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Anionic Liposomes Enhance and Prolong Adenovirus-Mediated Gene Expression in Airway Epithelia *in Vitro* and *in Vivo*

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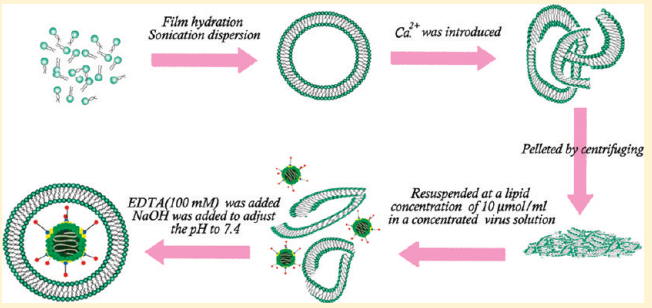
Supporting Information

ABSTRACT: Adenoviral vector mediated gene therapy has received extensive attention in airway disease treatment. However, the lack of the requisite coxsackie-adenovirus receptor (CAR) on the apical surface of airway epithelium and the host immune response to adenoviruses limit their *in vivo* application. In our study, we developed for the first time a novel formulation composed of anionic liposomes and adenoviruses (AL−Ad5) using a calcium-induced phase change method. The obtained formulation was employed to enhance the transduction efficiency of airway gene delivery. Our results indicated that primary cultured airway epithelial cells infected by AL−Ad5 displayed higher LacZ gene expression compared to naked adenovirus. Importantly, AL−Ad5 significantly improved and prolonged LacZ gene expression in murine airway tissues when delivered *in vivo* by intratracheal instillation. Additionally, it was found that anionic liposomes provided immunoprotection to the adenovirus from neutralizing antibody, thus slowing down the elimination of Ad5 particles meanwhile reducing the inflammatory reaction caused by the Ad5 vector. These results suggested that the combination of anionic liposomes with adenovirus may be a useful strategy to deliver therapeutic genes into the airway epithelia and is promising in clinical application.



KEYWORDS: adenovirus, anionic liposomes, gene therapy, airway epithelia, primary culture, antibody neutralization

INTRODUCTION

Lung disease causes morbidity, mortality, and economic burden. The most common forms of this disease include asthma, chronic obstructive pulmonary disease, pneumonia, interstitial and inhalation disorders, and pulmonary embolism.[1] Cystic fibrosis (CF) is a pulmonary disease that has received the most interest for gene therapy treatment.[2] This disease results from mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, and to date, around 1000 different mutations have been identified. The isolation of the *CFTR* gene in 1989 led to new therapeutic strategies for treating CF, which includes gene therapy and delivery of the *CFTR* gene targeted specifically to the airway epithelial cells. However, airway epithelial cells have been proven to be a difficult target for gene therapy. Several extracellular barriers, such as mucosa, lack of receptors, and immune surveillance, prevent successful delivery of gene therapy vectors. Furthermore, once these vectors are delivered to cells, additional hurdles need to be overcome, including DNA degradation, nuclear import and maintaining long-term transgene expression.[3]

Current findings have addressed barriers for the clinical applications of gene therapy, which assisted our study to develop new strategies targeting the airway epithelia. For example, plasmid-based vectors are susceptible to endosomal degradation once in the cells. Plasmid DNA is quickly degraded by Ca^{2+}-sensitive cytosolic nucleases, with a half-life of 50−90 min.[4,5] However, viral vectors are able to escape this process, which makes them ideally used for higher gene transfer efficiency. Additionally, epithelial tight junctions prevent uptake of viral vectors by the basolateral membrane of target cells, which are rich in viral receptors and have a higher rate of endocytosis. Disruption of tight junctions can be achieved by using Ca^{2+} chelator agents, such as EGTA or EDTA,[6,7] nonionic detergents and antibodies. It has been reported that complexing Ad vectors with polycations[8] or incorporating Ad in calcium phosphate precipitates[9] showed enhanced gene transfer efficiency to airway epithelia *in vitro* and *in vivo*. However, the inflammatory and toxic effects of cationic lipids *in vivo* are a concern. Scheule et al.[10] observed a dose-dependent pulmonary inflammation characterized by neutrophilic infiltrates and, to a lesser extent, macrophages and lymphocytes when the cationic lipid GL-67 was administered to mouse lungs *in vivo*. Moreover, upper-airway epithelial cells are negatively charged and possess a viscous mucus layer which often traps and neutralizes the positively charged surface of liposomal−DNA complexes. In Rosenecker's study,[11] topical gene administration of lipoplexes or polyplexes into the lung after intratracheal instillation or aerosolization caused interaction of

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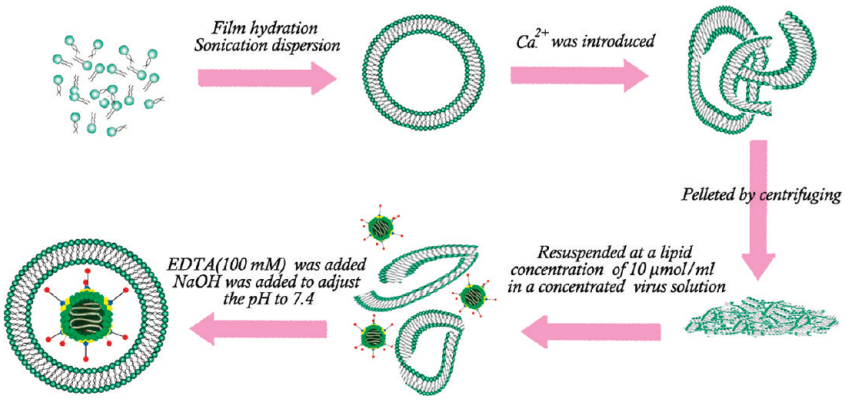

Figure 1. Schematic diagram for preparing the adenoviral anionic liposomes. Empty anionic liposomes were prepared using thin film hydration and sonication dispersion technique. Ca^{2+} was introduced by direct addition of 100 mM $CaC1_2$ to the empty anionic liposomes (final concentration 10 mM) and incubated at 37 °C for 1 h. The resulting precipitate was pelleted by centrifuging at 2500*g* for 10 min. The pellet was resuspended at a lipid concentration of 10 μmol/mL in a concentrated viral solution or TES buffer by vortexing for 10 min at room temperature. EDTA (100 mM) was added directly to this solution with a final concentration of 15 mM, and 10−20 μL of 100 mM NaOH was added to adjust the pH to 7.4. The solution was vortexed for 10 min at 37 °C, followed by incubation for 30 min.

the complexes with extracellular substances of the airway surface liquid. This interaction may lead to low transfection efficiencies *in vivo* after topical administration. Furthermore, pulmonary surfactant may inhibit cationic liposome-mediated gene delivery to respiratory epithelial cells.[12] Taken together, these limitations restrict the potential applications of cationic liposomal vectors in gene therapy.

