Targeting Viral-Mediated Transduction to the Lung Airway Epithelium with the Anti-inflammatory Cationic Lipid Dexamethasone–Spermine

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We formulated adenovirus (AdV) vectors with cationic steroid liposomes containing dexamethasone–spermine (DS)/dioleoylphosphatidylethanolamine (DOPE) in an effort to overcome the lack of apically expressed AdV vector receptors on airway epithelial cells and to reduce the inflammation associated with AdV vector exposure. An AdV vector (1 to 2.5 \times 10^{11} \text{ genome copies}) expressing human placental alkaline phosphatase or β-galactosidase (LacZ) was delivered alone or complexed with DS/DOPE, DC-Chol/DOPE, or dexamethasone to C57Bl/6 mice via intranasal instillation. Formulation of the AdV vector with DS/DOPE and DC-Chol/DOPE resulted in transgene expression targeted only to the airway epithelial cells with minimal expression in alveolar cells, while AdV alone caused high alveolar transduction. The DS/DOPE and dexamethasone formulations greatly reduced cellular infiltrates compared to AdV vector alone, while formulation with DC-Chol/DOPE did not. IFN-γ was significantly elevated at day 7 in mice receiving only the AdV vector compared to the AdV vector formulated with DS/DOPE, DC-Chol/DOPE, or dexamethasone. Lipid formulation of adeno-associated virus vector expressing LacZ also produced airway epithelial targeting, similar to the AdV vector. Viral vectors can be formulated with DS/DOPE to improve targeting to the airway epithelium in vivo and to attenuate vector-induced inflammation through the pharmacological activity of DS.

Key Words: glucocorticoid, liposome, adenovirus, cystic fibrosis, inflammation, epithelial cells

INTRODUCTION

Cystic fibrosis (CF) airway disease is a life-threatening genetic disease for which gene therapy presents a promising treatment option [1,2]. Adenoviral (AdV) vectors have been one of the most widely studied delivery systems for gene therapy of CF airway disease [3]. AdV vectors enter airway epithelial cells via a primary interaction with the coxsackie–adenovirus receptor (CAR) and secondary interactions with αv-integrins [4,5]. For ciliated airway epithelial cells, thought to be the target cell type for gene therapy of CF airway disease, the CARs are located on the basolateral membrane [4,6]. Consequently, these receptors are not entirely accessible to AdV vectors delivered directly to the airways, resulting in less than optimal binding and internalization of these vectors [4]. To overcome this inefficiency, high doses of AdV vector (≥10^{9} \text{ PFU per mouse lung}) have been used, transducing several cell types throughout the lung and causing substantial inflammatory response [7]. In lung, AdV vector-induced inflammation occurs within 24 h and peaks between day 5 and day 7. This inflammation is evidenced by CD4+ and CD8+ cellular infiltrates as well as the activation of NF-κB in AdV vector-infected cells and the subsequent release of inflammatory cytokines including IL-6, TNF-α, IFN-γ, and IL-12 [8–10]. The inflammation leads to a Th1-type immune response that eliminates positively transduced cells by 14 to 30 days and that also produces antibodies to the capsid of the AdV vector, which prevents successful re-administration [11,12].

Nonviral gene transfer vehicles such as cationic lipids and polymers have also been utilized in clinical trials for CF gene therapy [1–3]. Cationic molecules and liposomes associate with negatively charged AdV vector particles to facilitate entry into target cells that lack the
CAR and αv-integrins [4,13,14], thus improving transduction in vitro and in vivo [4,14–16]. Other molecules, such as cholesterol, have also been shown to increase transgene expression in CAR-deficient cells [17]. Cationic lipid molecules have been shown to increase AdV vector-mediated transgene expression in mouse nasal airway epithelial cells in vivo [15], although some investigators have demonstrated that certain routes of delivery or formulations with cationic molecules can result in lower total gene expression in vivo [18].

While cationic lipid/plasmid DNA complexes generally induce a less potent inflammatory response than viral vectors [19], nonviral transgene expression is not currently efficient enough for most clinical applications. Dexamethasone–spermine (DS) is an effective plasmid transfection reagent in vitro and in vivo when formulated with the neutral lipid dioleoylphosphatidylethanolamine (DOPE) [20]. Unlike other cationic lipids currently used for gene delivery, DS is pharmacologically active and induces nuclear localization of the glucocorticoid receptor in vitro and also reduces inflammation in vivo [20]. We tested formulations of DS/DOPE, DC–cholesterol (DC-Chol)/DOPE, and dexamethasone with AdV vectors in vitro in two epithelial cell lines, one with accessible CARs, A549 cells [21,22], and one without accessible receptors, MDCK cells [23,24]. We also evaluated these formulations in C57Bl/6 mice in vivo using a 50-μl dose of ~10^11 genome copies (GC) of vector and found that formulation with DS/DOPE facilitated targeting of transgene expression to the airway epithelium with significant inhibition of inflammatory cellular infiltrates.

