Aerosol-Mediated Delivery of AAV2/6-I κ B α Attenuates Lipopolysaccharide-Induced Acute Lung Injury in Rats

Ronan J. MacLoughlin,¹ Brendan D. Higgins,² James Devaney,³ Daniel O'Toole,³
John G. Laffey,⁴ and Timothy O'Brien¹

Abstract

Inhibition of the proinflammatory transcription factor NF- κ B has previously been shown to attenuate the inflammatory response in tissue after injury. However, the feasibility and efficacy of aerosolized adeno-associated viral (AAV) vector-delivered transgenes to inhibit the NF- κ B pathway are less clear. Initial studies optimized the AAV vector for delivery of transgenes to the pulmonary epithelium. The effect of repeated nebulization on the integrity and transduction efficacy of the AAV vector was then examined. Subsequent *in vivo* studies examined the efficacy of aerosolized rAAV2/6 overexpressing the NF- κ B inhibitor I κ B α in a rodent endotoxin-induced lung injury model. Initial *in vitro* investigations indicated that rAAV2/6 was the most effective vector to transduce the lung epithelium, and maintained its integrity and transduction efficacy after repeated nebulization. In our *in vivo* studies, animals that received aerosolized rAAV2/6-I κ B α demonstrated a significant increase in total I κ B α levels in lung tissue relative to null vector-treated animals. Aerosolized rAAV2/6-I κ B α attenuated endotoxin-induced bronchoalveolar lavage-detected neutrophilia, interleukin-6 and cytokine-induced neutrophil chemoattractant-1 levels, as well as total protein content, and decreased histologic indices of injury. These results demonstrate that aerosolized AAV vectors encoding human I κ B α significantly attenuate endotoxin-mediated lung injury and may be a potential therapeutic candidate in the treatment of acute lung injury.

Introduction

ACUTE RESPIRATORY DISTRESS SYNDROME (ARDS) is a common clinical syndrome that presents as severe lung inflammation, acute respiratory failure, noncardiogenic pulmonary edema, and associated inflammatory cell sequestration and cytokine release.¹ The pathogenesis of acute lung injury (ALI)/ARDS involves injury to both the pulmonary epithelium and vascular endothelium.² Although mortality from ARDS has declined since the 1980s, risk remains high, with underlying clinical causes, such as sepsis (43%) and pneumonia (36%), important determinants of outcome.³ The development of new therapeutic approaches therefore remains crucial. Nuclear factor (NF)- κ B is an important transcriptional regulator, which mediates the expression of hundreds of genes involved in inflammation, immunity, cell survival, and cancer.^{4,5} NF- κ B inhibition has been demonstrated to reduce indices of injury in preclinical models of

ALI, including acute *Escherichia coli*-induced pneumonia,⁶ ischemia–reperfusion,⁷ stretch-induced injury,⁸ endotoxemic sepsis,⁹ and cecal ligation and puncture-induced sepsis.¹⁰ However, NF- κ B also exerts important cytoprotective effects, promoting cell survival, resolution of inflammation, and wound repair.¹¹ The inhibition of NF- κ B may constitute a viable therapeutic approach, particularly in pneumonia-induced ALI/ARDS, where immune competence is essential to removal of pathogens.¹² Using gene therapy approaches to modulate the NF- κ B pathway may allow us to specifically target the lung epithelium, thereby reducing the systemic consequences of NF- κ B inhibition. Aerosol delivery constitutes a noninvasive administration approach to gene delivery, but the efficacy of these approaches has not been determined.

Acquired inflammatory respiratory disorders such as chronic obstructive pulmonary disease (COPD), asthma, or ARDS, which occur because of various polygenic and exogenous influences, may be candidates for transient gene

¹Regenerative Medicine Institute (REMEDI), National University of Ireland, Galway, Ireland.

²Physiology, National University of Ireland, Galway, Ireland.

³Anaesthesia, Clinical Sciences Institute, and Lung Biology Group, National Centre for Biomedical Engineering Sciences, National University of Ireland, Galway, Ireland.

⁴Department of Anesthesia, Keenan Research Centre in the Li Ka Shing Knowledge Institute, St. Michael's Hospital, Toronto M5B 1W8, Canada.

therapy approaches where short-term overexpression of protective genes or the suppression of damaging genes may attenuate inflammation.^{13,14} In clinical ARDS, NF- κ B pathway gene polymorphism activation can modify susceptibility to and severity of cases.^{15,16} Cell activation initiated by stimuli such as gram-negative bacterial endotoxin activates various signaling pathways, which converge to phosphorylate and activate the I κ B kinase complex proteins (IKK), which then phosphorylate and inactivate the I κ B proteins, which dissociates from NF- κ B. Activated NF- κ B translocates to the nucleus and binds to specific cognate-binding sequences in the promoter or enhancer regions of various target genes to initiate transcription.⁵ The evidence for potential therapeutic strategies emerging from inhibition of the NF- κ B pathway is demonstrated by decreasing injury in nonseptic ALI models, including pulmonary ischemia-reperfusion,¹⁷ endotoxemia,⁹ and smoke-induced pulmonary inflammation.¹⁸

Pulmonary aerosol deposition patterns may have significant implications for absorption profile and resulting pharmacokinetics, with deposition in the respiratory airways, typically resulting in greater absorption than that seen in the conducting airways.^{19–21} Direct liquid instillation to the pulmonary epithelium results in a heterogeneous pattern that deposits locally, predominantly in larger airways, which is of limited benefit when delivery to distal areas of the lung is required. Aerosolization offers the potential of a more diffuse, homogeneous pattern with deposition in the gas-exchanging smaller airways and alveolar regions.^{22,23}

Considering these issues, we wished to examine the feasibility and efficacy of aerosol delivery of transgenes to modulate the NF- κ B pathway in the pulmonary epithelium. Initial studies optimized the adeno-associated viral (AAV) vector for delivery of transgenes to the pulmonary epithelium. We then examined the effect of repeated nebulization on the integrity and transduction efficacy of the AAV vector. Subsequent *in vivo* studies examined the efficacy of aerosolized rAAV2/6 overexpressing the NF- κ B inhibitor, I κ B α , in a rodent endotoxin-induced lung injury model. We hypothesized that (1) the AAV vector would efficiently transduce the pulmonary epithelium *in vitro* and *in vivo*, (2) aerosolization of AAV vector would not diminish its structural integrity or transduction efficacy, and (3) aerosolized AAV-I κ B α would reduce the severity of endotoxin-induced lung injury in a rodent model.