Anionic liposomal DNA delivery vectors as an alternative to cationic liposomes have been reported in recent years.[13,14] Because anionic lipids are endogenetic components of eukaryotic cell membranes, they are less cytotoxic.[15] Patil et al.[16] have reported a novel anionic lipoplex DNA delivery system encoding a gene of interest with high transfection efficiency, serum stability and low toxicity. In our previous study, we also found that complexing anionic liposomes with adenovirus (AL−Ad5) could increase the efficiency of adenovirus-mediated gene transfer to CAR deficient cell lines.[17] We hypothesize that AL−Ad5 could also increase the gene expression mediated by adenovirus in murine airway epithelia, which are relatively devoid of CAR.[18] A primary culture model of differentiated murine tracheal epithelium was established by an air−liquid interface method, and gene transduction efficiency of both AL−Ad5 and the control Ad5/cationic liposome complexes (CL−Ad5) was investigated both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Adenovirus Vectors and Animals. An E1, E3-deleted type 5 adenovirus expressing the *Escherichia coli* LacZ gene was obtained from VGTC Gene Technology Company Ltd. Qbiogene (Carlsbad, CA). The vector was amplified in the 293 cell line using a modification of established methods[19] and purified from cell lysates by banding twice on CsCl gradients followed by dialysis. Concentration of the virus was determined by TaqMan qRT-PCR. All experiments were performed with freshly purified adenovirus.[20]

Specific pathogen-free C57BL/6N mice of both sexes, age 5−7 weeks, were used in this study. These mice were originally obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and bred in the Experimental Animal Center of Sichuan University (Chengdu, China). Mice were housed in a controlled environment and fed with standard rodent chow and water. The study was approved by the Animal Ethics Committee of Sichuan University West China Medical School, and all procedures with animals were conducted according to the guidelines of the local Animal Use and Care Committees of Chengdu and executed according to the National Animal Welfare Law of China.

Preparation of Complexes of Adenoviral Anionic Liposomes. The complexes of anionic liposomes/adenovirus (AL−Ad5) were prepared according to our previous report using the calcium-induced phase change method.[17] The complexes of cationic liposomes/adenovirus (CL−Ad5) were also prepared accordingly by mixing and incubating liposomes and Ad5 together. The schematic diagram depicted in Figure 1 outlines the prepared procedure for AL−Ad5.

Isolation and Culture of Tracheal Epithelial Cells. The primary culture of tracheal epithelia was carried out according to the air−liquid interface method as the report described.[21,22] Briefly, the trachea was isolated from the C57BL/6N mice and washed 3 times in PBS at room temperature (RT) for 5 min and then transferred into collection medium (1:1 mix of DMEM:Nutrient Mixture Ham's F-12 medium, 1% penicillin−streptomycin). Batches were incubated in dissociation medium (44 mM $NaHCO_3$; 54 mM KCl; 110 mM NaCl; 0.9 mM NaH_2PO_4; 0.25 μM $Fe(NO_3)_{3-9}$, 1 mM sodium pyruvate, 42 μM phenol red, pH 7.5, containing 1% penicillin−streptomycin, 1.4 mg/mL Pronase, and 0.1 mg/mL DNase) at 37 °C for 60 min, and digestion was stopped with fetal calf serum (FCS). The resultant cell suspensions were pooled and centrifuged at 1,000 rpm (120*g*) for 5 min at room temperature. The tracheal epithelial cells were collected, washed, and seeded at 4×10^5 cells in 200 μL of culture medium inside the Transwell insert, with 600 μL of blank medium outside (Costar Transwell clear, tissue culture-treated polyester membrane 24-well plate inserts, 0.4 μm pore; Corning Costar, High Wycombe, U.K.). The Transwell inserts were precoated with type VI acid-soluble human placental collagen (Sigma-Aldrich, Poole, U.K.). On day 4, the medium inside of the insert was removed along with any nonadherent cells and debris, and the medium outside (bathing the basolateral surface) was replaced with 600 μL of differentiation medium (collection medium with 2% Ultroser-G (USG) serum substitute, Pall 15950-017, Pall France/Division Pall BioPharmaceuticals) and changed twice weekly.

Electrophysiological Studies. To monitor the transepithelial resistance (Rte) of cultured murine tracheal epithelium, we used a Millicell-ERS (Electrical Resistance System, Millipore) after the addition and equilibration of 200 μL of USG medium prewarmed

to 37 °C to the apical surface. Rte was measured on days 4, 8, 14, and 21 after seeding.

Scanning Electron Microscopy. SEM was performed on primary cultures of murine tracheal epithelium and tracheal specimens from 5 week old mice ($n = 3$). For SEM, the specimens were washed with PBS and then fixed first in 2.5% glutaraldehyde in PBS and second in 1% osmium tetroxide in PBS. Subsequently, the specimens were dehydrated through an alcohol series, treated with hexamethyldisilazane, and allowed to air-dry overnight. Primary culture specimens (cut from the plastic insert) and tracheal specimens were mounted on stubs with carbon adhesive disks and silver DAG, sputter coated, and viewed in a JSM-5900LV (JEOL, Japan) scanning electron microscope.

To quantitatively assess the different cell populations present in the primary cultures of murine tracheal epithelium, morphometric analyses were performed on SEM images of primary cultures on days 4, 8, 21, and 28 after seeding and on tracheal specimens from 5 week old mice. We counted the number of ciliated and nonciliated cells in ≥4 fields of 100 cells and ≥2 specimens for each time point.

Fluorescence Immunohistochemistry. Primary cultures of murine tracheal epithelium were characterized with mouse monoclonal anti-human pan cytokeratin (Sigma-Aldrich, C2562) which recognizes human cytokeratins 1, 4, 5, 6, 8, 10, 13, 18, and 19. Primary culture specimens were washed in PBS, fixed in 1:1 acetone−methanol for 5 min, washed in PBS, and cut from the culture insert. The blocking solution, PBS with 2% normal goat serum, 0.2% Tween, 20 mg/mL of bovine serum albumin, and 7% glycerol, was prepared. The specimens ($n = 3$) were incubated with blocking solution for 20 min before incubation for 1 h at room temperature with the primary antibody (1:100) diluted in blocking solution. Negative controls were performed by omitting the primary antibodies. After a wash with PBS, a 30 min incubation was performed with the secondary antibody (FITC-labeled goat anti-mouse IgG, Santa Cruz Biotechnology, Inc.) diluted 1:100 in blocking solution. After being washed in PBS, the specimens were stained with 4,6-diamidino-2-phenylindole nuclear stain (DAPI, ROCHE, USA) to detect the positions of nuclei and mounted on glass slides with antifade mounting medium (Sigma). Specimens were viewed with a Leica TCS SP5 AOBS confocal microscopy system (Leica, Germany).

