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ORIGINAL ARTICLE

Pulmonary delivery of adenovirus vector formulated with dexamethasone–spermine facilitates homologous vector re-administration

AR Price[1,4], MP Limberis[2,4], JM Wilson[2] and SL Diamond[1,3]

[1]Department of Bioengineering, Institute for Medicine and Engineering, Vagelos Research Laboratory, University of Pennsylvania, Philadelphia, PA, USA; [2]Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, PA, USA and [3]Department of Chemical and Biomolecular Engineering, Institute for Medicine and Engineering, Vagelos Research Laboratory, University of Pennsylvania, Philadelphia, PA, USA

Gene transfer to lung has been hindered by inflammatory and immunological responses activated to the gene-transfer agent or transgene products. In prior work, adenovirus vector delivered to the lung with the cationic glucocorticoid, dexamethasone–spermine (DS) had improved targeting to conducting airway epithelium and reduced cellular infiltration. In this study, the effect of formulation on homologous adenovirus vector re-administration was studied in C57Bl/6 mice. Formulation of an adenovirus vector expressing LacZ with DS/dioleoylphosphatidylethanolamine (DOPE) delivered at day 0 allowed re-administration of adenovirus vector expressing alkaline phosphatase at day 21. Formulation with 3β [N-(N′, N′-dimethylaminoethane) carbamoy] cholesterol (DC-Chol) DC-cholesterol (DC-Chol))/DOPE or dexamethasone in the first dosing at day 0 resulted in moderate alkaline phosphatase expression at day 24. Neutralizing antibodies against adenovirus vector in serum at day 28 were greatly reduced by all three formulations in mice receiving a single dose of adenovirus at day 0. Also, homologous adenovirus vector re-administration at day 14 produced less neutralizing antibody at day 28 when adenovirus was formulated with DS/DOPE at day 0. The use of DS/DOPE at day 0 dramatically reduced CD4 and CD8 T-cell infiltration in mice receiving adenovirus at day 0 followed by vector re-administration at day 14. Transgene-specific T-cell activation was markedly reduced by the DC-Chol/DOPE formulation. Overall, DS/DOPE) facilitated homologous vector re-administration through a combination of liposomal and glucocorticoid mechanisms.

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Introduction

Gene-transfer vectors based on adenovirus (AdV), which is naturally tropic for the lung, are well suited for lung-directed gene therapy of monogenic diseases such as cystic fibrosis lung disease. However, strong immune responses are elicited by AdV vector delivery both in mice[1,2] and humans.[3] Also, the success of gene therapy in lung has been limited by the low transduction efficiency of the target ciliated airway epithelial cells. Although ciliated airway epithelial cells express the required coxsackie-AdV receptor and αv-integrins for viral uptake, AdV vectors have poor access to these receptors located on the basolateral surface of cells due to the presence of tight junctions[4–7] This physical barrier can be overcome by using high vector doses or transiently disrupting the epithelial integrity.[8,9] High doses of AdV vector, however, result in increased AdV-specific immune responses and consequently enhanced clearance of positively transduced cells.[10]

The temporal sequence of immune responses to AdV vector delivery can be monitored by quantifying cytokines, cellular activation and neutralizing antibody (NAB) production. Delivery of AdV vector results in the activation of Toll-like Receptor 4 and signaling through MyD88 as well as TRIF/TICAM-1, and initiating a type I interferon response.[11] Furthermore, for the most commonly used serotypes of AdV (Hu5), the coxsackie-AdV receptor and αv-integrin receptors are necessary for AdV vector entry to the cell[12,13] and are likely involved in the immune response cascade to AdV vectors.[14] A series of signaling pathways that include mitogen-activated protein kinases, extracellular responsive kinase, p38 and Jun kinase, are activated by AdV vector binding and internalization, followed by the activation of nuclear factor kappa B.[12] Depending on the cell type infected, these pathways result in the secretion of inflammatory mediators, including interleukin (IL)-6, tumor necrosis factor (TNF)-α, interferon (IFN) γ, macrophage inhibitory protein (MIP)-2, MIP-1α, monocyte chemotactic peptide-1 (MCP-1), regulated upon activation normal T-cell expressed and secreted (RANTES), interferon-induced protein

Correspondence: Dr SL Diamond, Department of Chemical and Biomolecular Engineering, Institute for Medicine and Engineering, University of Pennsylvania, 3340 Smith Walk, 1024 Vagelos Research Laboratories, Philadelphia, PA 19104, USA.
E-mail: sld@seas.upenn.edu
[4]These authors contributed equally to this work.
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(IP-10) and the upregulation of adhesion molecules, such as intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 (VCAM-1)[1,14–16] The cytokines released produce a Th1-dominant response against AdV vector genes and the encoded transgene product, resulting in cytotoxic T lymphocyte-mediated elimination of the positively transduced cells within 2–3 weeks.[1,2,16] A humoral response is also induced resulting in the generation of NAB to AdV capsid proteins, preventing successful homologous AdV vector re-administration.[2,17–19]

The understanding of vector-activated immune responses is further complicated when AdV vectors are delivered to the lung,[2] as there is precedent for a difference in the localized versus the systemic response to foreign antigens. Indeed, Matsuse *et al*.[20] showed that intranasal delivery of IL-12 resulted in divergent pulmonary and systemic effects. It is also well documented that lung delivery of AdV vector evokes distinct pulmonary and systemic immune responses as indicated by the Th2 phenotype of lung- or spleen-derived CD3+ T cells versus the Th1 cell phenotype of lower respiratory lymph-node-derived CD3+ T cells.[21] Also, van Ginkel *et al*.[21] found that lower respiratory lymph-node-derived CD4+ T cells displayed higher proliferation in response to heat-inactivated wild-type AdV vector or β-galactosidase (β-gal) than lung- or spleen-derived CD4+ T cells.[21]

We have previously shown that the formulation of AdV vector with liposomes comprised of the anti-inflammatory cationic lipid dexamethasone–spermine (DS) and the neutral lipid dioleoylphosphatidylethanolamine (DOPE), when delivered to the lung, targets transgene expression to the conducting airway epithelial cells and significantly reduces cellular infiltration, compared to delivery of AdV vector alone.[22] Improved targeting of AdV vector to the conducting airway epithelial cells by DS was due to the physical–chemical properties of liposomes in general, while the reduction in cellular infiltration was due to the anti-inflammatory properties of DS.[22] DS was also anti-inflammatory in a murine intraperitoneal thioglycollate challenge model and reduced IFNγ production at day 1 following plasmid lipoplex delivery to lung.[23]

In the current study, our goal was to investigate the nature of the immune response, both pulmonary and systemic, to AdV vector delivered in various formulations. Specifically, we used single and repeated AdV vector delivery to mouse lung to define the nature of the immune response and compare and contrast the effects that formulations of DS/DOPE, 3β [*N*-(*N*′, *N*′-dimethylaminoethane) carbamoy] cholesterol (DC-Chol) DC-cholesterol (DC-Chol)/DOPE or dexamethasone have on that response.

