Pulmonary overexpression of inhibitor κBα decreases the severity of ventilator-induced lung injury in a rat model

M. Hayes1,2, G. F. Curley1,3, C. Masterson1,2, M. Contreras1,2, B. Ansari1,2, J. Devaney1,2, D. O’Toole1,2 and J. G. Laffey1,3*

1 Lung Biology Group, Regenerative Medicine Institute and 2 Anaesthesia, School of Medicine, Clinical Sciences Institute, National University of Ireland, Galway, Ireland
3 Department of Anaesthesia, Keenan Research Centre for Biomedical Science, St Michael’s Hospital, University of Toronto, Toronto, Canada
* Corresponding author. E-mail: laffeyj@smh.ca

Background. Activation of the nuclear factor-κB (NF-κB) pathway is central to the pathogenesis of lung injury and inflammation. We determined whether targeted overexpression of inhibitor-κBα (IκBα) in the lung could decrease the severity of ventilator-induced lung injury (VILI).

Methods. Anaesthetized adult male Sprague–Dawley rats were randomly allocated to undergo intratracheal instillation of: (i) vehicle alone (surfactant, n=10); (ii) 1 × 1010 adenovirus-associated virus encoding IκBα (AAV-IκBα, n=10); (iii) 5 × 1010 AAV-IκBα (n=10); and (iv) 1 × 1010 AAV-Null (n=5). This was followed by 4 h of injurious mechanical ventilation. Subsequent experiments examined the effect of IκBα overexpression in animals undergoing ‘protective’ mechanical ventilation.

Results. IκBα overexpression increased survival duration at both the lower [3.8 h (0.4)] and higher [3.6 h (0.7)] doses compared with vehicle [2.7 h (1.0)] or the null transgene [2.2 h (0.8)]. IκBα overexpression reduced the alveolar–arterial oxygen gradient (kPa) at both the lower [53 (21)] and higher [52 (19)] doses compared with vehicle [75 (8.5)] or the null transgene [70 (15)], decreased alveolar neutrophil infiltration, and reduced alveolar concentrations of interleukin (IL)-1β and IL-10. The lower IκBα dose was as effective as the higher dose. IκBα overexpression had no effect in the setting of protective lung ventilation.

Conclusions. Inhibition of pulmonary NF-κB activity by IκBα overexpression reduced the severity of VILI in a rat model.

Keywords: acute respiratory distress syndrome; gene therapy; somatic; ventilation; mechanical; nuclear factor-κB

Accepted for publication: 30 April 2014

The contribution of ventilator-induced lung injury (VILI) to the disease burden of acute respiratory distress syndrome (ARDS) is underscored by the observation that ventilation strategies that reduce lung stretch save lives.1 Mortality from ARDS has decreased through advances in supportive care for sepsis, trauma and pneumonia, and the use of ‘lung protective’ ventilation strategies.2 However, VILI continues to be an important contributor to morbidity and mortality in patients with ARDS, with evidence suggesting that regional alveolar over-distension persists even with conventional lower stretch ventilation strategies.3,4 Directly targeting the injury pathways activated by lung stretch might be a useful alternative approach to reducing VILI.

Nuclear factor-κB (NF-κB), a key transcriptional regulator in the setting of inflammation and injury, has been implicated in the pathogenesis of stretch-induced injury.5 Inhibition of NF-κB reduces injury in pre-clinical ARDS models, including ischaemia–reperfusion,6 endotoxaemia,7 and caecal ligation and puncture-induced sepsis.8 Strategies to non-specifically block NF-κB, such as systemic pharmacological blockade9 or NF-κB antibodies,10 have been demonstrated to reduce VILI severity. However, NF-κB also exerts important cytoprotective effects, promoting cell survival, resolution of inflammation and wound repair,11 and enhancing the host response to bacterial infection.12 Whether targeted inhibition of NF-κB in the lung, minimizing systemic effects, can reduce VILI is not known.

Given these issues, we determined whether inhibition of pulmonary NF-κB activity, via overexpression of the gene encoding inhibitor-κB (IκBα), could modulate the severity of VILI. We hypothesized that IκBα overexpression would attenuate the severity of the lung injury induced by high-stretch ventilation. We further hypothesized that there would be a positive dose–response effect of inhibiting NF-κB, with a greater therapeutic effect at higher IκBα doses.
Methods

A total of 50 specific-pathogen-free adult male Sprague–Dawley rats (350–450 g) were used. All work was approved by the National University of Ireland Galway Research Ethics Committee (Res: 008–08) and conducted under license from the Department of Health, Ireland (Ref: B100-3151).