Materials and Methods

rAAV2, rAAV2/5, and rAAV2/6 manufacture

The I κ B α superrepressor, incorporating S32A and S36A mutations, is not susceptible to phosphorylation at these serines and therefore resistant to subsequent degradation. A plasmid carrying this mutated gene was a kind gift from R. Zwacka (National University of Ireland, Galway, Ireland).

Empty pTRUF and pTRUF-I κ B α plasmids were packaged with the relevant AAV serotype vectors through dual transfection, with pDP helper-free REP/CAP plasmids (Plasmid Factory, Bielefeld, Germany), into HEK293T cells and isolated by subsequent iodixanol density gradient centrifugation. After DNase and proteinase K digestion, viral titer was quantified by real-time PCR (GeneAmp PCR 9600 system; PerkinElmer, Waltham, MA), using primers directed against the cytomegalovirus (CMV) promoter, as compared

with a standard curve constructed with the pTRUF plasmid at defined copy number. Primers used were as follows: CMV promoter (sense, 5'-TGGAAATCCCCGT GAGTCAA-3'; antisense, 5'-CATGGTGTATGCGGTTT TGG-3').

Animal care

Specific-pathogen-free adult male Sprague Dawley rats (Harlan, Bicester, UK) weighing between 400 and 525 g were used in these experiments. All experimental work was approved by the Animal Care Research Ethics Committee of the National University of Ireland, Galway and conducted under license granted by the Department for Health, Ireland. On arrival, the rats were allowed to acclimatize for at least 1 week under standardized conditions of temperature (21–22°C), humidity (45–55%), and light (12 hr light:12 hr dark schedule). Animals had access to rat chow and tap water *ad libitum*.

Aerosol administration to rats

Animals were anesthetized with intraperitoneal ketamine (Ketalar, 80 mg·kg⁻¹; Pfizer Healthcare, Dublin, Ireland) and inhalational isoflurane (Abbott Laboratories, Dublin, Ireland) at a concentration of 2% in oxygen (fraction of inspired oxygen [FiO₂], 1.0) and then intubated as previously described.²⁴ Depth of anesthesia throughout the procedure was assessed every 10 min by monitoring the response to paw clamp. The rats were mechanically ventilated with a small animal ventilator (SAR-830/P; CWE, Ardmore, PA), with room air (FiO₂, 0.21), a respiratory rate of 45 breaths·min⁻¹, a tidal volume of 6 ml·kg⁻¹, a positive end-expiratory pressure of 2 cmH₂O, and a 2:1 inhalation-to-exhalation ratio. To minimize lung derecruitment, a recruitment maneuver consisting of a positive end-expiratory pressure of 10 cmH₂O for 25 breaths was applied before aerosolization began. The animal was ventilated for a further 2 min after recruitment to ensure the airway pressures were stable, and then a dose of 1×10¹² DNase-resistant particles (drp) of the relevant AAV serotype, in 0.3 ml of normal saline, was nebulized into the lung. A volume of 0.3 ml of normal saline was administered to sham controls. After completion of the first cycle of aerosolization, any rainout solution was collected from the nebulizer T-piece and recycled into the nebulizer medication cup. This step ensured minimal wastage of the rAAV dose. After the dose was completely delivered to the lungs, the animals were extubated, anesthesia was discontinued, and the rats were allowed to recover and subsequently returned to HEPA-isolated cages for a period of 5 days. The nebulizer actuation was set to deliver for the first 25% of the inhalation maneuver only. Airway pressures were monitored throughout aerosolization, using an MP30 BIOPAC Student Lab data acquisition module (BIOPAC Systems, Goleta, CA).

Endotoxin/vehicle instillation

The experimental injury model was based on those previously reported, with several modifications.^{25,26} Five days after vector/vehicle instillation, rats were reanesthetized and endotoxin (lipopolysaccharide [LPS]; 5 mg·kg⁻¹) derived from *Escherichia coli* serotype 055:B5 (Fluka, Poole, UK) in 0.3 ml of normal saline or vehicle was instilled intratracheally.

Primary cell isolation and cell line culture

Animals were anesthetized with intraperitoneal ketamine (Ketalar, 80 mg·kg⁻¹; Pfizer Healthcare) and xylazine (Xylapan, 8 mg·kg⁻¹; Vétoquinol, Dublin, Ireland) and then killed by exsanguination. Primary type II epithelial cells isolated from Sprague Dawley rats (PT2SD) were cultured for a minimum of 24 hr before subsequent experimental use.²⁷ A549 cells (adenocarcinomic human alveolar basal epithelial cells; European Collection of Cell Cultures, Porton Down, UK), L2 bronchial epithelial cells (ATCC, Middlesex, UK), and PT2SD cells were used to assess transduction by AAV serotypes *in vitro*. All cells were grown to confluence on plastic plates or tissue culture flasks (Corning, New York, NY) at 37°C in a humidified incubator saturated with a gas mixture containing 5% CO₂ in air.

Determination of β -galactosidase expression levels in the lung posttransduction

Expression levels of β -galactosidase in tissue were characterized with a standardized assay kit (Galacto-Star chemiluminescent reporter gene assay system; Applied Biosystems, Bedford, MA) according to the manufacturer's instructions.