Results

Transgene expression following single or repeated administration of AdV vector to lung

A central problem with viral gene delivery is the inability to achieve significant levels of transgene expression upon homologous vector re-administration due to blocking of transduction by serum-circulating NAB and immunological clearance of vector through cytotoxic T lymphocyte activation upon the second administration. Using various formulations for the first instillation of AdV–LacZ (10^{11} particle/mouse, IN, day 0), we then re-administered mice at day 21 with the same serotype AdV vector (without formulation) expressing a different transgene (alkaline phosphatase; AlkP) to directly evaluate the impact of the first AdV vector dosage on the second AdV vector dosage (Figure 1a). This experimental design would result in the activation of significant levels of serum-circulating NAB to the AdV vector capsid and potentially activate cytotoxic T lymphocytes specific to the vector capsid and/or the transgene resulting in decreased AlkP gene expression.

Three days following AdV–AlkP vector delivery (study day 24), AlkP gene expression was detectable in all treatment groups. As expected, delivery of AdV–LacZ vector without formulation at day 0 resulted in a striking 95% reduction ($P = 0.04$) in AlkP gene expression at day 24 compared to phosphate-buffered saline (PBS)-sham delivery at day 0 (Figure 1b), confirming a strong immunological response following the initial administration of AdV–LacZ vector. Formulation of the AdV–LacZ vector with DS/DOPE at day 0 resulted in a significantly higher ($P = 0.05$) level of AlkP gene expression at day 24 (3 days post-AdV–AlkP vector instillation) compared to that achieved when mice were pre-administered with either AdV–LacZ alone or in formulation with DC-Chol/DOPE at day 0. AlkP gene expression was also numerically greater with DS/DOPE formulation compared to dexamethasone formulation; however, the difference did not reach statistical significance. β-gal expression, from the first AdV vector dose was undetectable (as expected) in all groups by day 24. Formulation of AdV–LacZ vector with DS/DOPE at day 0 allowed homologous AdV vector re-administration at day 21, resulting in AlkP gene expression that was comparable to that achieved after a single administration of AdV–AlkP vector at day 21 in mice pretreated with PBS-sham at day 0.

Cytokine and chemokine response to AdV vector delivery

To understand the benefit of formulation of the initial AdV vector dose, the acute response to AdV–LacZ vector administration was evaluated by determining the serum cytokine and chemokine profiles in the groups treated with AdV–LacZ vector alone and AdV–LacZ vector formulated with DS/DOPE. Sera samples taken 6 h post-AdV vector instillation were pooled by treatment group ($n = 3$) and evaluated using the multi-analyte profiling facility at Charles River Laboratories (Austin, TX, USA) (www.rulesbasedmedicine.com). A total of 59 different serum cytokines and chemokines were measured, with 26 of these depicted in Figure 2 as a percentage of the level found in the PBS-sham group. The remaining 33 serum cytokines and chemokines that were assayed but not shown in Figure 2 were either not detectable or unchanged by any treatment compared with the PBS-sham treatment group. Substantial elevations of IL-11, IL-4, IL-5, lymphotactin, MCP3, MIP-1β and TNFα were induced by delivery of AdV vector alone; however, when the AdV vector was formulated with DS/DOPE, the levels of IL-11, IL-4, IL-5, lymphotactin, MCP3, MIP-1β, and TNFα were markedly reduced. In contrast, IL-6, IL-7 and IP-10 were elevated by the use of the DS/DOPE