Infection of Polarized Primary Cultures of Murine Tracheal Epithelium Cells by Naked Ad5, AL−Ad5 and CL−Ad5. Fourteen days after seeding, the polarized primary cultures of murine tracheal epithelium cells were rinsed twice with PBS and then infected by adding 50 μL of the formulation of naked Ad5 (MOI 40), AL−Ad5 and CL−Ad5 (total lipids 0.2 mg/mL, MOI 40) to the apical surface and the basolateral surface of the cultures, respectively. After 4 h, the suspension was removed, the cells were rinsed twice with PBS, and 0.2 mL of the culture medium was added to the insert and 0.6 mL to the outside of the insert. One day after infection, the transduction efficiency was assayed by quantifying the LacZ gene expression, in which the β-galactosidase activities were determined using the β-galactosidase enzyme assay system (Applied Biosystems) and the total protein content of the lysates was measured by a BCA assay (Pierce, USA) using a standard of bovine serum albumin (BSA). Infectivity was quantified as picograms of β-galactosidase (based on a standard curve for β-galactosidase activity) per microgram of total cell protein (pg/μg protein). The data represented the mean ± SD of three wells and was representative of three independent experiments.

***In Vivo* Animal Study.** C57BL/6N mice (6−8 weeks old, $n = 5$) were anesthetized with 100 mg/kg body weight ketamine hydrochloride (Sigma Aldrich) and 10 mg/kg body weight xylazine (Sigma Aldrich) by means of intraperitoneal injection,[23] and the trachea was exposed through a midline incision. Prepared formulations were administered intratracheally at a dose of 0.5×10^{10} particle/mice in 50 μL of each respective formulation. The incision was then closed, and the mice were returned to their cages to recover. Mice were sacrificed by cervical dislocation at 1, 2, 4, or 6 weeks following viral instillation. Blood was collected via a retro-orbital venipuncture utilizing a heparinized (1%) capillary tube.

Neutralizing Antibody Titers. To determine the neutralizing capacity of antisera, the ability of antisera to prevent the capacity of recombinant adenoviral vectors to infect and transduce 293T cells (80% subconfluent) was assayed according to the following procedure.[24] Antisera were diluted from 1/20 to 1/2560 by a 1/2 serial dilution with DMEM medium, and the stock recombinant adenoviral vector expressing the β-galactosidase (Ad5−LacZ) was diluted with blank DMEM medium. 50 μL of the serially diluted antiserum samples were mixed at a 1:1 ratio with the diluted adenovirus (MOI 20) and incubated for 1 h at 37 °C. The mixture was then placed on 293T cells in a 96-well plate and incubated for 4 h at 37 °C to permit transduction. Afterward, the supernatant was removed and replaced with 200 μL of fresh DMEM with 10% newborn calf serum and further incubated at 37 °C in 5% CO_2. After incubation for 24 h, the LacZ gene expression was measured by X-Gal staining. Briefly, cells were fixed with 1% formaldehyde−0.2% glutaraldehyde in 100 μL of PBS for 10 min at room temperature. After the cells were washed twice with 200 μL of PBS, they were incubated at 37 °C in 50 μL of 2.5 mM X-Gal (5-bromo-4-chloro-3-indolyl-β-galactosidase, Invitrogen, Grand Island, NY) reaction mixture containing 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, and 2 mM magnesium chloride in PBS. After 4 h of incubation, the positive blue cells were observed under inversion microscopy and the optical density was measured on an enzyme-linked immunosorbent assay plate reader (model 550, Biorad, USA) at 490 nm. The result was expressed as the relative transduction efficiency at a dilution of 1/80.

Histological Examination. After euthanasia by overinhalation of ether, the trachea and lungs were excised ($n = 5$). For routine histology, 5 μm sections of formalin-fixed, paraffin-embedded tissue were prepared and stained with hematoxylin and eosin (H&E). A histology scoring system was used as previously described.[25] In short, the following parameters were scored on a scale of 0 to 4: interstitial inflammation, endothelialitis, bronchitis, edema, hemorrhage, and necrosis. The severity of injury was judged according to the following criteria: no injury = 0; injury to 25% of the field = 1; injury to 50% of the field = 2; injury to 75% of the field = 3 and diffuse injury = 4. The histology score was expressed as the sum of the score for all parameters. All microscopic sections were analyzed in a blind fashion.

Determination of β-Galactosidase Gene Expression *in Vivo*. Mice were killed by asphyxiation with CO_2, and samples of trachea or lung were excised. For histochemical analysis, tissue biopsies were embedded in optimal cutting temperature compound and snap-frozen in liquid nitrogen precooled methylbutane. Cryostat sections (8 μm) were fixed in 4% PFA in PBS (pH 7.5) for 20 min on ice and incubated with X-Gal for overnight at 37 °C. After staining, the tissue biopsies were rinsed with PBS, dehydrated in a graded series of alcohol, and stained with eosin. The β-galactosidase stained cells were identified by light microscopy (Olympus AX-70).

To quantify β-galactosidase expression, the tissues were homogenized in lysis buffer and then immediately centrifuged at 3000*g* (Beckman Coulter, USA) for 10 min. The supernatant was removed and stored at −80 °C prior to analysis of transgene expression. The β-Gal levels were measured using a sensitive (detection limit 40 pg/mL) enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (Boehringer Mannheim, Mannheim, Germany). Protein concentrations in the homogenates were measured with a bicinchoninic acid (BCA) protein assay solution (Pierce, Rockford, IL). Results are expressed as picograms of β-galactosidase per milligram of total protein.