**RESULTS**

**Lipoplex Formulation of AdV for Targeting to Mouse Airway Epithelium**

To determine whether DS/DOPE was effective at increasing AdV vector-mediated gene transduction to airway epithelial cells and reducing associated inflammation, we delivered an AdV vector expressing human placental alkaline phosphatase (AlkP) (2.5 × 10^11 GC) to mice by intranasal instillation either alone or in a complex with DS/DOPE. At day 1 or 7, the lungs were removed and one lung was homogenized and the other frozen for cryosectioning as described under Materials and Methods.

Analysis of lung homogenates for AlkP revealed that gene expression was reduced by 4-fold on day 1 (11.0 ± 5 pg/g vs 46 ± 30 pg/g) and 146-fold on day 7 (1.2 ± 0.5 pg/g vs 180 ± 148 pg/g) when the AdV vector was formulated with DS/DOPE (51 μg DS/DOPE with 10^11 GC AdV). Histochemical analysis of lung sections stained for AlkP revealed that, although total AlkP staining was substantially decreased by the formulation of the AdV vector with DS/DOPE, the lipoplex resulted in the marked targeting of AlkP expression to the airway epithelial cells, whereas AdV vector alone resulted in transgene expression in both alveoli and airways at both days 1 and 7 (Fig. 1). Furthermore, hematoxylin/eosin (H&E) staining showed extensive cellular infiltration at both time points when the AdV vector was delivered alone. In contrast, when we formulated the AdV vector with DS/DOPE no irregularities were seen in the lung compared to PBS controls (Fig. 1).

These results indicate that DS/DOPE was effective at reducing the inflammation in the lung while targeting transgene expression to the airway epithelial cells.

The targeting effects of DS/DOPE on AdV vector delivery to mouse lung epithelium shown in Fig. 1 were likely due to the physical–chemical effects of liposomes. To test this possibility, we chose DC-Chol as a representative sterol-based reference cationic lipid to DS. At day 1 post-instillation of the AdV-LacZ vector (10^11 GC) with DS/DOPE or DC-Chol/DOPE (Figs. 2A–2D), both liposome formulations led to targeting of the transgene expression mainly to airway epithelial cells, though not only so. Consistent with our prior studies of plasmid delivered with DS/DOPE to mouse lung that reduced inflammation relative to DC-Chol/DOPE [20], the delivery of AdV-LacZ vector with DS/DOPE produced less cellular infiltration than that obtained with DC-Chol/DOPE (Figs. 2A–2D).

Analysis of the lung homogenates revealed no statistically significant differences between DS/DOPE and DC-Chol/DOPE formulations for transgene expression, IFN-γ, or IL-4 at day 1 (Table 1). Based on the above experiments, we conclude that the targeting action of DS/DOPE is a property achieved with liposomes in general and does not require the pharmacological activity of DS. One possible explanation for the reduced cellular infiltrate observed with DS/DOPE formulated AdV vector (Fig. 1) is the lower total gene expression obtained by this formulation. However, DC-Chol/DOPE caused a marked cellular infiltration at a similarly low total transgene expression (Figs. 2B and 2D). This comparison suggests the pharmacological action of DS reduced cellular infiltration caused by the AdV vector.

To quantify our qualitative observations of inflammation in the lung, we randomly numbered and ordered representative slides from each group (AdV-, AdV + DS/DOPE-, AdV + DC-Chol/DOPE-, AdV + dexamethasone-, and PBS-treated) and then they were analyzed by a pathologist blinded to the treatment groups. Scores were defined as 0 (normal, no inflammation) to +++ (extensive inflammation). A short description, along with a score, was provided by the pathologist for each slide. The results of the semiquantitative scoring agreed well with our own observations described above. The AdV-LacZ vector appeared to be less inflammatory (+/+ at day 1, + at day 7) than the AdV-AlkP vector (++ at day 1, +++ at day 7). The AdV-AlkP vector-treated tissue was characterized by mononuclear infiltrates and congestion of blood vessels, and at day 7 there was evidence of alveolar hemorrhage. As expected, scores at day 7 were consistently higher than...
scores at day 1. At day 7 there were no signs of inflammation of the airways treated with either AdV vector formulated with DS/DOPE (0, normal) or dexamethasone (0, normal). However, for airways treated with the AdV vector formulated with DC-Chol/DOPE at day 7 there was mild (+) monocytic infiltrate and congestion. The DC-Chol/DOPE-formulated AdV-LacZ vector-treated airways had levels of inflammation similar to those seen when the AdV-LacZ vector was delivered alone at both days 1 and 7 (+/− at day 1, + at day 7). This evidence strongly supports our hypothesis that the pharmacological activity of DS is responsible for the reduction in inflammation when AdV vector is formulated with DS/DOPE.

