consistent with previously observed spikes of keratinocyte derived chemokine and interleukin-6 in nasal lavage following intranasal delivery of vector or vehicle.

Depletion of neutrophils did not improve transduction efficiency of GP64-FIV. Our data suggest that macrophages and neutrophils are not the major gene transfer barrier in the airways. In addition, no improvement in efficiency of FIV-mediated transduction was observed in IFNαβR−/− mice as compared with the matched wild-type controls. IFNαx and β are crucial factors that induce proteins involved in innate defense against viral infection. They also drive maturation of dendritic cells and activation of natural killer cells. Our observations suggest that GP64-FIV transduction is not limited by IFNαβ responses. However, Brown et al. previously demonstrated that systemic administration of VSVG-pseudotyped HIV vector to mice by tail-vein injection triggered an acute IFNαβ response in the liver and spleen. In their study, significantly increased transduction was observed in the hepatocytes of IFNαβR−/− mice compared with the nude mice. The use of a different viral envelope is unlikely the explanation for the differences between studies because Brown et al. also demonstrated that GP64-pseudotyped HIV vector triggered an IFNαβ response. It is possible that the different lentiviral species, tissue targets, or routes of administration could elicit different host immune responses.

In addition to innate immune responses, we examined the role of adaptive immunity as a possible barrier. We observed no significant difference in luciferase gene expression in RAG-1 deficient mice transduced with GP64-FIV compared with the matched wild-type controls. Similar results were observed in SCID mice. These data indicate that the adaptive immune response is not a major impediment to airway gene transfer. Again, these data support our previous observation that readministration of GP64-FIV does not result in effective systemic or local neutralizing antibodies.

Recently, Limberis et al. demonstrated that VSVG-pseudotyped HIV vector carrying green fluorescent protein activated as well as envelope-specific T cells in murine lung airways. Their findings contrast with the result from our RAG-1 deficient mice study; however, possible explanations include the choice of reporter gene, vector administration protocol, or envelope pseudotype. In fact, their study showed that among the four pseudotyped vectors, the VSVG envelope resulted in the most robust T-cell response. Based on our experience to date, the GP64 envelope protein has less potential to trigger adaptive immune responses in mice. Further study will be required to determine the effects of the GP64-pseudotyped lentivirus on host immune response in larger animal models.

We previously demonstrated that GP64-FIV has broad tropism in the nasal airways of mice. Based on the relative anatomical distribution of submucosal glands and bronchiolar exocrine (Clara) cells, the upper airways of mice are generally considered to be a more suitable representation of the conducting airways in humans. We and others have reported that GP64-pseudotyped lentiviral vector transduces both ciliated and nonciliated cells. Offactory epithelial cells are known targets of GP64-FIV and may contribute to the nasal tropism; however, olfactory epithelial cells are not represented in the cell culture model. Further characterization of cells transduced with GP64-FIV may help us understand the preference of GP64-FIV transduction to the nasal airways compared with the pulmonary airways in mice. For example, future studies will examine the successful completion of steps involved in lentiviral transduction such as entry, fusion in low pH endosomes, reverse transcription, and integration. In addition, there are many additional variables to examine such as envelopes and lentiviral species.

Here, we replicate previous observations that GP64-FIV has a superior transduction efficiency in murine nasal respiratory epithelia as compared with the intrapulmonary airway epithelial cells. Comparison of GP64-FIV-mediated transduction efficiencies in primary cells derived from nasal and tracheal epithelia minimized potential variables such as phagocytic cells, adaptive immune responses, mucociliary clearance, and surface area. Administering a different vector, GP64-VSV, still showed preference of nasal transduction.

These results lead us to speculate that differential expression of cellular factors specific for FIV transduction between nasal and lung epithelia likely underlie the observed differential transduction efficiencies. Replication and transgene expression from the VSV vector occurs in the cytoplasm; however, expression from a lentiviral vector utilizes much different cellular pathways. The FIV genome is reverse transcribed, imported to the nucleus, and integrated into the host genome. Several studies have demonstrated that HIV-1 is unable to replicate in murine cell lines due to restrictions at multiple steps. It is possible that host factors necessary
for efficient FIV transduction are not expressed in murine intrapulmonary airway epithelial cells. Determining which step(s) are restricted during lentivirus vector transduction is of future interest. There may be host cell type–specific lentiviral restriction factors that are more abundant in the intrapulmonary airway epithelial cells compared with the nasal epithelial cells. Several host restriction factors are known to inhibit lentivirus infection. There may be yet undiscovered lentiviral-specific restriction factors that are induced in murine lung epithelial cells.

Future studies will address the mechanism of this difference in nasal versus intrapulmonary epithelial cells. Microarray analysis of gene expression in primary cultures of septal and tracheal epithelial cells may point to candidate restriction factors. In addition, contrasting the transduction of cell lines in the NCI-60 panel, which is a collection of 60 extensively characterized cancer cell lines by GP64-FIV and GP64- VSV may also reveal candidate proteins involved in lentiviral gene transfer. These studies may provide valuable information concerning cellular tropism of lentiviral vectors and help guide preclinical studies in pigs and larger animal models.