RT-PCR of *I κ B α*

Approximately 200 mg of recovered lung tissue was homogenized in 2 ml of TRIzol reagent (Sigma-Aldrich, Arklow, Ireland), using a TissueRuptor homogenizer (Qiagen, Germantown, MD). RNA was isolated through subsequent chloroform phase separation and isopropanol precipitation. One microgram of RNA was used to generate cDNA, using a reverse transcription kit (Promega, Madison, WI). Relative levels of transgene were quantified by real-time PCR (GeneAmp PCR 9600 system; PerkinElmer), using primers directed against human *I κ B α* and normalized relative to a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeper control. Primers used were as follows: human *I κ B α* (sense, 5'-GTCAAGGAGCTGCAGGAGAT-3'; antisense, 5'-CCA TGGTCAGTGCCTTTTCT-3') and rat GAPDH (sense, 5'-CTGGCATTGCTCTCAATGACA-3'; antisense, 5'-TTACT CCTTGGAGGCCATGTA-3').⁸

Western blot for *I κ B α*

Approximately 200 mg of recovered lung tissue was homogenized in 2 ml of phosphate-buffered saline (PBS) containing cOmplete, Mini, EDTA-free proteinase inhibitor cocktail (Roche Diagnostics, Burgess Hill, UK), using a TissueRuptor homogenizer (Qiagen). Insoluble material was precipitated at 12,000×g for 10 min in a minifuge, and protein content was assessed by bicinchoninic acid (BCA) assay. Fifty micrograms of protein was then applied to a 4–20% gradient polyacrylamide gel, using denaturing loading buffer and boiling, and electrophoresed. Protein was then transferred to nitrocellulose in a mini Trans-Blot cell (Bio-Rad, Mexico City, Mexico) and nonspecific sites were blocked with 5% (w/v) nonfat milk in PBS. Blots were probed with rabbit anti-*I κ B α* antibody (Cell Signaling Technology, Danvers, MA) followed by an anti-rabbit IgG-horseradish peroxidase (HRP) conjugate antibody (Sigma-Aldrich). Blots were soaked in SuperSignal chemiluminescent substrate

(Thermo Fisher Scientific, Waltham, MA) and imaged. Densitometry was performed with ImageJ software and band intensity was normalized by subsequent blotting with a β -actin-HRP antibody (Sigma-Aldrich).²⁸

Tissue sampling and assays

After 5 days, heparin (400 IU·kg⁻¹; CP Pharmaceuticals, Wrexham, UK) was administered intravenously, and the animals were killed by exsanguination. Immediately post-mortem, the heart–lung block was dissected from the thorax and bronchoalveolar lavage (BAL) collection and analysis was performed as previously described.²⁴ The concentrations of interleukin (IL)-6, and cytokine-induced neutrophil chemoattractant (CINC)-1 in BAL fluid, were determined in commercially available enzyme-linked immunosorbent assays (R&D Systems Europe, Abingdon, UK). The total protein infiltrate present in BAL fluid was determined with a BCA assay kit (Pierce, Thermo Fisher Scientific, Rockford, IL).

Histologic and stereologic analysis

The left lung was isolated and fixed for morphometric examination as previously described.²⁹ The extent of histologic lung damage was determined by blinded assessors using quantitative stereological techniques as previously described.³⁰ Briefly, the vertical axis of each left lung was identified and the lung was cut perpendicular to this axis into 4-mm-thick slices with a sharp blade beginning at a position chosen by random number within the first slice. These tissue slices were then embedded in paraffin and sections (10 μ m) from each slice were mounted on slides and stained with hematoxylin and eosin. One field of view from each lung section was chosen, by means of the random selection of grid co-ordinates from a fixed point at the extreme lower left tip of the lung section. Each field was examined by light microscopy (model BX51; Olympus, Mason Technologies, Dublin, Ireland) at $\times 10$ magnification, and the images were digitally captured and stored in 8-bit (256 level) format and displayed with analySIS^D imaging software (version 1.20; Olympus Europa, Hamburg, Germany). A 100-point counting grid was then digitally superimposed over the image and a touch count was performed for each of the following: acinar tissue, nonacinar tissue, and airspace. Acinar volume fraction represented all alveolar tissue including epithelium, endothelium, connective tissue, and inflammatory cells. Nonacinar fraction meant bronchial or vascular space, and airspace described the gaseous component of the lung.

Assessment of pulmonary epithelial gene transfer and expression

Transduced rat lung epithelia were characterized with monoclonal pan-cytokeratin antibody [C-11] (phycoerythrin [PE]) (Abcam, Cambridge, UK). Appropriate PE-conjugated immunoglobulin isotype controls were also used (Abcam). Green fluorescent protein (GFP) generated by the instilled AAV-delivered GFP *I κ B α* plasmid construct was measured in the fluorescein isothiocyanate (FITC) channel. A single-cell suspension was obtained by collagenase digestion of rat lung homogenate. For staining, cells were first washed with fluorescence-activated cell-sorting (FACS) buffer (PBS

containing 2% fetal calf serum and 0.01% NaN₃ [Sigma-Aldrich]) and then stained on ice for 30 min in 1:20 antibody FACS buffer solution. Unbound antibody was removed by washing twice with FACS buffer and cells were resuspended in FACS buffer for analysis with a FACSCanto flow cytometer (BD Biosciences, Oxford, UK).

Compensation parameters were established on the cytometer, using appropriately stained single cells. Unlabeled beads (CaliBRITE unlabeled beads; BD Biosciences) were added to FACS samples for detection of absolute number of cells, and dead cells were identified with propidium iodide (PI) staining solution. Data were analyzed with FlowJo software (Tree Star, Ashland, OR) and results are presented as a percentage of cell population.

Statistical analyses

All data were checked for normality before statistical analysis. Data distribution was assessed using Kolmogorov–Smirnov tests. Data were analyzed using an unpaired Student *t* test and are shown as means $\pm$ standard deviation. To compare differences between groups, one-way analysis of variance (ANOVA) coupled with the Bonferroni multiple comparison test was used. Lung histology was analyzed by two-way ANOVA, with group as the first factor and histologic classification (tissue, airspace, extraacinar) as the second factor. Asterisks indicate level of significance (**p* < 0.05; ***p* < 0.01; ****p* < 0.0001). All statistics were performed with GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA).