Further evidence to support that the pharmacological activity of DS was related to the observed reduction in scores at day 1. At day 7 there were no signs of inflammation of the airways treated with either AdV vector formulated with DS/DOPE (0, normal) or dexamethasone (0, normal). However, for airways treated with the AdV vector formulated with DC-Chol/DOPE at day 7 there was mild (+) monocytic infiltrate and congestion. The DC-Chol/DOPE-formulated AdV-LacZ vector-treated airways had levels of inflammation similar to those seen when the AdV-LacZ vector was delivered alone at both days 1 and 7 (+/− at day 1, + at day 7). This evidence strongly supports our hypothesis that the pharmacological activity of DS is responsible for the reduction in inflammation when AdV vector is formulated with DS/DOPE.

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cellular infiltration came from staining cryosections for the presence of CD8+ T cells at days 1 and 7. Lungs treated with DS/DOPE-formulated AdV vector showed little to no positive staining for CD8+ T cells. However, lungs that were treated with only AdV vector resulted in a significant incidence of CD8+ cells (Figs. 2E–2J). These data further support the hypothesis that the pharmacological activity of DS inhibits the immune response to the AdV vector when delivered to the lung by intranasal instillation.

To investigate further the hypothesis that DS/DOPE targeting of AdV vector to airway epithelium is unrelated to its pharmacological activity, we evaluated transgene expression from the AdV-LacZ vector at day 7 in mouse lung for DS/DOPE, DC-Chol/DOPE, or dexamethasone (Figs. 3A–3D). Mostly alveoli were transduced when AdV-LacZ vector was delivered alone or with dexamethasone (Figs. 3A and 3D), but mostly airway epithelial cells were transduced with DS/DOPE or DC-Chol/DOPE (Figs. 3B and 3C). At day 7, formulations of AdV-LacZ vector reduced transgene expression compared to AdV-LacZ vector alone and caused significant reduction of IFN-γ, but not IL-4 (Table 1). Table 1 shows that IFN-γ was significantly elevated for AdV vector alone at day 7 compared to all other groups (P < 0.02), but that IL-4 levels were not significantly different for any group at this time point.

**Lipoplex Formulation of AAV-LacZ for Targeting to Mouse Airway Epithelium**

To assess whether the effect of targeting transgene expression was unique to the AdV vector, AAV-LacZ vector was formulated with DS/DOPE, DC-Chol/DOPE, or dexamethasone. Since inflammation is not as severe a side effect with AAV delivered to the lung compared to AdV vector instillation, we evaluated gene expression at 21 days post-instillation. We determined LacZ gene expression in the lung homogenates and fixed and stained lung sections for LacZ gene expression. Similar to the AdV vector, total gene expression was substantially reduced when the AAV vector was formulated with DS/DOPE (89.7 ± 33.5 pg/mg, n = 5), DC-Chol/DOPE, or dexamethasone. Since inflammation is not as severe a side effect with AAV delivered to the lung compared to AdV vector instillation, we evaluated gene expression at 21 days post-instillation. We determined LacZ gene expression in the lung homogenates and fixed and stained lung sections for LacZ gene expression. Similar to the AdV vector, total gene expression was substantially reduced when the AAV vector was formulated with DS/DOPE (89.7 ± 33.5 pg/mg, n = 5), DC-Chol/DOPE (116 ± 133 pg/mg, n = 5), or dexamethasone (279 ± 247 pg/mg, n = 5) compared to gene expression when the AAV vector was delivered alone (5000 ± 5560 pg/mg, n = 3). However, formulation of AAV vector with cationic lipids,
but not dexamethasone alone, resulted in transgene expression targeted mainly to the airway epithelium (Figs. 3E–3H).