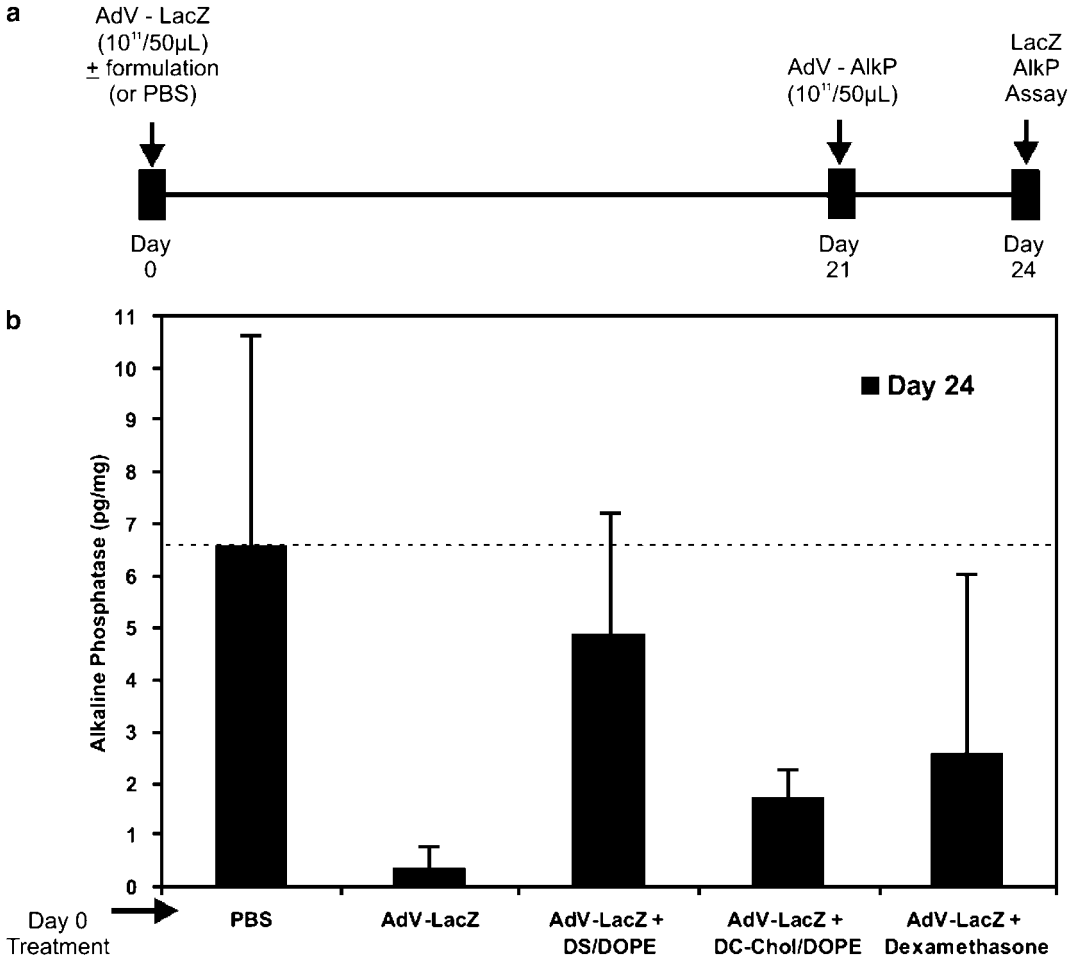

Figure 1 Effect of adenoviral vector formulation on re-administration of vector. Dosing regimen for AdV–LacZ vector (with or without formulation) or phosphate-buffered saline (PBS)-sham dosing at day 0 followed by homologous re-administration at day 21 with AdV–alkaline phosphatase (AlkP). (**a**) At day 24, AlkP gene expression was observed in the day 0 phosphate-buffered saline (PBS)-sham treatment group but was reduced by ~95% in the day 0 AdV–LacZ treatment group. (**b**) The use of dexamethasone–spermine/dioleoylphosphatidylethanolamine (DS/DOPE) and two other formulations at day 0 resulted in significantly higher levels of AlkP compared to AdV vector delivered alone at day 0 ($P<0.05$).

formulation compared to the levels observed with AdV vector alone. Cytokine indicators of natural killer and T cells, such as IL-4 and IL-5, respectively, were increased by greater than two-fold for AdV vector alone versus AdV vector formulated with DS/DOPE. IL-11 was increased ~6-fold for AdV vector alone compared to AdV vector formulated with DS/DOPE. KC/GROα, an indicator of inflammation, was reduced in mice treated with AdV vector formulated with DS/DOPE compared to the PBS-sham and AdV vector alone treatment groups. Other markers of neutrophil and lymphocyte recruitment (MCP3, MIP-1β and TNF-α) were also higher in mice treated with AdV vector alone.

Since the levels of serum cytokines and chemokines, produced as a result of an inflammatory or immunological response to AdV vector, are transient and can peak within hours to days post-vector instillation, the single 6-h time point used to assay these in serum was a semi-quantitative indicator of the systemic response to the AdV vector. To further understand the dynamics of the acute response to AdV vector with formulation, we measured the levels of IL-6 and TNF-α in sera at 6 h, and 1, 3 and 8 days post-vector instillation. When AdV was formulated with DS/DOPE, DC-Chol/DOPE or dexamethasone, there was a nonsignificant trend towards reduced levels of IL-6 in sera at 6 h compared to mice treated with AdV vector alone (Figure 3a), and similar to those observed 6 h after PBS-sham delivery. Delivery of PBS to lung resulted in baseline production of IL-6, presumably attributed to the stress of the anesthesia and delivery procedure. Interestingly, formulation of the AdV vector with DS/DOPE resulted in relatively low IL-6 levels at day 3, which was not the case for AdV vector formulated with either DC-Chol/DOPE or dexamethasone, both of which produced an unexplained IL-6 elevation at day 3. While the multi-analyte profiling detected at day 3 a striking sixfold induction of TNF-α by delivery of AdV vector alone, which was completely absent when AdV vector was formulated with DS/DOPE (Figure 2), the detection limit of the commercial TNF-α enzyme-linked immunosorbent assay (ELISA) was not as sensitive as that of the multi-analyte profiling and we could not detect any changes in TNF-α secretions at 6 h and day 1. Additionally, serum levels of TNF-α in response to AdV vector (with or without formulation) at day 3 were not significantly elevated over

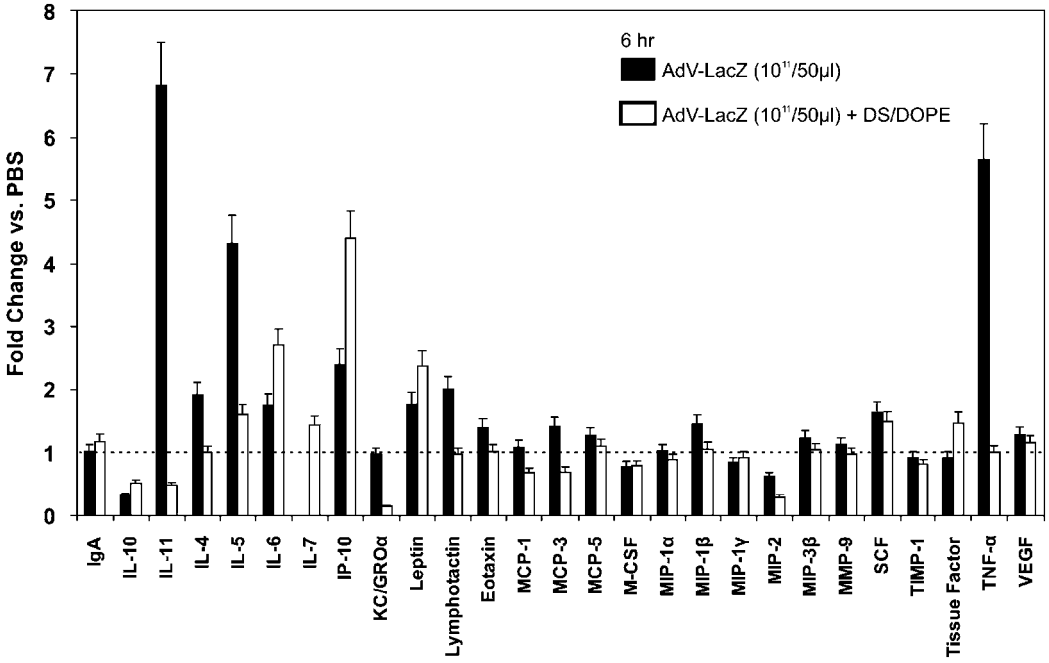

Figure 2 Cytokine and chemokine profiles of pooled sera 6 h post-AdV vector instillation. Pooled sera from three treatment groups (AdV–LacZ alone, AdV–LacZ with dexamethasone–spermine/dioleoylphosphatidylethanolamine (DS/DOPE) and phosphate-buffered saline (PBS-sham control; $n=3$ mice each) were analyzed by Luminex assay (see Materials and methods) and individual protein values were then normalized to the PBS-sham control group. Values are presented as 'fold change over PBS'. The dotted line represents the value of '1' where either AdV vector cohort—with or without DS/DOPE—was equal to the PBS-sham control.

PBS-sham levels by ELISA determination (~250 pg/ml) (Figure 3b). Similarly, TNF-α levels in response to AdV vector (with or without formulation) at day 8 were not significantly elevated over PBS-sham levels (~800 pg/ml), except for a modest 1.7-fold elevation over PBS-sham levels with the use of DC-Chol/DOPE. The cytokine levels detected by ELISA in serum, in response to AdV vector instillation, were not particularly high for any of the formulations relative to the control PBS-sham treatment.