Preparation of adeno-associated virus

The IxBα- SuperRepressor (IxBα-SR) gene (1566 bp) was ligated into the pAAV-MCS vector (Agilent Technologies Inc., Santa Clara, CA, USA) and plasmid size confirmed by gel electrophoresis and validated by sequencing (Eurofins MWG Operon, Ebersberg, Germany). IxBα-FLAG and adeno-associated virus (AAV) serotype 6 RepCap plasmid DNA were generated and sent to Virapur for AAV production (Virapur, San Diego, CA USA). Viral vector particle titres were determined by quantitative real-time PCR, aliquoted, and stored at −80 °C. As required, an aliquot was thawed and added to 75 μl of the porcine surfactant Curosurf® (120 mg ml⁻¹) (Trinity-Chiesi Pharmaceuticals Limited, Cheadle, UK), and a final instillate volume of 300 μl was made up with phosphate-buffered saline (PBS). For those animals receiving vehicle only, the instillate was 75 μl of Curosurf® mixed with 225 μl of PBS.

Vector instillation

Animals were placed in a clear plastic box, and anaesthetized by inhalation induction with isoflurane (2%) and an intraperitoneal injection of 40 mg kg⁻¹ ketamine (Pfizer, Kent, UK). After confirmation of depth of anaesthesia i.e. lack of pedal withdrawal to a noxious stimulus, laryngoscopy was performed, and the trachea intubated with a size 16 i.v. catheter (BD Insyte®; Becton Dickinson Ltd, Oxford, UK). Animals were randomized to intratracheal instillation of the surfactant–PBS mixture containing: (i) vehicle alone (surfactant); (ii) 1 × 10¹⁰ AAV-IxBα; (iii) 5 × 10¹⁰ AAV-IxBα; and (iv) 1 × 10¹⁰ AAV-Null. After vector instillation, a procedure that lasted for <15 s, animals were extubated, and allowed to recover from anaesthesia and subsequently carefully monitored for signs of discomfort or distress. Butorphanol 0.25–0.4 mg kg⁻¹ was administered subcutaneously for any signs of distress in the recovery period. Relevant aspects of the ARRIVE guidelines were adhered to as appropriate.

Injurious and protective lung ventilation protocols

At 96 h after vector instillation, anaesthesia was induced with intraperitoneal ketamine 80 mg kg⁻¹ (Ketalar, Pfizer, Cork, Ireland) and xylazine 8 mg kg⁻¹ (Xylapan, Vetoquinol, Dublin, Ireland). After demonstration of the absence of a response to paw clamp, the dorsal penile vein and right carotid artery were cannulated, and tracheostomy was performed. Anaesthesia was maintained with i.v. Saffon® (Scherering-Plough, Welwyn Garden City, UK), and after confirmation of depth of anaesthesia, cisatracurium (0.5 mg; Nimbex®, GlaxoSmithKline, Dublin, Ireland) was administered. The lungs were mechanically ventilated (Model 683 Ventilator, Harvard Apparatus, Kent, UK) with an inspired gas mixture of F1O₂ of 0.3, respiratory rate of 90 bpm, tidal volume of 6 ml kg⁻¹, and PEEP of 2.5 cm H₂O, with recruitment manoeuvres applied every 15 min. Peak inspiratory pressures were 7–8 cm H₂O at baseline.

Body temperature was maintained at 36–37.5 °C, and systemic arterial pressure, peak airway pressure, and temperature were measured continuously. Arterial blood gas sampling was performed, and static inflation lung compliance was measured at baseline and at hourly intervals, as previously described. The following baseline values were required for progression to the ventilation protocol: Pao₂ of >16 kPa, HCO₃⁻ of >20 mM, and temperature of 36.0–37.5 °C.

Injurious lung ventilation series

Mechanical ventilation settings were adjusted to: peak inspiratory pressure (PImp) 30 cm H₂O; respiratory rate 18 bpm; PEEP 0 cm H₂O, and the animals were ventilated for 4 h. These ventilator settings for VILI were shown to produce severe lung injury. A PImp of 30 cm H₂O produced a tidal volume of ~40 ml kg⁻¹ in uninjured animals.

Protective lung ventilation

Mechanical ventilation was adjusted to the volume-controlled mode with the following settings: tidal volume 6 ml kg⁻¹, respiratory rate of 80 bpm, and PEEP of 2.5 cm H₂O for a 4 h period.

Experiment termination criteria

The experimental protocol was terminated before 4 h if mean arterial pressure decreased below 30 mm Hg for >15 min or for hypoxaemia (arterial PO₂ <6 kPa), indicating severe injury. In these animals, physiological data from the previous hourly assessment were taken as final data. Samples were obtained from all animals for all assays and for the histological analysis. This approach ensured that we had physiological data and samples for analysis from every animal studied.