Results

Determination of optimal AAV vector serotype for transduction of pulmonary epithelium: In vitro studies

The transduction efficiencies of rAAV2, rAAV2/5, and rAAV2/6 expressing LacZ were compared in three different pulmonary epithelial cell lines: A549 cells, L2 bronchial epithelial cells, and PT2SD cells. The transduction efficiency of the three rAAV serotypes is shown (Fig. 1A) (*n* = 10 for each group). Across all cell types tested, rAAV2/6-LacZ resulted in the highest transduction efficiency (*p* < 0.0001). The least efficient was rAAV2/5-LacZ, which consistently had the lowest β -galactosidase activity levels across the cell types.

AAV vector aerosolization preserves structural integrity and transduction efficacy: In vitro studies

The process of aerosol generation during both ultrasonic and jet nebulization is known to create heat and shear forces, respectively, thus potentially affecting the formulation being nebulized. Using an alternative aerosol generator technology, an actively vibrating mesh nebulizer (Aeroneb[®] Lab; Aerogen, Galway, Ireland), the effect of two rounds of nebulization on the titer of the rAAV serotypes under investigation was determined by real-time PCR. The effect of nebulization on 1×10^{12} drp in 0.3 ml of sterile 0.9% saline is shown in Fig. 2A. After collection of nebulized vector, each sample was immediately subjected to a second round of nebulization, mimicking our protocol for intratracheal aerosol administration. On characterization, the titer of rAAV2-LacZ decreased by $17.66 \pm 1.44\%$ (*p* < 0.0001), the titer of rAAV2/5-LacZ decreased by $5.67 \pm 1.46\%$ (*p* < 0.01),

FIG. 1. (A) *In vitro* transduction efficiency of rAAV2, rAAV2/5, and rAAV2/6 in PT2SD, A549, and L2 bronchial epithelial cell types, 48 hr postadministration (*n* = 10 per group). (B) Transduction efficiency of rAAV2, rAAV2/5, and rAAV2/6 in whole lung 5 days postadministration (*n* = 5 animals per group). Significance established at ****p* < 0.0001. All significance was established relative to rAAV2/6. Data are presented as means $\pm$ standard deviation.

and a significant titer reduction was also recorded for rAAV2/6-LacZ, $7.66 \pm 1.44\%$ (*p* < 0.0001) (*n* = 5 for each group).

The effect of two rounds of nebulization on the *in vitro* transduction efficiency of the rAAV vectors is shown in Fig. 2B. Transduction efficiency was quantified by measuring the activity levels of β -galactosidase in A549 cells, after coin-cubation. A significant decrease in the β -galactosidase activity levels (5.47 relative light units [RLU]/ μ g protein) was noted for rAAV2-LacZ (*p* < 0.01). The β -galactosidase activity levels also decreased significantly when rAAV2/5-LacZ was nebulized twice (2.58 RLU/ μ g protein; *p* < 0.05) (relative light units standardized per microgram of protein). However, no reduction in transduction efficiency was seen after two rounds of nebulization of rAAV2/6-LacZ, for which a 3.59-RLU/ μ g protein decrease in β -galactosidase activity was recorded (*n* = 5 for each group).

In vivo studies

Transduction efficiency of AAV serotypes. Each rAAV serotype being evaluated was delivered by aerosolization at a dose of 1×10^{12} drp in 0.3 ml of sterile 0.9% saline (Fig. 1B). Five days postadministration, the transduction efficiency of rAAV2/6-LacZ (4.02 RLU/ μ g protein) was significantly higher than that of both rAAV2-LacZ (2.17 RLU/ μ g; *p* < 0.0001) and rAAV2/5-LacZ (0.66 RLU/ μ g; *p* < 0.0001) (*n* = 5 animals for each group). These results are in broad agreement with the performance recorded in the isolated Sprague Dawley primary type II epithelial cells tested *in vitro*.

FIG. 2. (A) Effect of two rounds of nebulization on the titer of rAAV2, rAAV2/5, and rAAV2/6 ($n=5$ for each group). (B) Effect of nebulization on the transduction efficiency of rAAV2-, rAAV2/5-, and rAAV2/6- β -galactosidase in A549 cells ($n=5$ per group). Significance established at * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.0001$. Significance was established relative to unnebulized sample. Data are presented as means $\pm$ standard deviation.

Aerosol-delivered AAV- $\text{I}\kappa\text{B}\alpha$ inhibits endotoxin-induced lung injury. rAAV2/6 vector encoding the $\text{I}\kappa\text{B}\alpha$ or null gene was administered to rats via aerosolization 5 days before insult, in order to allow time for appreciable expression of the transgene. LPS or vehicle was administered intratracheally on day 5, and animals were killed 24 hr later.

$\text{I}\kappa\text{B}\alpha$ was delivered and expressed in the lung. Across all treatment groups, exogenous human $\text{I}\kappa\text{B}\alpha$ gene expression was analyzed by quantitative RT-PCR after LPS or vehicle administration (Fig. 3A). In the $\text{I}\kappa\text{B}\alpha$ gene+LPS injury treatment group there was a 127.82 ± 3.21 -fold increase in $\text{I}\kappa\text{B}\alpha$ levels compared with the sham group. This increase can be attributed to exogenous human $\text{I}\kappa\text{B}\alpha$ in rat lung after rAAV2/6-mediated delivery. Three pooled samples of two animals each were analyzed in triplicate.

Homogenized lung tissue was examined for total $\text{I}\kappa\text{B}\alpha$ protein levels by Western blotting followed by densitometry (Fig. 3B). As expected, LPS injury and null gene+LPS injury groups exhibited reduced levels of $\text{I}\kappa\text{B}\alpha$ as part of an inflammatory NF- κB pathway response. This degradation was significantly ameliorated in the $\text{I}\kappa\text{B}\alpha$ gene+LPS injury treatment group ($p < 0.05$ vs. null gene+LPS injury group) and, indeed, protein levels were increased beyond that seen in the sham controls ($p < 0.05$).