Cationic Lipids Increase AdV Vector-Mediated LacZ Transduction in A549 Cells

To determine whether formulation of the AdV-LacZ vector with cationic lipids could increase transduction efficiency in vitro, we formulated the AdV vector with DS/DOPE, DC-Chol/DOPE, and dexamethasone and used it to transduce A549 cells. A549 cells have accessible CARs and αv-integrins allowing the entry of the AdV vector [21,22]. At 24 h post-transduction we harvested the cells and measured β-galactosidase activity. We used AdV vector at an m.o.i. of $10^4$ or $10^5$ GC per cell and formulated it with 300 µl of 0.5 mM DS, DS/DOPE (1:1), DC-Chol/DOPE (1:1), or dexamethasone at each vector dose. After 2 h of incubation, we removed the transduction medium and replaced it with fresh growth medium. The DS/DOPE formulation enhanced gene transduction at an m.o.i. of $10^1$ (Fig. 4A) but not at $10^4$ GC (Fig. 4B), indicating an advantage of DS/DOPE over DC-Chol at low viral load.

AdV-LacZ Vectors Transduce MDCK Cells When Formulated with Cationic Liposomes

If the AdV vector, when formulated with DS/DOPE or other cationic lipids, were entering the cell by “lipofection” (i.e., a non-receptor-mediated pathway), then cells that are normally resistant to AdV uptake would be expected to be transduced by cationic-lipid-formulated AdV vectors and not dexamethasone-formulated vectors. MDCK cells were used to test this hypothesis, since they form tight junctions that restrict access of AdV vector to the basolateral surface where the CARs are located [23,24]. In testing AdV vector formulations, a range of lipid concentrations (0, 10, 100, 500 µM) was added in 300 µl of Opti-MEM to an m.o.i. of $10^4$ GC of AdV vector per well. Both DS/DOPE- and DC-Chol/DOPE-formulated AdV vector showed high levels of LacZ gene expression, while dexamethasone did not significantly improve LacZ gene expression compared to levels obtained with the AdV vector alone (Fig. 4C). These results suggest that, when formulated with liposomes, the AdV vector was lipofected into cells and thus may have bypassed the normal requirement for specific surface receptors.

DISCUSSION

Despite extensive research in using AdV vectors for CF gene therapy, limited transduction of the target ciliated airway epithelial cells as well as immune responses to the vector have hindered efforts to achieve clinical success. DS/DOPE liposomes significantly improved targeting of
the transduction to the lung airway epithelia and also reduced the cellular infiltration observed with the use of AdV vectors. We confirmed that a similar sterol-based liposome, DC-Chol/DOPE, resulted in comparable targeting of the AdV vector to the airways, but also resulted in cellular infiltration associated with AdV vector delivery. Also, while dexamethasone effectively limited cellular infiltration, it did not aid in targeting of AdV vector-mediated gene expression to the airway epithelia.

We have shown that AdV and AAV vectors, when formulated with liposomes, can target vector-mediated transgene expression to airway epithelial cells, both in vivo and in vitro, due to the process of lipofection. Specifically, formulating the AdV vector with liposomes resulted in a significant increase in LacZ gene expression in cells with available CARs at a very low m.o.i. of 10^4, suggesting that this formulation could allow for much smaller AdV vector doses to be used. In vivo, this reduction in AdV vector dose could reduce the inflammation and resulting adaptive immune response typically associated with high vector doses. Although the in vivo results using AAV vectors formulated with liposomes resulted in very low gene expression, this may have been due to formulation issues, which we are currently investigating. Since the AAV vector is a much smaller viral particle (~20 nm) compared to AdV vectors (~100 nm), a much lower liposome concentration may have been required to formulate the AAV vector efficiently. The transgene expression levels within each group that received AAV vector were highly variable, but we have consistently observed that AAV-mediated gene expression levels do vary significantly within a treatment group when this route of instillation is used.