Quantitative Analysis of Adenovirus in Trachea and Lung Tissue. To quantify the genome copy number of adenovirus by TaqMan qRT-PCR, the trachea and lung tissue samples were homogenized in lysis buffer and centrifugated at 10000*g* for 15 min. Viral DNA was isolated from the tissue extracts using the High Pure Viral Nucleic Acid Kit (Roche, Basel, Switzerland) according to the manufacturer's recommendations. The forward primer (CAAAGACTGGTTCCTGGTACAAATG), reverse primer (GTGTAGGATGCCCACCTGTTG) and the probe (5′-FAM AGTATCATCCACCACCTGACGGCTCBHQ1-3′) were designed and synthesized according to a conserved region of the species C adenovirus hexon gene (nucleotides 21127 to 21304 of Ad5; GenBank accession number NC_001406). The TaqMan qRT-PCR amplification was carried out in 25 μL reaction mixtures consisting of 10 μL iQ supermix (Bio-Rad), 250 nM each primer, 250 nM TaqMan probe and 1 μL of DNA template. Thermocycling profiles for it consisted of 1 cycle of 95 °C for 3 min followed by 45 cycles of 95 °C for 15 s, 54.3 °C for 20 s and 72 °C for 10 s in a Bio-Rad iQ5. This amplification yields a 177-bp product. Quantification of adenovirus copy number was performed using a standard curve consisting of dilutions of adenovirus DNA from 1,000,000 to 1 copy.

Statistical Analysis. Data were expressed as the means ± standard deviation of at least three independent experiments. Significant differences between sample means were calculated using Stastica, version 6.0 (StatSoft, USA) by one-way ANOVA (analysis of variance) followed by post hoc testing with Dunnett's method. Data were considered to be statistically significant if $P < 0.05$ (*) and very significant if $P < 0.01$ (**).

RESULTS

Characterization of the Cultured Primary Mouse Tracheal Epithelial Cells. Primary murine tracheal epithelial cells were cultured by an air−liquid interface method described in the experimental methods. The transepithelial resistance (Rte) was used as a parameter for assessing the confluency of primary culture murine tracheal epithelial cells. We used a Millicell-ERS (Electrical Resistance System, Millipore) to monitor these cells after the addition of 200 μL of USG medium prewarmed to 37 °C to the apical surface for equilibration. Measurements were taken according to the manufacturer's protocol on days 4, 8, 14, and 21 after seeding. The result indicated that cells remained dry from day 4, with a transepithelial resistance of 2000−3000 $\Omega \cdot cm^2$, decreasing and stabilizing at about 1000 $\Omega \cdot cm^2$ by day 14 (Table S1 in the Supporting Information). Cilia were visible from approximately day 8. These values of Rte were consistent with other reports.[21,22]

Scanning electron microscopy (SEM) was performed on primary culture murine tracheal epithelial cells on days 4, 8, 14, 21, and 28 ($n = 3$) and tracheal specimens from 5 week old animals ($n = 3$) of the same strain used to isolate epithelial cells for primary culture (Figure 2). On day 4, a flattened epithelium was observed (Figure 2a), with no features of differentiation. This was characteristic of early stage primary culture models in other species, such as cultured rat tracheal epithelial cells. However, SEM analyses on days 8 and 21 revealed 13 ± 2% ($n = 3$ observations) and 21 ± 3% ($n = 3$ observations) ciliated cells, respectively (Figures 2b, 2d). By day 28, the percentage of ciliated cells was 30 ± 9% ($n = 2$ observations, Figure 2e). This value agrees with the percentage of ciliated cells that we observed in murine tracheal specimens by SEM (32 ± 5%; $n = 2$ observations; Figure 2f).

To characterize the cell types that constituted the primary culture murine tracheal epithelial cells, we performed immunohistochemical analyses on day 8 using a mouse monoclonal anti-human pan cytokeratin primary antibody (anti-cytokeratins 1, 4, 5, 6, 8, 10, 13, 18, and 19) which was used to characterize the epithelial nature of the primary culture murine tracheal epithelial cells. Epithelial cells were characterized with antibodies raised against different cytokeratins, a family of intermediate filaments involved in the cytoskeleton of epithelial cells. The expression profile of these cytokeratins is variable and dependent on epithelial cell subtype and the stage of differentiation.[26] Figure 3 shows that the anti-human pan cytokeratin antibody revealed the positive tracheal epithelia.

Gene Expression in Primary Cultured Cells. Cells were treated from the apical and basolateral surfaces, respectively. It is worth mentioning that, when infected from basolateral sides, the epithelium was turned upside down and 50 μL of formulation solution was carefully applied to the bottom of the Costar filter. The *in vitro* transduction was done over two independent sets of experiments. Their results are similar, and one set of results is described in Figure 4.

From the X-Gal staining (Figure 4A) and quantitive detection of β-galactosidase (Figure 4B), we observed higher LacZ gene expression when the cells were infected on the apical surface by AL−Ad5 and CL−Ad5 compared to cells infected with naked adenovirus. Figure 4B indicated that the average amounts of β-galactosidase transduced by AL−Ad5 or CL−Ad5 from the apical side was 5.15×10^5 and 4.90×10^5 pg of β-Gal/μg of protein, respectively. Their expression levels were about 6-fold higher than that of β-galactosidase transduced by naked Ad5, which is 8.40×10^4 pg of β-Gal/μg of protein. When the cells were infected from the basolateral surface, LacZ gene expression level transduced by AL−Ad5 or CL−Ad5 kept almost unchanged ($p > 0.05$), while the transduction mediated by naked Ad5 was significantly higher compared with the treatment from the apical side. This result suggested that adenovirus had the ability to infect polarized airway epithelia via the basolateral side, which may be due to the presence of CAR located at the basolateral membrane.[18] In addition, EDTA could enhance the gene expression of naked Ad5; however, the higher transduction efficiency of AL−Ad5 was due not only to the residual EDTA but also to a contribution by the formation of Ad5−liposome complexes (Figure S1 in the Supporting Information).

***In Vivo* Transduction Efficiency of Formulated Adenovirus in the Lung and Trachea of C57BL/6N Mice.** To determine whether *in vitro* transduction efficiency in primary cultured cells is an accurate predictor of formulation performance *in vivo*, 0.5×10^{10} particles of adenovirus in each formulation were delivered by intratracheal injection to C57BL/6N mice ($n = 5$). Lung and tracheal cells were tracked by X-Gal staining one week after