Analysis by FACS was performed on rat lung cotransduced with $\text{I}\kappa\text{B}\alpha$ and GFP at a titer of 1×10^{10} drp in 0.3 ml of saline at 96 hr in order to assess the efficiency of pulmonary epithelial cell transduction by the rAAV2/6 vector employed in the current study. A PE-labeled epithelial specific antibody (pan-cytokeratin) was used to identify epithelial cells from the lung cell suspension (Fig. 4A–F), and FITC excitation (Fig. 4G) was used to calculate the percentage of cells that were transduced with GFP. Of the epithelial cells stained by the cytokeratin–PE antibody in lungs that received rAAV 2/6- $\text{I}\kappa\text{B}\alpha$, 21% were positive for GFP (Fig. 4F), whereas overall 4.3% of events were positive for GFP.

$\text{I}\kappa\text{B}\alpha$ overexpression reduced lung inflammation. Endotoxin produced a significant inflammatory response compared with vehicle. $\text{I}\kappa\text{B}\alpha$ overexpression attenuated—but did not eliminate—the pulmonary inflammatory response to endotoxin, attenuating the increase in alveolar concentrations of the key cytokines IL-6 and CINC-1 (Fig. 5A and B) and mitigating the increase in total alveolar inflammatory

FIG. 3. (A) RT-PCR detection of the exogenous $\text{I}\kappa\text{B}\alpha$ transgene in the lung 24 hr after LPS or saline sham administration. Each column represents three pools of two animals ($n=6$ animals) and is the mean of three experimental replicates $\pm$ standard deviation. (B) Total $\text{I}\kappa\text{B}\alpha$ expression levels 24 hr after LPS or saline sham administration to the lung and Western blot of $\text{I}\kappa\text{B}\alpha$ protein expression. Data are presented as the $\text{I}\kappa\text{B}\alpha$ -to-actin signal ratio normalized to sham control. Significance established at * $p < 0.05$ and ** $p < 0.01$. Significance was established relative to the $\text{I}\kappa\text{B}\alpha$ gene+LPS injury treatment group.

FIG. 4. FACS analysis of rat pulmonary epithelial cells transduced with AAV expressing $\text{I}\kappa\text{B}\alpha$ -GFP construct. (A–C) Control; (D–F) $\text{I}\kappa\text{B}\alpha$ and GFP (1×10^{10} drp in 0.3 ml). After 96 hr a PE-labeled epithelial specific antibody (pan-cytokeratin) was used to identify epithelial cells from a single-cell suspension of the lung by FACS. FITC excitation was used to calculate the percentage of cells that were transduced with GFP. Of the epithelial cells stained with the cytokeratin-PE antibody, 21% were positive for GFP (F), whereas only 1.2% were positive in the control (C). Data are presented as percentage of live cells present in the suspension. (G) summarizes the increased FITC excitation and hence $\text{I}\kappa\text{B}\alpha$ GFP expression in the transduced epithelial lung cells compared with the nontransduced control. Color images available online at www.liebertpub.com/hum

cell infiltration (Fig. 5C) and alveolar neutrophil infiltration (Fig. 5D). The total protein infiltrate present in BAL fluid from injured lungs also showed a significant improvement compared with injured animals (Fig. 6A).

Quantitative stereologic analysis demonstrated that overexpression of $\text{I}\kappa\text{B}\alpha$ significantly attenuated injury with respect to tissue density and airspace in the $\text{I}\kappa\text{B}\alpha$ gene + LPS injury treatment group. $\text{I}\kappa\text{B}\alpha$ overexpression maintained significant alveolar airspace fraction (LPS injury, $p < 0.0001$; $\text{I}\kappa\text{B}\alpha$ gene + LPS injury, $p < 0.01$) and a significantly reduced alveolar tissue fraction compared with other LPS-treated groups (LPS injury, $p < 0.01$; $\text{I}\kappa\text{B}\alpha$ gene + LPS injury, $p < 0.01$) (Fig. 6B). This demonstrates that in the case of NF- κB inhibition mediated by the $\text{I}\kappa\text{B}\alpha$ gene, alteration of the

alveolar wall as a result of edema and the accumulation of inflammatory infiltrates occurred to a lesser degree (Fig. 7).

Discussion

Key findings

rAAV-mediated overexpression of the NF- κB inhibitory protein, $\text{I}\kappa\text{B}\alpha$, both *in vitro* and *in vivo*, attenuates the severity of the inflammatory response in the setting of endotoxin-mediated ALI, thus providing proof that (1) optimized aerosol delivery of the rAAV vector and the subsequent localized expression of a suitable transgene in the lung is capable of having a therapeutic effect on the markers of acute lung injury in a preclinical setting, and that (2) the

FIG. 5. (A) IL-6 levels in BAL 24 hr after LPS administration. (B) CINC-1 levels in BAL 24 hr after LPS administration. (C) Total BAL cell counts recorded 24 hr after LPS administration. (D) Absolute neutrophil counts recorded 24 hr after LPS or vehicle administration. Significance established at $**p < 0.01$ and $***p < 0.0001$. Significance was established relative to the IκBα gene + LPS injury treatment group. Data are presented as minimal value, median, interquartile ranges, and maximal value.

effect of overexpression of a superrepressor form of IκBα can sufficiently reduce the activity of NF-κB to indirectly regulate a variety of proinflammatory cytokines. Insights into the nuclear activity and biological function of IκBα are essential for targeting the NF-κB pathway for future therapeutic approaches to inflammation and lung injury.

In vitro and in vivo serotype optimization studies

Preliminary *in vitro* investigations using the immortalized A549 (human) and L2 (rat) cell lines and primary isolated

type II cells (PT2SD) confirmed the abilities of the tested AAV serotypes to transduce lung epithelial cells. PT2SD cells demonstrated a high correlation of transduction, as did the immortalized cell lines (Fig. 1A), with the rAAV2/6 serotype being similarly effective when targeting the Sprague Dawley rat pulmonary epithelium (Fig. 1B).