The Luminex offers a large profile of antigens and high sensitivity to detect a pattern of an immune response; however, the ELISA is fully calibrated and exploits a sandwich-based assay to maximize the specificity of the signal. It is important to note, however, that the Luminex multi-antigen analysis has no absolute calibration and is presented as a ratio to a historic control normal mouse serum sample and in our study was normalized as a ratio to the PBS-sham treated group. Thus, the data in Figure 2 are only semi-quantitative and could be subject to distortion when taking ratios of small numbers. In contrast, an ELISA was conducted on fewer antigens but more time points. For a specific antigen of investigation (such as IL-6), we place more value in multiple time points assayed for levels of IL-6 antigen by ELISA (Figure 3a) as opposed to Luminex profiling (Figure 2).

Effect of formulation on the generation of neutralizing antibodies against AdV vector

To understand the impact of lipid formulation on the production of NAB in serum following AdV vector delivery to lung, the levels of serum-circulating NAB of each treatment group were quantified. Specifically, the levels of serum-circulating NAB at day 28 following a single administration of AdV vector were dramatically reduced by formulation of AdV vector with DS/DOPE, DC-Chol/DOPE or dexamethasone (Figure 4a), indicating that either a cationic liposome formulation (DS or DC-Chol) or a glucocorticoid prevented the development of NAB.

Since DS/DOPE is a liposome formulation with glucocorticoid activity[22,23] that appeared to facilitate AdV vector re-administration (Figure 1), we also evaluated its effect on NAB production at day 28 following AdV vector re-administration. For the second administration at day 14, we used an AdV vector expressing human α1-antitrypsin (*h*AAT) to avoid a combined response to vector and transgene products (Figure 4b). The treatment group that received two doses of AdV vector with no formulation ($n=3$) had a high titer of serum-circulating NAB (1:3700). However, this high titer was attributed to a single outlier (1:10 240). As such, Figure 4b also displays the NAB data with this outlier omitted ($n^*=2$). As expected from the *h*AAT gene expression results in Figure 1, the use of DS/DOPE to formulate the AdV vector delivered at day 0 followed by a second dose of AdV vector alone (no formulation) at day 14 reduced serum-circulating NAB assayed at day 28. Formulation of the second dose of AdV vector with DS/DOPE delivered at day 14 further reduced the generation of NAB assayed at day 28.

Effect of AdV vector formulation on pulmonary T-cell-mediated response

In light of the results highlighted in Figures 1–4, which demonstrate the inflammatory and immunological

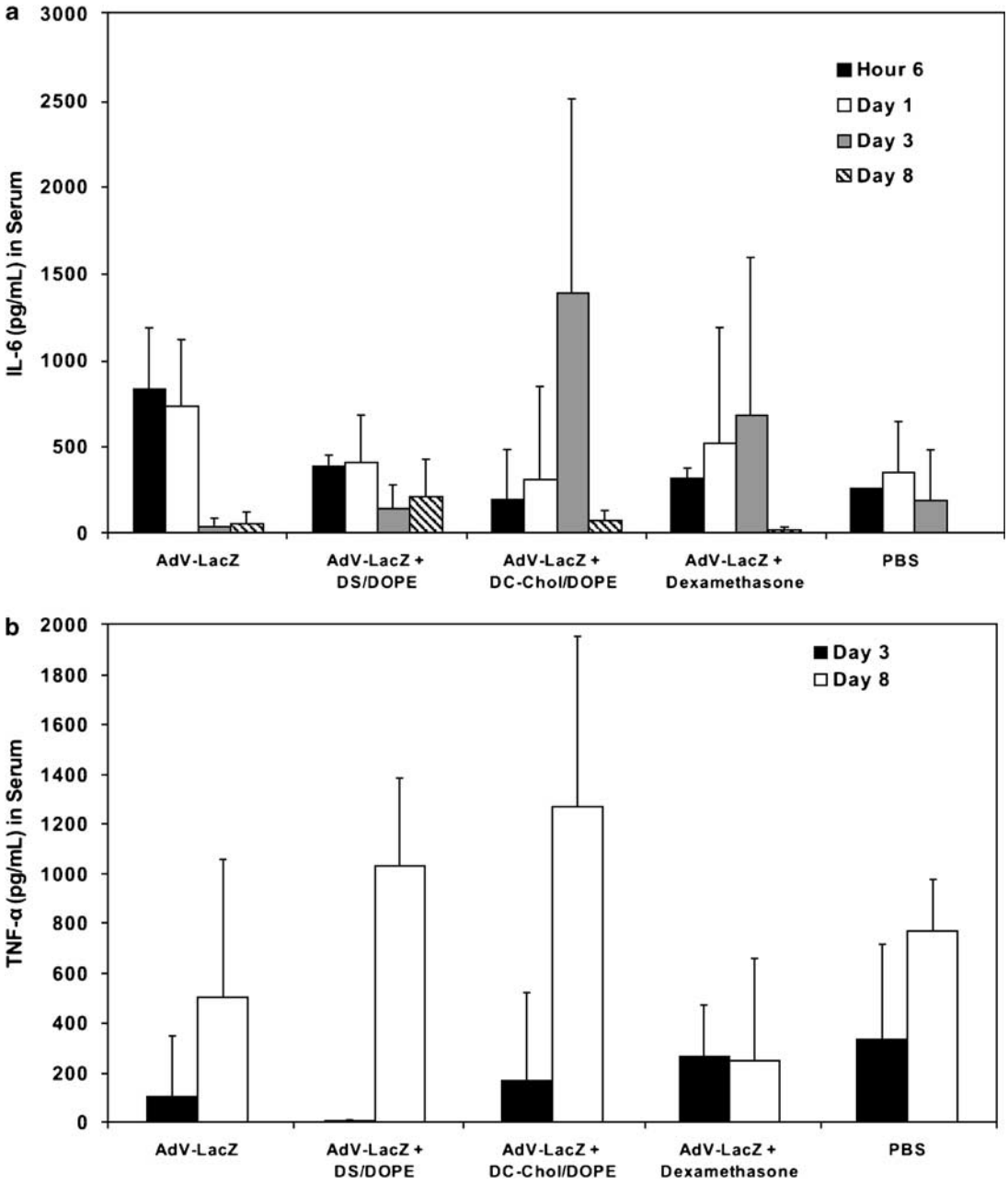

Figure 3 Sera levels of interleukin (IL)-6 (**a**) and tumor necrosis factor (TNF)-α (**b**) determined by enzyme-linked immunosorbent assay (ELISA) assay at various time points after AdV–LacZ (± formulation) or phosphate-buffered saline (PBS)-sham delivery to lung.