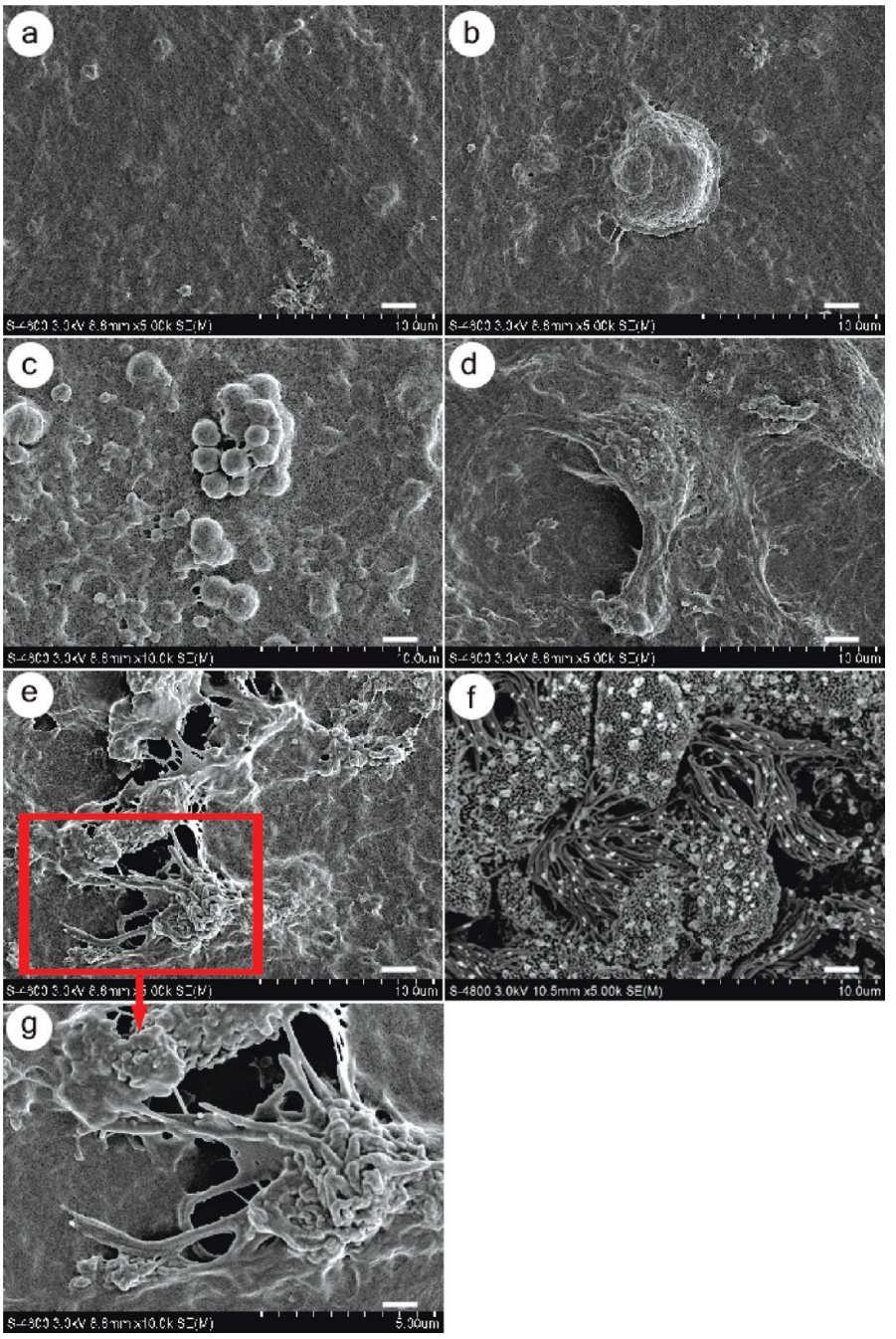

Figure 2. Scanning electron micrographs of primary cultures of murine tracheal epithelium on days 4 (a), 8 (b), 14 (c), 21 (d), and 28 (e) and a 5 week old mouse trachea (f). The specimens were fixed in 2.5% glutaraldehyde in PBS and 1% osmium tetroxide in PBS. Subsequently, the specimens were dehydrated through an alcohol series, treated with hexamethyldisilazane, air-dried overnight, mounted on stubs with carbon adhesive disks and silver DAG, sputter coated, and viewed in a JSM-5900LV (JEOL, Japan) scanning electron microscope ($n = 3$). The red box marked in (g) is enlarged in (e). On day 4, a flattened epithelium was observed (a), with no ciliated cells. Typical features of differentiated cells were seen in panels b, c, d, e and g. The bar of panels a−f was 2 μm, and the bar of panel g was 1 μm.

instillation, and β-galactosidase expression was measured at 1, 2, 4, and 6 week time points. The X-Gal staining (Figure S2 in the Supporting Information) showed that the lungs had positively stained cells localized primarily in large airways with minimal inflammation. In the lungs and trachea of animals treated with the CL−Ad5 formulation, few stained cells were seen. Figures 5A and Figure 5B demonstrated that the amount of β-galactosidase in the trachea and lungs of mice treated with naked adenovirus were 1189 ± 33.5 and 315 ± 13.5 pg of β-Gal/mg of protein, respectively, at the one week time point. However, animals treated with the AL−Ad5 formulation had significantly ($P < 0.05$) increased β-galactosidase expression reported at 1499 ± 37.8 and 355 ± 13.2 pg of β-Gal/mg in the trachea and lungs, respectively. On the other hand, compared to the naked adenovirus and CL−Ad5, AL−Ad5 formulation could significantly prolong transgene expression. At the two week time point, the