Our results suggest that the initial inflammation and further immune activation associated with these entry steps of AdV vectors may be significantly reduced by the formulation of AdV vector with cationic liposomes. DS/DOPE liposomes could supplement the utilization of an alternate cell entry pathway via its pharmacological activity, shown in this study by the complete inhibition of cellular infiltration to the lung at days 1 and 7 after AdV vector delivery with DS/DOPE-formulated AdV vector. Comparison of IFN-γ levels between DC-Chol/DOPE- and DS/DOPE-formulated AdV vector did not result in significant differences even though H&E staining indicated higher amounts of cellular infiltration with DC-Chol/DOPE-formulated AdV vector. In previous studies with plasmid delivery to the lung, DS/DOPE did, however, significantly reduce IFN-γ levels in comparison to DC-Chol/DOPE [20]. AdV vector, when delivered to airway epithelium in vivo, results in the secretion of several chemokines, such as RANTES, IP-10, and MIP-2, as well as the upregulation of adhesion and costimulatory molecules such as ICAM-1 [8]. Therefore, the cellular infiltration observed with AdV vector delivered alone and when formulated with DC-Chol/DOPE could be associated with a cytokine or chemokine other than IFN-γ. Furthermore, DS is capable of reducing the expression of adhesion molecules on cultured endothelium (data not shown), which may suggest that some reduction in cellular infiltration was due to the action of DS on endothelium. Delivering dexamethasone along with the AdV vector has been shown to increase gene transduction and reduce the immune response to AdV vector in the lung [25]. DS/DOPE-formulated AdV vector is advantageous over dexamethasone since this formulation appears to enhance transduction targeting to the airway epithelial cells. Furthermore, DS/DOPE liposomes locally deliver targeted pharmacologically active dexamethasone-based DS cospatially with the AdV vector as opposed to the diffuse dosing that would occur with dexamethasone alone, possibly reducing side effects associated with systemic glucocorticoid delivery. Another potential benefit of formulating the AdV vector with DS is the duration of transgene expression, which may be longer than the 14 to 30 days expected with AdV vector delivery since there may be a significant repression of the immune response.

In this study we have demonstrated that formulation of DS/DOPE liposomes with AdV vectors significantly reduces inflammation when delivered to the lungs of mice in vivo and also appears to eliminate the necessity of the CAR and α5-integrins on the apical membrane of the target ciliated airway cell surface to allow efficient gene transduction. Furthermore, the pattern of transgene expression achieved with this novel formulation, limited almost exclusively to airway epithelial cells, is ideal for the treatment of the CF airway disease, which requires correction of the ciliated airway epithelial cells that line the conducting airways.

**Materials and Methods**

**Materials.** DS was prepared as described previously [20]. Briefly, dexamethasone–mesylate (Steraloids, Newport, RI, USA), Traut’s reagent (Pierce, Rockford, IL), and spermine (Sigma, St. Louis, MO, USA) were reacted in a 1:1:5 molar ratio in a one-step reaction to form DS. DS was then purified using HPLC (Hamilton PRP-1 column) and freeze dried as DS trifluoroacetic acid salt (MW 1133). DOPE and DC-Chol were purchased from Avanti Polar Lipids (Alabaster, AL, USA). DS/DOPE and DC-Chol/DOPE liposomes were prepared as previously described [20]. Briefly, a solution of DOPE/ethanol was evaporated to form a lipid film. DS or DC-Chol in a solution (0.5 to 1.5 mM) of sterile PBS for in vitro experiments or sterile water for in vivo experiments was added in a 1:1 molar ratio to the DOPE film. The lipids were then vortexed thoroughly and sonicated (103 W for 15 min) at room temperature in a water bath sonicator (Fisher FS9H) to form liposomes. Dexamethasone (Sigma) was prepared by evaporation of a dexamethasone/ethanol solution and reuspension of the dexamethasone as described for DS and DC-Chol. All preparations were made fresh before each experiment and stored at 4 °C for less than 4 weeks.

**Virus preparation and formulation.** E1/E3-deleted replication-deficient, recombinant adenovirus vectors (2–4.5 × 10^12 GC/ml stock) expressing either LacZ or Alkβ reporter were created as previously described [26] by homologous recombination between the shuttle plasmid that carries the transgene and the 5’ and 3’ ends of the adenovirus genome with E1 deleted and 3’ end of H5.sub360 in which the E3 gene was inactivated by a small deletion. The recombinant viruses were propagated in 293 cells and

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purified by the standard CaCl₂ gradient sedimentation method. The replication-deficient AAV2/5Cre/CreLacZ contains the LacZ gene with a nuclear localization sequence at its N-terminus, under the transcriptional control of the chicken β-actin promoter. The AAV2/5 vector (~2 x 10¹² GC/ml stock) was produced by transcapsidation in 293 cells using a triple transfection method and purified by CaCl₂ gradient sedimentation as described [27].

For in vivo studies, DS/DOPE (51 µg total lipid/10¹¹ GC), DC-Chol/DOPE (40 µg total lipid/10¹¹ GC), or dexamethasone (29.4 µg/10¹¹ GC) was added to an equal volume of AdV-LacZ, AdV-AlkP, or AAV-Lacz vector and incubated at room temperature for 15 to 30 min prior to use. The in vitro studies used varying lipid concentrations as indicated in the text and lipid/vector complexes were prepared as described above.