response to AdV vector when formulated with DS/DOPE, we next evaluated the role of formulation on cellular responses. The localized pulmonary response at day 28 to AdV vector (±DS/DOPE formulation for the first or second vector instillation) was investigated by immunostaining for CD4 and CD8 T-cell infiltration in frozen lung sections (Figures 5a–f). Delivery of AdV–LacZ at day 0 followed by AdV–*h*AAT at day 14 (both doses delivered with no formulation) in either vector instillation resulted in a striking elevation of CD4 and CD8T lymphocytes surrounding the conducting airways at day 28 (Figures 5a and b). The magnitude of CD4 or CD8 T-cell infiltration at day 28 was reduced when the AdV–LacZ vector was formulated with DS/DOPE for the first delivery at day 0 even though the AdV–*h*AAT vector was delivered with no formulation at day 14 (Figures 5c and d). CD4 and CD8 T-cell infiltration at day 28 was further reduced when the AdV–LacZ vector was formulated with DS/DOPE for the first instillation at day 0 and the AdV–*h*AAT vector formulated with DS/DOPE for the second instillation at day 14 (Figures 5e and f). We have previously found that the DS/DOPE formulation promotes targeting of the AdV vector to the conducting airways.[22] This transduction pattern was also correlated with CD4 and CD8 T-cell infiltration, which was reduced and localized to the conducting airway epithelium when the delivered AdV vector was formulated with DS/DOPE. Similar reductions of the localized CD4 T-cell infiltration were observed for the dexamethasone formulation, but not for the DC-Chol/DOPE formulation (data not shown).

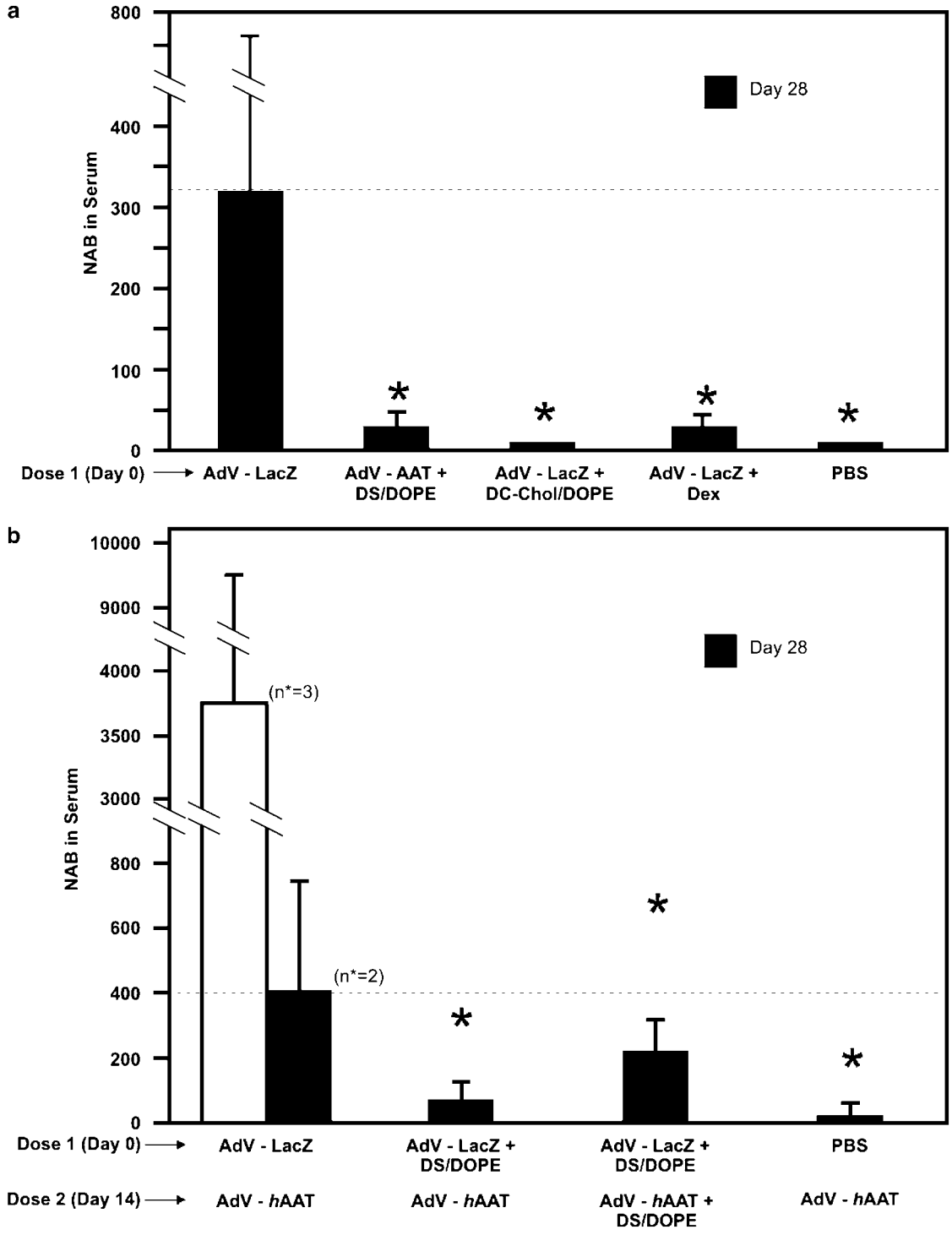

Figure 4 Neutralizing antibodies (NABs) against AdV vector in sera at day 28 (**a**) post-single vector administration at day 0 or (**b**) double administration at days 0 and 14. The titer of serum-circulating NAB for each sample was reported as the highest dilution producing $\leqslant 50$ % cells being LacZ-positive after exposure to wild-type AdV–LacZ (multiplicity of infection $= 10^4$) that had been pre-incubated in heat-inactivated diluted serum (1 h, 37 °C) ($n = 3$ mice per cohort). All lipid formulations (dexamethasone–spermine/dioleoylphosphatidylethanolamine (DS/DOPE), DC-cholesterol (DC-Chol)/DOPE and dexamethasone) resulted in suppression of NAB production at day 28 to near-background levels seen with phosphate-buffered saline (PBS)-sham control (**a**). Suppression of circulating levels of NAB by DS/DOPE formulation was observed after homologous vector re-administration at day 14 (**b**). In the vector re-administration study, one mouse of the AdV–LacZ (day 0)/AdV–human α1-antitrypsin (*h*AAT) (day 14) cohort displayed unusually high NAB titer, but removal of this value ($n^* = 2$ bar) still indicated that formulation of AdV–LacZ with DS/DOPE resulted in strong suppression of NAB production (*$P = 0.05$).