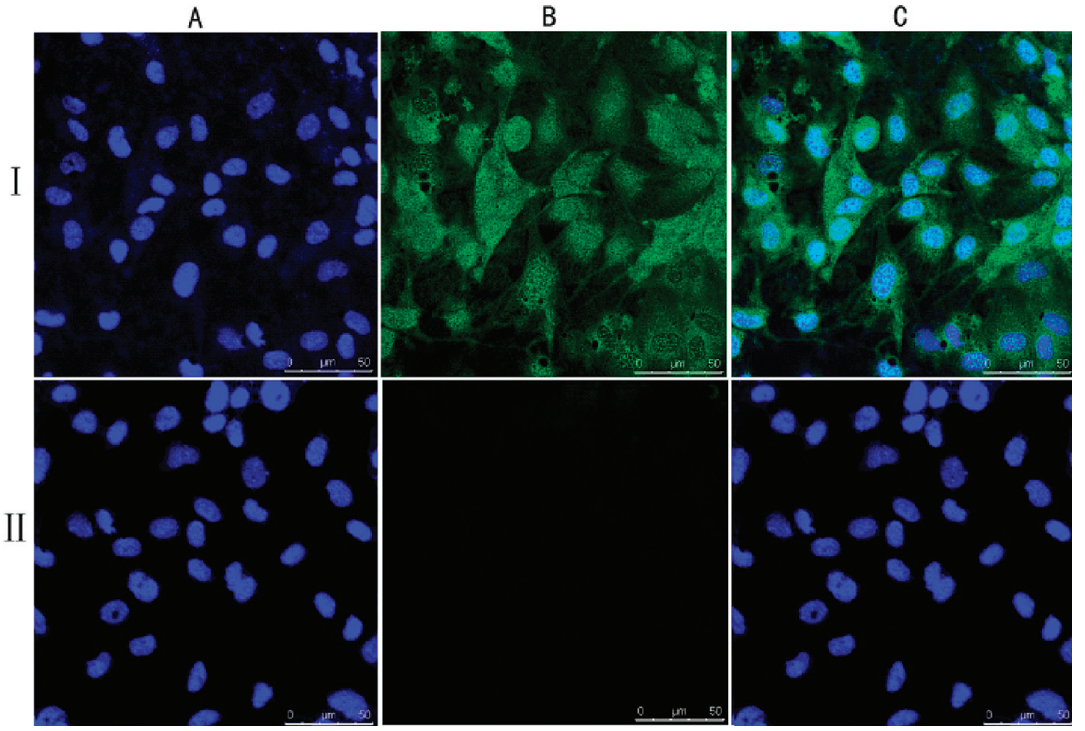

Figure 3. Immunohistochemical characterization of primary culture murine tracheal epithelial cells (n = 3): I positive, II negative control (no primary antibody); (A) blue channel, (B) green channel, (C) merged channels. Positive FITC signal is represented in green, with DAPI nuclear stain in blue.

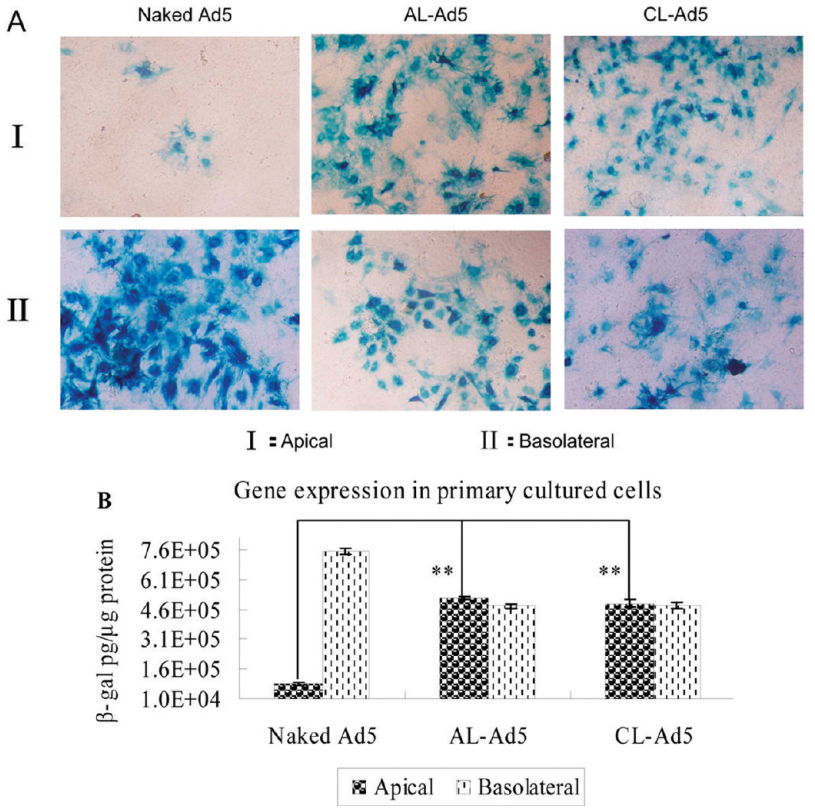

Figure 4. Transduction levels of polarized primary cultured tracheal epithelial cells. Primary cultures were transduced with naked Ad5, AL−Ad5 and CL−Ad5 (total lipids 0.2 mg/mL) containing Ad−LacZ applied to the apical or basolateral surface following by X-Gal staining (A) and quantitative detection of β-galactosidase gene expression (B). All transductions used an MOI of 40. Results are presented as mean ± standard deviation (SD) (n = 5), $^{*}P < 0.05$, $^{**}P < 0.01$, ANOVA.

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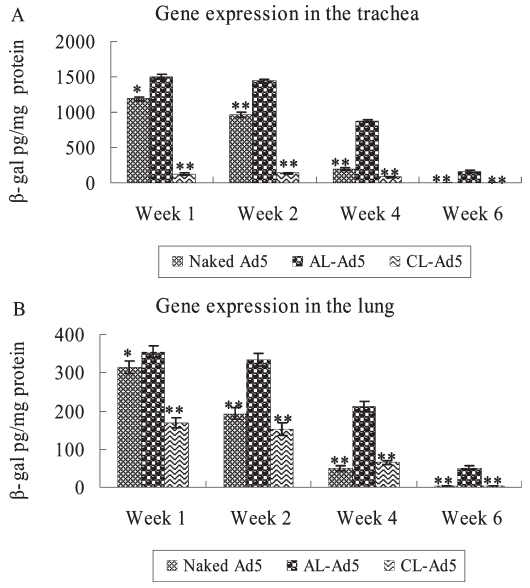

Figure 5. LacZ gene expression in trachea and lung of C57BL/6N treated with three different formulations (0.5×10^{10} particles/mice). (A) Quantitive detection of β-galactosidase expression by ELISA in the trachea and (B) in the lung at the different test time point after intratracheal instillation. Results are presented as mean ± standard deviation (SD) ($n = 5$), $^{*}P < 0.05$, $^{**}P < 0.01$, ANOVA.

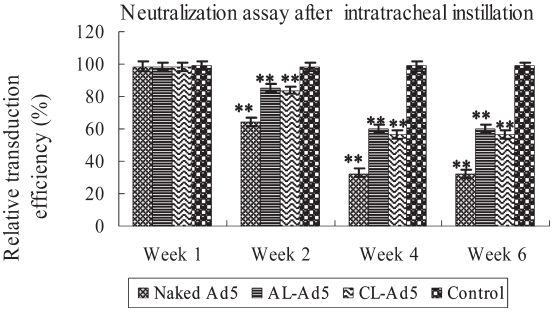

Figure 6. Antiadenoviral antibody titration of serum after intratracheal instillation of each formulation. Antisera were collected and diluted from 1/20 to 1/2560 by a 1/2 serial dilution and incubated with adenovirus for 1 h at 37 °C. The mixture was then placed on 293T cells in a 96-well plate and incubated for 4 h at 37 °C. Afterward, the mixture was removed and replaced with 200 μL of fresh DMEM with 10% newborn calf serum, and incubation was continued for 24 h at 37 °C in 5% CO_2. The results were expressed as the relative transduction efficiency at a dilution of 1/80 and presented as mean ± standard deviation (SD) ($n = 5$), $^{*}P < 0.05$, $^{**}P < 0.01$, ANOVA.

gene expression transduced by the naked adenovirus decreased by 19%, but those transduced by AL−Ad5 formulations remain unchanged (<5%). At the 4 week time point, LacZ expression in the trachea of mice treated with naked adenovirus significantly decreased by 83.9%, but only decreased by 41.9% using the AL−Ad5 formulations. Moreover, β-galactosidase expression remained at a relatively high level for 6 weeks after intratracheal instillation.