Cell culture and in vitro gene transfer studies. A549 (ATCC CCL-185) and MDCK (ATCC CCL-34) cells were grown in DMEM (GIBCO, Carlsbad, CA, USA) supplemented with 10% newborn calf serum (GIBCO), 2% penicillin/streptomycin, and 1% L-glutamine (GIBCO) and maintained at 37 °C and 5% CO₂. For transfections/transductions, MDCK and A549 cells were seeded at 2 x 10⁴ cells/well in 24-well plates and grown to confluency. Prior to transfection/transduction, wells were examined under the light microscope for general uniformity and health and then cells from at least one well were then harvested and a cell count was performed using a hemacytometer. All vector and liposome formulations were prepared in Opti-MEM and then 300 µl of the transfection/transduction mixture was added to PBS-rinsed cells. The mixture was incubated with the cells for 2 h at 37 °C, then removed, and fresh growth medium added. At 24 h after transduction, cells from each well were harvested and lysed using β-galactosidase reporter gene assay lysis buffer (Roche, Indianapolis, IN, USA) according to the manufacturer’s instructions. Samples were collected and centrifuged at maximum speed for 2 min and analyzed immediately or stored at −80 °C. LacZ gene expression was assayed by using 50 µl of cell extract per well in a 96-well plate using a chemiluminescence β-galactosidase reporter gene assay kit (Roche). Signal was detected by integrating light production from the chemiluminescent substrate for 5 s on a fluorescence/luminescence plate reader (Fluoroskan Ascent FL, Labsystems).

In vivo delivery to mouse lungs. C57Bl/6 mice (6 to 8 weeks of age) were anesthetized by an intraperitoneal injection of a 1:1 mixture of PBS and OCT embedding compound. Lungs were harvested at 1, 7, or 21 days post delivery to mouse lungs. For dosing, mice were suspended from their dorsal incisors (hind quarters supported) and a dose of 10¹¹ GC of AdV or AAV vector was delivered as a 50-µl bolus into the nostrils using a gel-loading tip (Finnpipette). Lungs were harvested at 1, 7, or 21 days and inflated with a 1:1 mixture of PBS and OCT embedding compound.

One lobe was then submerged in OCT, frozen in isopentane cooled with liquid nitrogen, and cryosectioned (10 µm). Sections were fixed in 4% glutaraldehyde in PBS (Electron Microscopy Sciences, Hatfield, PA, USA) and stained with X-gal substrate (5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside) for 16 h at 37 °C to reveal LacZ-positive cells, NBT/BCIP (NBT/BCIP) were air dried and fixed in cold (−20 °C) acetone for 5 min. Once dried the sections were blocked with 1% goat serum/PBS for 15 min. The primary antibodies [rat anti-CbD8 (BD Pharmingen, 550281)] were then added at a 1:20 dilution in PBS/1% goat serum and incubated at room temperature for 1 h. Following three 5-min washes in PBS, the secondary antibody (FITC anti-rat) was added at a 1:200 dilution in PBS/1% goat serum and incubated in the dark at room temperature for 30 min. Following three 5-min washes in PBS, the sections were mounted in Vectashield with DAPI and viewed under a fluorescence microscope.

Transgene and cytokine detection. Mouse IFN-γ and IL-4 enzyme-linked immunosorbent assays (ELISA) (Pierce–Endogen, Rockford, IL, USA) were performed on lung homogenates according to the manufacturer’s instructions. AdV vector-mediated LacZ gene expression in lung homogenate was analyzed in vitro and in vivo, respectively, using either the β-galactosidase chemiluminescence gene reporter assay (Roche) or the β-galactosidase ELISA kit (Roche) according to the manufacturer’s instructions. All values were normalized to total protein content in the sample using the Bio-Rad protein assay (~0.5 µg/ml per sample).

Statistical analysis. Values are reported as means ± standard deviation. Treated groups of mice were n = 4, unless otherwise indicated. All quantitative analyses were performed in duplicate or triplicate for each lung sample. In vitro studies were performed in triplicate wells and transgene quantification was performed for each well in duplicate. Statistical analysis was performed using JMP software. Analysis of variance tests were performed to determine difference between groups; P ≤ 0.05 was considered significant. Within groups, the Student t test was used to determine significance when P ≤ 0.05.

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