To assay the systemic cellular response, we used the IFNγ enzy,e-linked immunosorbent spot (ELISPOT) assay to evaluate CD8 T-cell activation using the H_2-K^b immuno-dominant β-gal CD8 T-cell epitope ile-cys-pro-met-tyr-ala-arg-val (ICPMYARV[24]). At day 11 post-vector instillation, spleens were harvested. In contrast to the

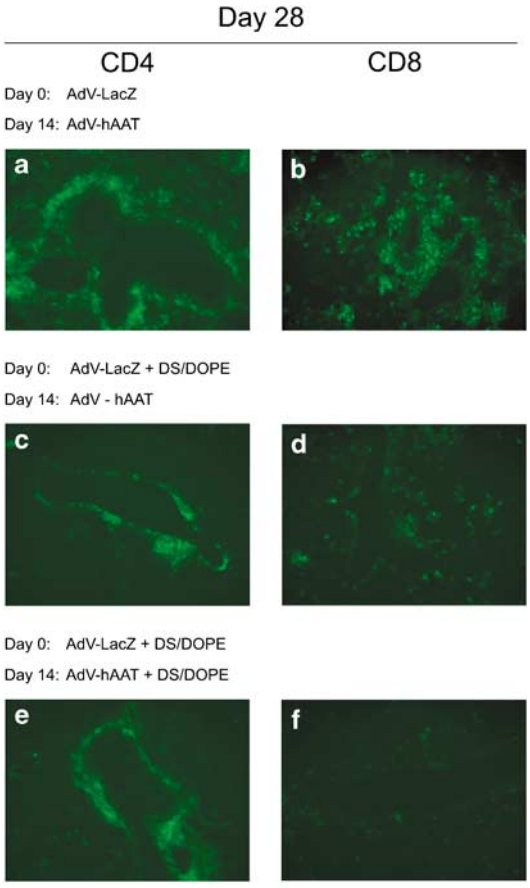

Figure 5 Localized immune responses following vector delivery to lung. Immunostaining of CD4 and CD8 T-cell infiltration in lung sections at day 28 following re-administration of AdV vectors (**a** and **b**) without dexamethasone–spermine/dioleoylphosphatidylethanolamine (DS/DOPE) formulation, (**c** and **d**) with DS/DOPE formulation only at the first instillation at day 0 and (**e** and **f**) with DS/DOPE formulation used for both instillations at days 0 and 14. CD4 T-cell staining (**a**, **c** and **e**) and CD8 staining (**b**, **d** and **f**) indicated that the use of the DS/DOPE formulation reduced CD4 and to a lesser extent CD8 T-cell infiltration.

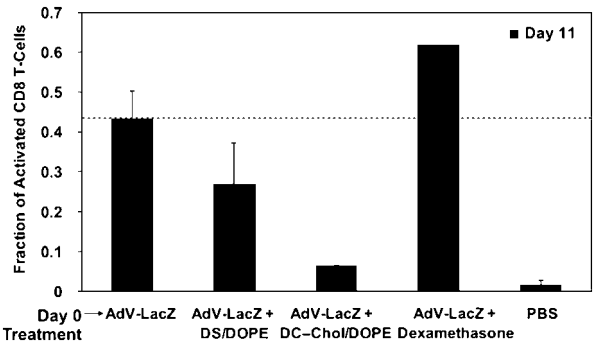

Figure 6 Activation of transgene-specific CD8 T cells following AdV vector delivery to lung. The fraction of activated splenocytes (number of activated CD8 T cells per well divided by total number of cells per well) at day 11 post-administration of vector formulation is shown. No significant difference between AdV–LacZ and AdV–LacZ formulated with dexamethasone–spermine/dioleoylphosphatidylethanolamine (DS/DOPE) was detected. DC-cholesterol (DC-Chol)/DOPE-formulated AdV vector resulted in less CD8 T-cell activation, while the use of the dexamethasone formulation caused a slight elevation of CD8 T-cell activation over control treatment group (phosphate-buffered saline (PBS)-sham).

results observed for localized T-cell infiltration (Figure 5), the number of IFNγ-secreting splenocytes at day 11 was reduced only slightly by the DS/DOPE formulation and not at all by the dexamethasone formulation. Interestingly, there was marked reduction of CD8 T-cell activation when the AdV vector was formulated with DC-Chol/DOPE (Figure 6). This finding suggests that localized T-cell activation in response to AdV vector is affected primarily by localized delivery of glucocorticoid formulation (DS and dexamethasone) and only secondarily by liposome formulation (DS but not DC-Chol). The systemic antigenic response to the transgene product β-gal was reduced mostly by the liposomal formulation (DC-Chol), while the serum-circulating NAB production was reduced by both the liposome and glucocorticoid formulations (DS, DC-Chol and dexamethasone).

Discussion

Immune responses to both viral and non-viral vectors are considered a significant impediment to successful lung-directed gene therapy. AdV vectors elicit cellular and humoral immune responses, resulting in loss of transgene-expressing cells and blocking of homologous vector re-administration, respectively. Lipoplexes have been recently shown to activate a significant inflammatory response; however, the components do not cause a significant immune response when delivered alone.[25] Another barrier to successful gene therapy is delivery of the gene specifically to the target cells. AdV vectors are efficient at transducing multiple cell types in lung, including conducting airway epithelial cells. However, as we have shown in a previous study,[22] AdV vector formulated with DS/DOPE promotes transduction of the conducting airways, which remain the therapeutic target for genetic lung diseases such as cystic fibrosis.

We hypothesized that reduction of cellular infiltration could have significant implications to the generation of immune responses and potentially allow homologous vector re-administration. We have shown that both AdV formulated with DS/DOPE and plasmid DS/DOPE lipoplexes induce low immune responses when delivered *in vivo*.[22,23] In this study, we examined the immune responses to AdV vector formulated with DS/DOPE in an attempt to ascertain how this formulation alters the properties of the AdV vector. Specifically, we evaluated which features of the reduction of cellular infiltration were due to the liposome formulation and which were due to the anti-inflammatory activity of DS.

One indication that the immune response to AdV vector formulated with DS/DOPE was substantially reduced was the successful AdV vector re-administration. In this study, we demonstrated significant levels of AlkP gene expression upon re-administration of the AdV–AlkP vector formulated with DS/DOPE. Very little transgene expression was apparent following re-administration of AdV vector delivered without formulation in the first or second instillation. Decreased levels of AlkP gene expression following re-administration were observed for DC-Chol/DOPE and dexamethasone formulations, suggesting that the combination of the

liposome formulation and glucocorticoid activity was necessary for successful re-administration.