Materials and methods

Vector production. The FIV vector system utilized in this study expressed firefly luciferase in the FIV3.3RSV backbone. Autographa californica multinucleocapsid nucleopolyhedrovirus glycoprotein GP64-pseudotyped FIV vector particles were generated by transient transfection, concentrated 250-fold by centrifugation, and titered using real-time PCR as previously described. Glycoprotein-deleted recombinant VSV expressing firefly luciferase was kindly gifted by Dr. Michael Whitt (University of Tennessee). Pseudotyping of the FIV vector system utilized in this study was removed, cells were washed once with 1X phosphate-buffered saline, and further incubated at 37 °C, 5% CO2, for 7 days before cells were imaged using Xenogen IVIS-200 (PerkinElmer, Waltham, MA). Murine airway cultures were derived from ~8 week-old female mice. From ~15 mice, we disassociated cells from pooled nasal septa. In parallel, we collected and pooled the trachea from the same group of mice. After seeding cells on the Transwell filters (Corning Life Sciences, Lowell, MA), cells were allowed to differentiate for 4 weeks.

In vivo delivery of viral vector. Six- to 12-week-old Balb/c and C57Bl/6 mice were purchased from National Cancer Institute (Bethesda, MD). C57Bl/6 Rag−/− mice were kindly supplied by Dr. Stanley Perlman (The University of Iowa). SCID/NCr mice (Balb/c background, 01S11) were purchased from National Cancer Institute. C57Bl/6 IFNβR−/− mice were kindly gifted by Dr. John T. Harty (The University of Iowa). This study was approved by the Institutional Animal Care and Use Committee at the University of Iowa.

Mice were first anesthetized by intraperitoneal injection of ketamine/xylazine (87.5 + 2.5 mg/kg). Approximately 1 × 107 TU of FIV vector formulated with 1% methylcellulose (1:1) in a total volume of 50 µl was delivered to the nasal epithelium via direct instillation or to the trachea epithelia using a 24-gauge teflon catheter. Methylcellulose increases viral vector gene transfer efficiency in nasal and intrapulmonary airways without disrupting transepithelial resistance in human airway epithelial cells. Adenovirus vector was delivered at approximately 1 × 107 TU in a 50 µl volume with 1% methylcellulose (1:1). Before methylcellulose formulation and delivery, the VSV vector was normalized by Western blot using VSV matrix antibody.

Neutrophil depletion. Neutrophil depletion was achieved using mouse monoclonal RB6-8C5 antibody (BE0075; Bio X Cell, West Lebanon, NH). 24 hours before and 24 and 72 hours after the first dose of viral vector administration, mice were anesthetized by intraperitoneal injection of ketamine/ xylazine and 100 µg of RB6-8C5 or rat IgG2b isotype control (BE0090, Bio X Cell) was administered via nasal instillation or tracheal intubation.

Bioluminescence imaging. Mice were injected intraperitoneally with 200 µl of D-luciferin (15 mg/ml in 1X phosphate-buffered saline; Xenogen) using a 25-gauge needle. After 5 minutes, mice were imaged using the Xenogen IVIS charge-coupled device camera while under 1–3% isoflurane anesthesia. For the primary airway cultures in 24-well culture plates, 100 µl of D-luciferin was applied apically and imaged using the Xenogen IVIS. Imaging data were analyzed and signal intensity was quantified using Xenogen Living Image software (Xenogen). The negative controls in all in vivo bioluminescent assays were naïve strain matched mice. We included negative controls in every assay and report only background subtracted levels of experimental groups.

BAL collection and cell counts. At the appropriate end point, mice were euthanized by hypercarbia. After euthanasia, the trachea was exposed and a small incision in the trachea was made. Polyethylene tubing (1.22 mm outer diameter) was inserted through the incision and 3 ml of BAL fluid was
collected. Total cells in the BAL fluid samples obtained from each mouse were washed and counted with a hemocytometer. For differential cell counts, cells were applied to slides by cytospin and visualized with Giemsa stain. A total of 100 cells per lavage were counted under the light microscope.

**Electron microscopy.** Scanning electron microscopy was used to examine cell morphology after fixation in 2.5% glutaraldehyde (0.1 mol/l sodium cacodylate buffer, pH 7.4) for 30 minutes. After serial alcohol dehydration, samples were treated with hexamethyldisilazane (Polysciences, Warrington, PA) and mounted on stubs. Samples were examined under a Hitachi S-4800 electron microscope.

**Statistics.** All numerical data are presented as the mean ± SE. Statistical analyses were performed using two-tailed Student’s t-test with unequal variance.

**Supplemental material**

**Figure S1.** Balb/c mice were pretreated with 100 µg of RB6 antibody or IgG2b isotype control (IgG) 24 hours before and 24 hours and 72 hours after the first dose of GP64-FIV.

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