The effect of nebulization on the integrity of rAAV serotypes has not been adequately addressed previously. Although a significant reduction in titer for all three rAAV serotypes tested was observed, the transduction efficiency of the AAV2/6 vector before and after nebulization was

FIG. 6. (A) Total protein concentration in BAL samples recorded 24 hr after LPS administration. Significance was established relative to the I κ B α gene+LPS injury treatment group. Data are presented as minimal value, median, and maximal value. (B) Stereologic assessment of the extent of histologic injury across the treatment groups in LPS and sham injured lung. Significance established at * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.0001$. Significance was established relative to the I κ B α gene+LPS injury treatment group. Data are presented as means $\pm$ standard deviation.

unchanged, even after two cycles of aerosolization, suggesting the vector was not damaged by the nebulization process (Fig. 2A and B). These results are in agreement with a previously published characterization of the effect of nebulization on rAAV2 and rAAV2/5 using vibrating mesh nebulizer technology.³¹ If no damage to the vector was seen *in vitro*, it would be expected that delivered AAV2/6 would retain its activity *in vivo*.

Aerosol characteristics and behavior dictate that in a small-bore diameter system, such as that used presently, droplet size is a factor of vital importance in the system's delivery performance. We have previously demonstrated that the experimental system used in the present study delivers a high percentage of the original dose to the lungs of a mechanically ventilated rat. This is achieved through the combined use of a low flow rate and low residual volume

FIG. 7. Photomicrographs (original magnification, $\times 10$) of representative sections of hematoxylin–eosin (H&E)-stained lung tissue from each of the treatment protocols demonstrating the histologic level of ALI. (A) Normal lung (sham). (B) LPS-induced ALI demonstrating inflammation of tissue, and infiltration of macrophages and neutrophils (LPS injury). (C) Vector control treatment (null gene+LPS injury). (D) Attenuation of ALI as indicated by a reduced level of tissue inflammation concomitant with reduced neutrophil and macrophage counts (I κ B α gene+LPS injury). Color images available online at www.liebertpub.com/hum

breath-actuated vibrating mesh nebulizer, generating a small droplet size appropriate for alveolar deposition.³²

Demonstration that nebulized AAV-I κ B α reduces LPS injury

The presence of rAAV2/6-mediated exogenous human I κ B α was confirmed by quantitative PCR. A 127.82 ± 3.21 -fold increase ($p < 0.0001$) was noted in I κ B α levels 24 hr after LPS administration (Fig. 3A). As a result of the activation of NF- κ B by LPS, the amount of unbound I κ B α at the local site is known to decrease as it is degraded by the 26S proteasome. This change in I κ B α protein levels was confirmed by Western blot (Fig. 3B).

The efficacy of pulmonary epithelial cell transduction by the rAAV2/6 vector employed in this study was measured by flow cytometry 96 hr after instillation to calculate the percentage of lung epithelial cells that were transduced with GFP and I κ B α . Of the epithelial cells stained with the cytokeratin-PE antibody and that received rAAV 2/6-I κ B α , 21% were positive for GFP (Fig. 4F), whereas for the lung as a whole, 4.3% of events were positive for GFP, indicating targeted delivery to the pulmonary epithelium. Previous studies have shown the efficacy of rAAV2/6 serotype-delivered I κ B α in lung injury models such as *E. coli*-induced injury⁶ and ventilator-induced injury,³³ with both studies demonstrating attenuation of indices of injury and inflammation. The results of the present study further indicate the potential for significant levels of super-repressor transduction and hence the improved indices of injury seen here.

As evidenced by assays of the proinflammatory cytokines IL-6 and CINC-1, the increased concentration of I κ B α protein was capable of binding unbound NF- κ B that had been released as a result of the proinflammatory signal and so attenuated the proinflammatory cascade regulated by NF- κ B (Fig. 5A and B). Proinflammatory IL-6 levels were seen to be reduced significantly in the prophylactic I κ B α gene + LPS injury treatment group in comparison with the control injury groups ($p < 0.0001$) (Fig. 5A). Previous reports of NF- κ B inhibition have shown a reduction in IL-6 expression levels.³⁴ Although the reduction in IL-6 levels noted in the present study is quantitatively small, studies in murine sepsis and clinical ARDS have linked attenuation of IL-6 to improved survival.^{35,36} The reduction in IL-6 levels correlated with that seen in CINC-1 levels (Fig. 5B).

Concomitant with the reduction in CINC-1 levels, a significantly lowered absolute number of neutrophils was recorded in the rAAV2/6-I κ B α treatment group in comparison with both control injury groups (LPS injury, $p < 0.0001$; I κ B α gene + LPS injury, $p < 0.01$) (Fig. 5D). CINC-1 acts as a functional chemoattractant for neutrophils in inflammation, and therefore reduced CINC-1 levels may provide some explanation for this reduced neutrophil count. This is consistent with previous observations.³⁷ The total BAL cell count was also significantly lower in the rAAV2/6-I κ B α treatment group compared with control injury groups ($p < 0.01$) (Fig. 5C). Significantly less protein was present in the BAL of rAAV2/6-I κ B α -treated animals compared with both injury control groups (LPS injury, $p < 0.01$; I κ B α gene + LPS injury, $p < 0.05$) (Fig. 6A), suggesting that overexpression of superrepressor I κ B α is protective, ame-

liorating disruption caused to the alveolar epithelium and vascular endothelium.

Quantitative stereologic analysis demonstrated significantly less histologic injury with respect to tissue and airspace in the I κ B α /LPS treatment group; and a significantly greater alveolar airspace fraction (LPS injury, $p < 0.001$; I κ B α gene + LPS injury, $p < 0.01$) and a significantly reduced alveolar tissue fraction were recorded when compared with the LPS injury groups (LPS injury, $p < 0.01$; I κ B α gene + LPS injury, $p < 0.01$) (Fig. 6B). The greater loss of alveolar architecture and thickening of alveolar septae in LPS-injured lungs results in impaired gas exchange, compared with the protection afforded by I κ B α treatment. The closer-to-normal histology present in the I κ B α gene treatment group should allow for improved gaseous exchange in the lungs and, thus, attenuate lung injury-associated hypoxemia.