Dexamethasone is not associated with the AdV vector particle via electrostatic interactions, in contrast to DS/DOPE or DC-Chol/DOPE. In these experiments, we attempted to determine which aspects of the reduced immune response to the AdV vector were due to the liposomal nature of DS/DOPE and which were due to the anti-inflammatory (glucocorticoid) nature of DS/DOPE. Liposomes without the neutral lipid are not as effective for transfection as those which include neutral lipid. However, the use of DS instead of dexamethasone would have made difficult the differentiation between the two properties of DS/DOPE (liposome versus glucocorticoid) that were contributing to a reduced immune response. Additionally, given the local nature of our *in vivo* delivery system (directly to the lung), the dexamethasone would have been mainly delivered to the apical side of cells lining the lung airways rather than systemically. Therefore, a comparison of AdV with dexamethasone and AdV with DS/DOPE facilitates the evaluation of the consequence of steroid delivered along with AdV.

We showed that the acute response to the AdV vector, at 6 h post-vector administration, was reduced for the AdV vector formulated with DS/DOPE. This result correlates well with previous work showing reduced cellular infiltration at days 1 and 7 post-administration of AdV vector formulated with DS/DOPE.[22] In the present study, the reduced immune response is evidenced as a sharp decrease in TNFα levels at 6 h post-administration of AdV vector formulated with DS/DOPE. TNFα is produced predominantly by macrophages and mast cells, and plays a pivotal role in the induction of further cytokine secretion. Our data suggest that AdV vector formulated with DS/DOPE decreases certain early mediators of the inflammatory response resulting in a significantly blunted adaptive immune response to the AdV vector. IL-11, a cytokine that promotes growth and differentiation of cells involved in the immune response, was decreased by more than sixfold following delivery of the AdV vector formulated with DS/DOPE compared to AdV vector delivered alone. Interestingly, the levels of only one chemokine (IP-10) were elevated 6 h post-delivery of AdV vector formulated with DS/DOPE. IP-10 has been shown to be involved in the recruitment of T cells.[26] However, we did not observe an increase in T-cell-mediated responses against the transgene expressed by the delivery of AdV formulated with DS/DOPE versus that expressed by AdV vector alone.

The titer of serum-circulating NAB is another important parameter that influences the success of gene therapy. In this study, we were able to show reduced NAB production when AdV vector was formulated with DS/DOPE. We speculate that NAB production was not completely eliminated due to the partial dissociation of the DS/DOPE–AdV vector complexes in the negatively charged environment of the lung. While the systemic T-cell response to the encoded transgene (β-gal) was not significantly reduced by formulation of AdV vector with DS/DOPE, the localized T-cell response was significantly reduced as evidenced by the significant reduction in CD4 and CD8 T-cell infiltration present in the lungs of mice treated with AdV vector formulated with DS/DOPE.

Various cationic lipids such as DC-Chol or 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) or *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA) when used in lipoplexes are known to be weak activators of complement.[27] The alternative complement pathway is operative in lung, but less is known about complement activation after intranasal delivery of lipoplexes. Also, plasmid lipoplexes with DOTAP has recently been shown to elevate systemic cytokines (IL-6, IL-12 and TNFα) after intravenous delivery when compared to AdV.[25] However, Sakurai *et al*.[25] did not address the delivery of the individual components of the lipoplex (DOTAP/Chol alone or pDNA alone). In comparison, we have previously shown reduced levels of inflammatory mediators as well as reduced levels of cellular infiltration when delivering DS/DOPE lipoplexes[23] and when delivering AdV vector formulated with DS/DOPE.[22]

In conclusion, we have demonstrated that formulation of AdV vector with a liposome reduces some key aspects of the immune response to the AdV vector, including NAB production and select cytokine production at specific time points. The unique pharmacological activity of DS/DOPE further contributed to the reduction of the immune response to the AdV vector delivered to the lung. This was especially apparent at the localized delivery site, where CD4 T-cell infiltration was significantly reduced only by formulation of the AdV vector with DS/DOPE or dexamethasone. While DS/DOPE did not completely eliminate the T- and B-cell immune responses to AdV vector delivered to the lung, the significant reduction in both T- and B-cell activation did allow for the successful re-administration of AdV vector to lung.

Materials and methods

Materials

Dexamethasone–spermine was prepared as described previously.[22] Briefly, dexamethasone-mesylate (Steraloids, Newport, RI, USA), Traut's reagent (Pierce, Rockford, IL, USA) and spermine (Sigma, St Louis, MO, USA) were reacted in a 1:1:5 molar ratio in a one-step reaction to form DS, which was purified using high-performance liquid chromatography (Hamilton PRP-1 column) and freeze-dried as DS trifluoroacetic acid salt (1133 Da). DOPE and DC-Chol were purchased from Avanti Polar Lipids (Alabaster, AL, USA). DS/DOPE and DC-Chol/DOPE liposomes were prepared as described previously.[22] Briefly, a solution of DOPE/ethanol was evaporated to form a lipid film. DS or DC-Chol in a solution (0.5–1.5 mM) of sterile PBS for *in vivo* experiments was added in a 1:1 molar ratio to the DOPE film. The lipids were then vortexed thoroughly and probe-sonicated (103 W for 15 min) at room temperature (RT) in a water bath sonicator (Fisher FS9H) to form liposomes. Dexamethasone (Sigma) was prepared by evaporation of a dexamethasone/ethanol solution and resuspension of the dexamethasone as described for DS and DC-Chol. All preparations were made fresh before each experiment or stored at 4 °C for less than 1 month.

Virus preparation and formulation

E1/E3-deleted replication deficient, recombinant AdV vectors ($2–4.5 \times 10^{12}$ particle/ml) expressing either β-gal or *h*AAT were generated as described previously.[28] The recombinant viruses were propagated in 293 cells and

purified by the CsCl gradient sedimentation method. For *in vivo* studies, DS/DOPE (51 μg total lipid), DC-Chol/DOPE (40 μg total lipid) or dexamethasone (29.4 μg) was added to an equal volume of AdV–LacZ or AdV–*h*AAT vector (10^{11} p) and incubated at RT for 15–30 min prior to use.

In vivo *delivery to mouse lungs*

C57Bl/6 mice (6–8 weeks of age) purchased from Charles River Laboratories were anesthetized by an intraperitoneal injection of a 3:2 mixture of xylazil (20 mg/ml)/ketamine (100 mg/ml). For dosing, mice were suspended from their dorsal incisors (hind quarters supported) and a dose of 10^{11} p of AdV–LacZ vector was delivered as a 50 μl bolus to the both nostrils using a gel-loading tip (Finnpippette). Serum was collected at intermediate time points via retro-orbital bleed. In some experiments, a second dose of AdV–*h*AAT was delivered. At necropsy, the lungs and serum were harvested for further analysis. One lobe of the lung was inflated with PBS/optimal cutting temperature (OCT) (1:1) mixture, covered in OCT and frozen in isopentane cooled with liquid nitrogen, and cryosectioned (10 μm). Sections were fixed and processed as described previously.[29] The other lung lobe was placed in 2 ml of Lysis Buffer (Roche, Indianapolis, IN, USA) and placed on ice. Lungs were homogenized as described previously,[22] and the supernatant was transferred to a sterile Eppendorf tube until analysis of transgene and cytokine expression. Serum was collected and stored at −20 °C.