Ad5 Neutralization Measured by Transgene Inhibition Method. In Figure 6, we show the results of a neutralization assay upon the serum collected at the indicated time point from C57BL/6N mice treated with the different formulations by intratracheal instillation ($n = 5$). The relative transduction efficiency (RTE) was taken to describe the titer of neutralizing

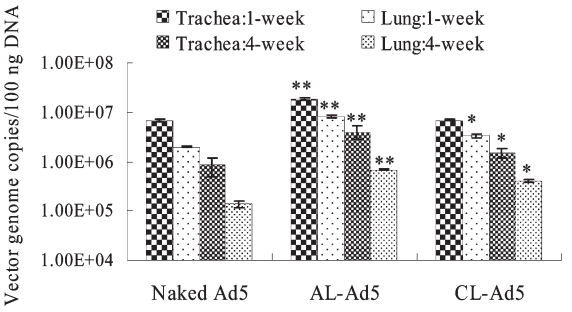

Figure 7. Quantification of vector genomes in tracheae and lungs harvested at the 1 and 4 week time points from mice treated with naked Ad5, AL−Ad5 and CL−Ad5, respectively. The samples were subjected to vector genome quantification by TaqMan qRT-PCR. Results are presented as mean ± standard deviation (SD) ($n = 5$), $^{*}P < 0.05$, $^{**}P < 0.01$, ANOVA.

antibody (NAB), which was reverse consensus with RTE, higher RTE with less NAB. The antisera collected at one week post-injection from all of the tested groups displayed little inhibition of gene expression, which may be due to low titers of antiadenoviral antibodies in the antisera. At the two week time point, on comparing the two groups of AL−Ad5 and naked adenovirus, we found that the AL−Ad5 group had 32% greater average gene expression (85.0 ± 3.1% versus 64.5 ± 2.5%) than the naked adenovirus. No difference was found between AL−Ad5 and CL−Ad5 groups ($p > 0.05$). The cells treated by antisera collected at the four week time point from the naked adenovirus group showed a decreased gene expression with a percent of 32.6 ± 2.3%, while the relative transduction efficiency in the AL−Ad5 and CL−Ad5 group was 60.3 ± 2.5% and 56.2 ± 2.1%, respectively. From four weeks to six weeks, the neutralizing antibody kept stable.

Vector Genome Detection in the Trachea and Lung Tissue after Instillation. At one-week and four-week time points after instillation, the tracheae and lungs were harvested. To demonstrate the presence of adenovirus in those tissues, TaqMan qRT-PCR was used to quantify viral copies in the samples.

The result showed that adenovirus DNA was detected in the tracheae and lungs of mice treated with AL−Ad5, CL−Ad5 and naked Ad5 but not in those of mice treated with PBS (data not shown). As shown in Figure 7, at the one week time point in the trachea the genome copy number from the AL−Ad5 group was significantly higher than that from the naked Ad5 and the CL−Ad5 group; there is no significant difference between the naked Ad5 and the CL−Ad5 group in the trachea. In the lung tissue, the genome copy number of the AL−Ad5 group was also the highest one among the tested groups. These results indicated that the initial vector distribution in trachea and lung mediated by AL−Ad5 was much higher than that mediated by naked Ad5 or CL−Ad5. At the four week time point, compared with the genome copy in the naked Ad5 group, higher genome copy numbers were observed in the AL−Ad5 and CL−Ad5 groups, especially in the AL−Ad5 group, indicating both anionic and cationic liposomes can partially protect the adenovectors from elimination by immune cells.

Histology Findings. In each group the n number of mice was 5, and for each mouse at least ten sections (5 μm) were analyzed by a pathologist who was blinded to the experimental protocol. Figure 8 shows the histological findings of trachea and lung tissues from the mice treated with PBS, naked Ad5, AL−Ad5 and CL−Ad5 respectively.

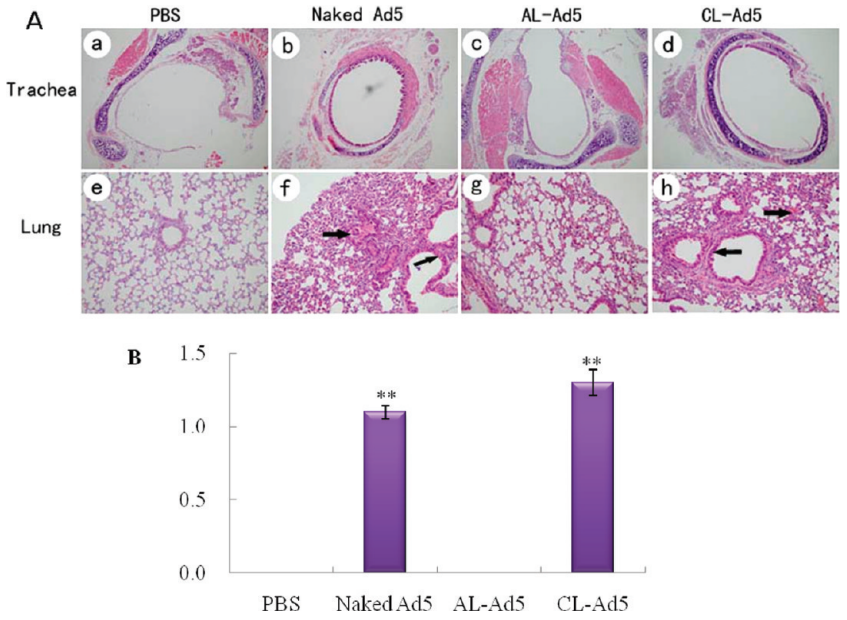

Figure 8. Histology findings of the tracheae and lungs taken from C57BL/6 mice sacrificed at 4 week after being treated with various formulations of PBS, naked Ad5, AL−Ad5 and CL−Ad5 ($n = 5$). (A) Representative histological sections of the trachea and lung. Original magnification: panels a−d, ×100; panels e−h, ×200. Black arrows in panel indicate the areas of damage. (B) Histological score for the semiquantitative assessment of lung inflammation. Results are presented as mean ± standard deviation (SD), $^{**}P < 0.01$, ANOVA.