Despite the reduction in proinflammatory signals seen across all experiments, as well as reduced histologic injury, the indices of proinflammatory signals for the rAAV2/6-I κ B α /LPS treatment group were significantly higher than those measured in uninjured animals. LPS is a potent activator of proinflammatory signals and thus it is extremely difficult to completely abrogate its proinflammatory effect. Despite the considerable research to date in LPS-induced ALI, there are no reports of complete reversal of LPS-induced injury. While I κ B α inhibits only the canonical pathway of NF- κ B, a number of other mechanisms may be responsible for cytokine production.

Limitations

This study involved administration of vector 5 days before the initiation of lung injury. In the clinical setting, lung injury is generally well established at presentation. It is not clear what effect I κ B α overexpression might have if introduced after lung injury or if the inflammatory process already underway would hinder I κ B α transduction. However, there are certain situations, such as prior major intra-abdominal or cardiovascular procedures, in which an insult, such as the development of endotoxemia, is a relatively frequent occurrence.³⁸ Intratracheal delivery of transgene after the development of ALI/ARDS may also be difficult, particularly in a context of atelectasis and edema, reducing the therapeutic usefulness of intrapulmonary delivered therapies. Although gene transfer in this environment has been shown to be successful,^{39–41} it is likely to be associated with reduced gene transfer efficiency. Also, the time course of ARDS is significantly longer than the 24 hr assessed here. Differences between treatment groups that were not apparent at 24 hr may be more obvious after a number of days. Further experiments with a longer treatment period might be useful in giving insights into the likely clinical impact of pulmonary I κ B α therapy for ARDS. The optimal timeframe for gene expression may also be longer than 5 days. However, a study of this nature would require a different model of injury than LPS. We are unable to furnish any data relating to any potential influence of LPS on transgene expression in the current model.

As type II pneumocytes represent approximately 14% of the total alveolar population and cover approximately 5% of the total lung surface area,⁴² there are limitations to their use *in vitro*. Finally, because of major anatomic and

physiological differences between rats and humans, aerosol-based therapeutic strategies would need to be evaluated in large animal studies before translation to clinical applications.

In conclusion, we have demonstrated the effectiveness of aerosol delivery of rAAV2/6-I κ B α , which inhibits the proinflammatory effects of NF- κ B in the setting of endotoxin-induced ALI. There is no evidence that nebulization resulted in any degradation of viral activity. This innovative therapeutic approach provides the proof of concept for a single prophylactic delivered intervention and could serve as the basis for a potential protective strategy, based on aerosol-based vector delivery, for patients against the establishment of ARDS.

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Author Disclosure Statement

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References

- Ware LB, and Matthay MA. The acute respiratory distress syndrome. *N Engl J Med* 2000;342:1334–1349.
- Johnson ER, and Matthay MA. Acute lung injury: epidemiology, pathogenesis, and treatment. *J Aerosol Med Pulm Drug Deliv* 2010;23:243–252.
- Eisner MD, Thompson T, Hudson LD, et al. Efficacy of low tidal volume ventilation in patients with different clinical risk factors for acute lung injury and the acute respiratory distress syndrome. *Am J Respir Crit Care Med* 2001;164:231–236.
- Gilmore TD. Introduction to NF- κ B: players, pathways, perspectives. *Oncogene* 2006;25:6680–6684.
- Park GY, and Christman JW. Nuclear factor κ B is a promising therapeutic target in inflammatory lung disease. *Curr Drug Targets* 2006;7:661–668.
- Devaney J, Curley GF, Hayes M, et al. Inhibition of pulmonary nuclear factor κ -B decreases the severity of acute *Escherichia coli* pneumonia but worsens prolonged pneumonia. *Crit Care* 2013;17:R82.
- Ishiyama T, Dharmarajan S, Hayama M, et al. Inhibition of nuclear factor κ B by I κ B superrepressor gene transfer ameliorates ischemia–reperfusion injury after experimental lung transplantation. *J Thorac Cardiovasc Surg* 2005;130:194–201.
- Contreras M, Ansari B, Curley G, et al. Hypercapnic acidosis attenuates ventilation-induced lung injury by a nuclear factor- κ B-dependent mechanism. *Crit Care Med* 2012;40:2622–2630.
- Matsuda N, Hattori Y, Takahashi Y, et al. Therapeutic effect of *in vivo* transfection of transcription factor decoy to NF- κ B on septic lung in mice. *Am J Physiol Lung Cell Mol Physiol* 2004;287:L1248–L1255.
- Matsuda N, Hattori Y, Jesmin S, et al. Nuclear factor- κ B decoy oligodeoxynucleotides prevent acute lung injury in mice with cecal ligation and puncture-induced sepsis. *Mol Pharmacol* 2005;67:1018–1025.
- Rahman A, and Fazal F. Blocking NF- κ B: an inflammatory issue. *Proc Am Thorac Soc* 2011;8:497–503.
- Lavon I, Goldberg I, Amit S, et al. High susceptibility to bacterial infection, but no liver dysfunction, in mice compromised for hepatocyte NF- κ B activation. *Nat Med* 2000;6:573–577.
- Kolb M, Martin G, Medina M, et al. Gene therapy for pulmonary diseases. *Chest* 2006;130:879–884.
- Geiger J, Aneja MK, and Rudolph C. Vectors for pulmonary gene therapy. *Int J Pharm* 2010;390:84–88.
- Adamzik M, Frey UH, Rieman K, et al. Insertion/deletion polymorphism in the promoter of *NFKB1* influences severity but not mortality of acute respiratory distress syndrome. *Intensive Care Med* 2007;33:1199–1203.
- Zhai R, Zhou W, Gong MN, et al. Inhibitor κ B- α haplotype GTC is associated with susceptibility to acute respiratory distress syndrome in Caucasians. *Crit Care Med* 2007;35:893–898.
- Ishii T, Wallace AM, Zhang X, et al. Stability of housekeeping genes in alveolar macrophages from COPD patients. *Eur Respir J* 2006;27:300–306.
- McMillan DH, Bagloli CJ, Thatcher TH, et al. Lung-targeted overexpression of the NF- κ B member RelB inhibits cigarette smoke-induced inflammation. *Am J Pathol* 2011;179:125–133.
- Vogel P, Rivera VR, Pitt ML, et al. Comparison of the pulmonary distribution and efficacy of antibodies given to mice by intratracheal instillation or aerosol inhalation. *Lab Anim Sci* 1996;46:516–523.
- Codrons V, Vanderbist F, Ucakar B, et al. Impact of formulation and methods of pulmonary delivery on absorption of parathyroid hormone (1–34) from rat lungs. *J Pharm Sci* 2004;93:1241–1252.
- Pang Y, Sakagami M, and Byron PR. The pharmacokinetics of pulmonary insulin in the *in vitro* isolated perfused rat lung: implications of metabolism and regional deposition. *Eur J Pharm Sci* 2005;25:369–378.
- Osier M, and Oberdorster G. Intratracheal inhalation vs intratracheal instillation: differences in particle effects. *Fundam Appl Toxicol* 1997;40:220–227.
- Leong BK, Coombs JK, Sabaitis CP, et al. Quantitative morphometric analysis of pulmonary deposition of aerosol particles inhaled via intratracheal nebulization, intratracheal instillation or nose-only inhalation in rats. *J Appl Toxicol* 1998;18:149–160.
- Costello J, Higgins B, Contreras M, et al. Hypercapnic acidosis attenuates shock and lung injury in early and prolonged systemic sepsis. *Crit Care Med* 2009;37:2412–2420.
- Laffey JG, Honan D, Hopkins N, et al. Hypercapnic acidosis attenuates endotoxin-induced acute lung injury. *Am J Respir Crit Care Med* 2004;169:46–56.
- Hassett P, Curley GF, Contreras M, et al. Overexpression of pulmonary extracellular superoxide dismutase attenuates endotoxin-induced acute lung injury. *Intensive Care Med* 2011;37:1680–1687.
- Richards RJ, Davies N, Atkins J, et al. Isolation, biochemical characterization, and culture of lung type II cells of the rat. *Lung* 1987;165:143–158.
- O'Toole D, Hassett P, Contreras M, et al. Hypercapnic acidosis attenuates pulmonary epithelial wound repair by an NF- κ B dependent mechanism. *Thorax* 2009;64:976–982.
- Higgins BD, Costello J, Contreras M, et al. Differential effects of buffered hypercapnia versus hypercapnic acidosis