Multi-analyte profiling

The serum samples at the 6-h time point were pooled by group in equal volumes of 20 μl each, and subjected to Luminex-based multiplexed antigen profiling (Rules Based Medicine; Charles River Laboratories). A serum antigen profile was obtained for each of three groups: AdV vector, AdV vector formulated with DS/DOPE and PBS. Levels of 59 serum proteins were determined: apolipoprotein A1, CD40, CD40L, C-reactive protein, epidermal growth factor, endothelin-1, eotaxin, Factor VII, fibroblast growth factor (FGF)-basic, FGF-9, fibrinogen, granulocyte chemotoctic protein-2 (GCP-2), GM-colony-stimulating factor (CSF), growth hormone, glutathione transferase-α, haptoglobin, IFNγ, immunoglobulin A, IL-1 α, IL-1 β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-10, IL-11, IL-12p70, IL-17, insulin, IP-10, KC/GROα, leptin, Leukemia inhibitory factor (LIF), lymphotactin, MCP-1, MCP-3, MCP-5, M-CSF, macrophage-derived chemokine (MDC), MIP-1 α, MIP-1 β, MIP-1γ, MIP-2, MIP-3β, matrix metalloproteinase-9, myoglobin, oncostatin M (OSM), regulated upon activation, normal T-cell expressed and secreted, stem cell factor (SCF), serum glutamic oxaloacetic transaminase (SGOT), tissue inhibitor of metalloproteinase (TIMP-1), tissue factor, TNF-α, thrombopoietin (TPO), VCAM-1, vascular endothelial growth factor and von Willebrand factor.

Transgene and cytokine detection

Mouse TNF-α and IL-6 ELISAs (Endogen; Pierce) were performed on sera samples according to the manufacturer's instructions. AdV vector-mediated β-gal gene expression in lung homogenate was analyzed by using the β-gal ELISA kit (Roche) according to the manufacturer's instructions. All values were normalized to total protein content in the sample quantified using the Bio-Rad Protein Assay. ELISAs were performed on sera samples to detect *h*AAT transgene expression. For AdV vector-mediated AlkP gene expression in lung homogenate, the AlkP chemiluminescent detection kit was used (Roche) according to the manufacturer's instructions.

IFNγ ELISPOT assay

The assay was performed using the enzyme-linked immunosorbent spot (ELISPOT) Mouse Set (BD PharMingen, San Diego, CA, USA) according to the manufacturer's instructions. Briefly, a 96-well ELISPOT plate was coated with 5.0 μg/ml of anti-mouse IFNγ capture antibody overnight at 4 °C. The next day, wells were washed and blocked with complete culture medium for a minimum of 2 h at RT. Splenocytes and lymph-derived cells (isolated from the draining superficial cervical lymph nodes) (at seeding densities of 2×10^5 or 5×10^5 cells per well) from vector-treated mice were added to wells and stimulated with 100 μl (2 μg/ml) of the β-gal H2-K^b CD8 T-cell epitope ile-cys-pro-met-tyr-ala-arg-val (ICPMYARV) (Mimotopes, Victoria, Australia). Cells were incubated at 37 °C and 5% CO_2 for 18 h. Control cells were incubated either in the absence of peptide (negative) or with the nonspecific stimulator, phorbol myristate acetate (0.05 μg/ml) plus ionomycin (1 μg/ml; positive).[30] Following the overnight incubation, the wells were vigorously washed with water followed by PBS containing 0.05% Tween-20 and subsequently incubated with 2.0 μg/ml biotinylated anti-mouse IFNγ detection antibody for 2 h at RT. Following three PBS/0.05% Tween-20 washes, the wells were incubated with 5 μg/ml of streptavidin–horseradish peroxidase antibody for 1 h at RT. Wells were washed with PBS/0.05% Tween-20, followed by PBS and developed by adding AEC substrate (BD PharMingen) to all wells. Color development was monitored and stopped 5–10 min later by washing wells with distilled water. After drying overnight at RT, plates were read and counted using an ELISPOT reader.

NAB detection

'8431' cells were seeded onto flat-bottom 96-well cell culture plates at 5×10^4 cells per well. At 16 h post-seeding, the cells were infected with 200 particle of wild-type AdV/cell for 3 h at 37 °C. Sera samples were heat-inactivated at 56 °C for 35 min, diluted 1:10 in 50 μl of serum-free Dulbecco's modified Eagle's medium and diluted twofold in a 96-well round-bottom tissue culture dish. A total of 50 μl of serum-free Dulbecco's modified Eagle's medium containing AdV–LacZ (multiplicity of infection $= 10^4$) was added to both the diluted sera and a control serum sample and incubated for 1 h at 37 °C. The media containing the wild-type AdV was aspirated and replaced with 100 μl of the AdV/serum mix and incubated for 1 h at 37 °C. FCS/Dulbecco's modified Eagle's medium (DMEM) 20% (100 μl) was added to each well and incubated for 48 h at 37 °C. Transduction was evaluated by counting LacZ-positive cells in each well under high-power magnification. The titer of NAB for each sample was reported as the highest dilution with which less than 50% of the cells were LacZ positive.

CD4+ and CD8+ immunohistochemistry on lung cryosections

The lung sections (10 μm) were air-dried and fixed in −20 °C cold acetone for 5 min. Once dried the sections were blocked with 1% goat serum/PBS for 15 min. The primary antibodies (rat anti-CD8; BD Pharmingen, 550281 and rat anti-CD4; BD Pharmingen, 550280) were then added at a 1:20 dilution in PBS/1% goat serum and incubated at RT for 1 h. Following three 5 min washes in PBS, the secondary antibody (fluorescein isothiocyanate anti-rat) was added at a 1:200 dilution in PBS/1% goat serum and incubated in the dark at RT for 30 min. Following three 5 min washes in PBS, the sections were mounted in Vectashield with 4,6-diamidino-2-phenylindole and viewed under a fluorescence microscope.

Statistical analysis

Values are reported as mean ± s.d. Number of mice per group is three, unless otherwise stated. All quantitative analyses of lung samples were performed in duplicate or triplicate. Statistical analysis was performed using JMPTM software. Analysis of variance tests were performed to determine difference between groups, $P \leqslant 0.05$ was considered significant. Within groups, the Student's *t*-test was used to determine significance.

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