No histological changes were observed in the trachea tissues from the treated mice (Figure 8b−d) and the controlled mice (Figure 8a). Thickened alveolar walls, inflammatory cell infiltration and simple columnar epithelial cells in bronchial cavities were observed in the lung tissues of naked Ad5 treated mice (Figure 8f). Similarly, inflammatory cell infiltration and thickened alveolar walls were also observed in CL−Ad5 treated mice (Figure 8h). However, there were no obvious histological changes in control mice and AL−Ad5 treated mice (Figure 8e and Figure 8g). In addition, the histological score for the semiquantitative assessment of lung inflammation (Figure 8B) also indicated that the lung inflammation scores in the naked Ad5 group and the CL−Ad5 group were significantly increased, compared to the PBS group or the AL−Ad5 ($P < 0.01$). There is no obvious difference about the inflammation between different tissue sections of the same mouse.

DISCUSSION

Recombinant adenovirus mediated gene therapy is an exciting novel strategy in cystic fibrosis treatment. However, poor infection efficiency on airway epithelium is one of the major challenges for its practical and extensive application. Moreover, the clinical application of recombinant adenovirus is limited by the short time gene expression and the systemic side effects experienced by patients. Therefore, continuous localized gene expression is a desirable goal for the gene therapy of cystic fibrosis. In this study our objective was to develop a novel formulation which would not only reduce the host immune response to adenovirus but also enhance and prolong the adenoviral-mediated gene expression in the airway epithelium *in vitro* and *in vivo*.

Some airway epithelia cell lines such as CRL-9483 or 16HBE14o− cells were used to evaluate the efficiency of gene transfer,[27,28] but the air−liquid interface cultures of airway epithelial cells are more appropriate *in vitro* models to assess transduction efficiency in airway epithelia, which accurately represents the behavior of polarized, differentiated airway epithelial cells. Therefore, we set up a primary culture model of differentiated mouse tracheal epithelium on semipermeable support membranes. The characteristics of the primary culture model were investigated by scanning electron microscopy, immunohistochemistry, and electrophysiology, which confirmed the successful establishment of this model. The transduction results suggested the LacZ gene expression mediated by naked Ad5 was as poor as predicted for lack of CAR on the apical surface of airway epithelium. But both AL−Ad5 and CL−Ad5 increased the LacZ gene expression when infected from the apical side. For CL−Ad5, the positive charged surface enhanced vector binding to the negatively charged cell membranes, which led to higher transfection efficiency. For AL−Ad5, the result was consistent with our previous report that anionic liposomes increased the efficiency of adenovirus-mediated gene transfer to CAR deficient cells.[17]

It was reported that a cellular immune response could be induced after adenovirus delivery. One of the immune responses to adenoviral vectors is the induction of a humoral antibody response that neutralizes adenoviruses before they reach their target cells.[29,30] Even if such antibodies are absent initially, they may develop rapidly in humans following exposure to the vectors and preclude or severely handicap attempts at repeated administration of the vector as transgene expression decreased. To test whether AL−Ad5 could reduce the induction of the humoral antibody response, the antiadenoviral antibodies titer (neutralizing antibody) was assessed by the transgene inhibition method. We found the neutralizing antibody titer increased gradually over time, peaked at week 4, and was stable up to week 6. Importantly, we found the neutralizing antibody titer was much higher in the serum of mice treated with the Ad5 alone than that in the groups receiving liposome−vector complexes, AL−Ad5 or CL−Ad5. It was in agreement with the findings by Natsume et al.[31] and Steel et al.,[32] whose study developed complexes of adenovirus and cationic

liposmes. Our result suggested combination of anionic liposomes with adenovirus could also significantly decrease the generation of neutralizing antibody.

Clinical trials of gene therapy for cystic fibrosis suggest that current levels of gene transfer efficiency are probably too low to result in clinical benefit, largely as a result of the barriers faced by gene transfer vectors within the airways. As mentioned above, these barriers included mucosa, lack of receptors, and immune surveillance etc. In the studies described here, AL−Ad5 could significantly improve the gene transfer level mediated by Ad5 in trachea and lung tissues from week 1 until week 6 after single dose of intratracheal instillation (Figure 5). The enhanced gene expression levels were attributed to 2 reasons. First, anionic liposoms could facilitate the distribution of Ad5 in CAR deficient airway epithelium. According to the result of vector genome detected by TaqMan qRT-PCR in the trachea and lung tissues (Figure 7), higher genome numbers of Ad5 were detected in both tissues in the AL−Ad5 group at week 1, which resulted the elevated transgene level observed in this group at this time point. Second, in the present study, the adenovirus was coated by anionic liposomes to shield the adenoviral capsid and reduce the immune responses generated by adenoviral vectors. As shown in Figure 7, significantly higher genome copy numbers remained in trachea and lungs at week 4, suggesting AL−Ad5 could slow down the elimination of adenovirus by the immune systems. As a result, the transgene expression profiles achieved with AL−Ad5 were significantly extended beyond what was seen with naked viruses.

It has been reported[33] that Ad5 vector-induced 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 Ad5 vector, which prevents successful readministration. Price et al.[32] reported that adenoviral vectors formulated with cationic steroid liposomes could not attenuate vector-induced inflammation, unless in the presence of dexamethasone. Our histology findings also came to similar results that, even 4 weeks postinstillation, inflammatory cell infiltration and thickened alveolar walls were still observed in the lung tissues of mice treated with the complexes of Ad5 and cationic liposomes or treated with naked Ad5. However, the formulation of anionic liposomes with Ad5 vectors significantly reduced the inflammation reaction following intratrachea instillation *in vivo*. Additionally, CL−Ad5 and AL−Ad5 produced similar transduction efficiency on primary cultured murine airway epithelia; but when applied *in vivo*, CL−Ad5 led to much lower gene expression than AL−Ad5 did, which may be associated with their adhesion with the negative-charged components in the mucus and the inflammation reaction.

In the present study, the results indicated that the AL−Ad5 prepared by combination strategy protected Ad5 from recognition by immune cells, leading to significant reduction of the vector-induced inflammation and slowing down the elimination of vector genome, thus coming to the enhanced and prolonged gene expression. Meanwhile, it also appeared to eliminate the necessity of the CAR on the apical membrane of the target ciliated airway cell surface to allow improved gene transduction, which was beneficial to the application in cystic fibrosis gene therapy *in vivo*.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. The role of EDTA in formulation of naked adenovirus, the transepithelial resistance (Rte) determination of the primary murine tracheal epithelial cells and qualitative determination of β-galactosidase gene expression *in vivo*. This material is available free of charge via the Internet at http://pubs.acs.org.

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