- on shock and lung injury induced by systemic sepsis. *Anesthesiology* 2009;111:1317–1326.
30. Hopkins N, Cadogan E, Giles S, et al. Chronic airway infection leads to angiogenesis in the pulmonary circulation. *J Appl Physiol* 2001;91:919–928.
 31. Wilson L, Simmons R, and Uster P. Aerosol characteristics of adeno-associated viral vectors produced by an electronic micropump inhaler. Poster Presented at the Cystic Fibrosis Foundation (CFF) Annual Meeting. Anaheim, CA, Oct 16–19, 2003.
 32. MacLoughlin RJ, Higgins BD, Laffey JG, et al. Optimized aerosol delivery to a mechanically ventilated rodent. *J Aerosol Med Pulm Drug Deliv* 2009;22:323–332.
 33. Hayes M, Curley GF, Masterson C, et al. Pulmonary overexpression of inhibitor $\kappa B\alpha$ decreases the severity of ventilator-induced lung injury in a rat model. *Br J Anaesth* 2014;113:1046–1054.
 34. Hak Kim B, Lee K-H, Chung EY, et al. Inhibitory effect of chroman carboxamide on interleukin-6 expression in response to lipopolysaccharide by preventing nuclear factor- κB activation in macrophages. *Eur J Pharm* 2006;543:158–165.
 35. Lin WC, Lin CF, Chen CL, et al. Prediction of outcome in patients with acute respiratory distress syndrome by bronchoalveolar lavage inflammatory mediators. *Exp Biol Med* 2010;235:57–65.
 36. Guan S, Guo C, Zingarelli B, et al. Combined treatment with a CXCL12 analogue and antibiotics improves survival and neutrophil recruitment and function in murine sepsis. *Immunology* 2014. DOI: 10.1111/imm.12382 [Epub ahead of print].
 37. Sheikh N, Tron K, Dudas J, et al. Cytokine-induced neutrophil chemoattractant-1 is released by the noninjured liver in a rat acute-phase model. *Lab Invest* 2006;86:800–814.
 38. Laffey JG, Boylan JF, and Cheng DC. The systemic inflammatory response to cardiac surgery: implications for the anesthesiologist. *Anesthesiology* 2002;97:215–252.
 39. Factor P, Mendez M, Mutlu GM, et al. Acute hyperoxic lung injury does not impede adenoviral-mediated alveolar gene transfer. *Am J Respir Crit Care Med* 2002;165:521–526.
 40. Rooney CP, Denning GM, Davis BP, et al. Bronchoalveolar fluid is not a major hindrance to virus-mediated gene therapy in cystic fibrosis. *J Virol* 2002;76:10437–10443.
 41. Stammberger U, Uduchi AN, Kubisa B, et al. Non-viral gene delivery to atelectatic and ventilated lungs. *Ann Thorac Surg* 2002;73:432–436.
 42. Chen J, Chen Z, Narasaraaju T, et al. Isolation of highly pure alveolar epithelial type I and type II cells from rat lungs. *Lab Invest* 2004;84:727–735.

Address correspondence to:
Prof. Timothy O'Brien
Regenerative Medicine Institute
National University of Ireland
Galway
Ireland

E-mail: timothy.obrien@nuigalway.ie

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