Post-mortem analyses

At the end of the protocols, animals were killed by exsanguination under anaesthesia, and plasma was snap frozen. The heart–lung block was dissected from the thorax, bronchoalveolar lavage (BAL) was performed, and BAL fluid differential leucocyte counts and lung bacterial colony counts were completed. BAL fluid was centrifuged, and the supernatant was snap frozen and stored at −80 °C. BAL concentrations of tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6, IL-10, and cytokine-induced neutrophil chemoattractant 1 (CINC-1) were determined using ELISA (R&D Systems, Abingdon, UK) and BAL protein concentrations measured (Micro BCA™ Protein assay kit, Pierce, Rockford, IL, USA).

Assessment of histological injury

The left lung was isolated and fixed, and the extent of histological lung damage determined using quantitative stereological techniques. Briefly, after flushing of pulmonary circulation with normal saline, and perfusion with...
parafomaldehyde (6% wt vol⁻¹, in PBS), the left lung was inflated with paraformaldehyde, and the pulmonary artery and trachea ligated. After fixation, this lung was cut into 4 mm thick slices perpendicular to the vertical axis, these slices were embedded in paraffin and 7 μm sections from each slice mounted on slides and stained with haematoxylin and eosin. Images were randomly obtained at 10 times magnification from each slide, and the volume fractions of intra-acinar tissue and intra-acinar airspace determined by a blinded assessor using a point counting grid.¹⁵

Assessment of transgene expression
Lung homogenate IκBα-transgene expression was determined by qRT–PCR. Briefly, RNA was extracted from lung tissue, cDNA was synthesized, and quantitative PCR performed for IκBα-FLAG, normalized against a lamin control (Supplementary Appendix Table SA). IκBα protein was assessed using western blotting and ELISA. For western blot IκBα-FLAG analysis, total cell protein was extracted, protein concentration was determined, and samples were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to nitrocellulose.¹² Primary anti-human FLAG monoclonal antibody (Sigma-Aldrich, MO, USA) was used, with secondary antibody conjugated to horseradish peroxydase (Cell Signaling Technology, Danvers, MA, USA), and the membrane incubated with a chemiluminescent substrate (SuperSignal West Pico; Pierce). Lung homogenate IκBα concentrations were measured in all samples using ELISA (R&D Systems).

The effect of IκBα overexpression on activation of the NF-κB pathway was assessed by measurement of nuclear accumulation of the activated P65 subunit of NF-κB.⁵ Nuclear extracts were performed on homogenized rat lung tissue using a Nuclear and Cytoplasmic Protein Extraction Reaction Kit (Fisher Scientific Ireland, Dublin, Ireland), and NF-κB (p65) measured using an NF-κB Transcription Factor Assay Kit (Cayman Chemical Company, Ann Arbor, MI, USA).

Data presentation and analysis
Continuous responsive variables are summarized using mean (so) and median (inter-quartile range, IQR) as appropriate. The proportion of animals surviving was analysed using the χ² test. All other data were analysed by one-way analysis of variance (ANOVA), followed by Dunnett’s test, or by the Kruskal–Wallis followed by Dunn’s test, with the vehicle group used as the reference group for all comparisons. Assumptions underlying all models were checked using suitable residual plots. A P-value of <0.05 was considered statistically significant.

Results
Three animals were excluded before induction of injury due to failure to achieve baseline stability. In the VILI series, 35 animals were randomized to receive: (i) vehicle alone (surfactant, n=10); (ii) 1 x 10¹⁰ AAV-IκBα (n=10); (iii) 5 x 10¹⁰ AAV-IκBα (n=10); and (iv) 1 x 10¹⁰ AAV-Null (n=5); and all animals subsequently underwent injurious ventilation. In the protective ventilation series, 12 animals were randomized to receive: (i) vehicle alone; (ii) 1 x 10¹⁰ AAV-IκBα; (iii) 5 x 10¹⁰ AAV-IκBα; and (iv) 1 x 10¹⁰ AAV-Null (n=3 per group).

Ventilator-induced lung injury
Baseline characteristics
There were no differences between groups at baseline (Table 1). High-stretch ventilation induced a severe lung injury and increased animal mortality.

IκBα transfection
Pulmonary instillation of AAV-IκBα produced a dose-dependent increase in lung IκBα gene transcription (Fig. 1a) and protein production (Fig. 1a and c). VILI significantly reduced intracellular IκBα concentrations compared with protective ventilation and this reduction was ablated by overexpression of IκBα at both doses (Fig. 1c). IκBα overexpression decreased VILI-induced NF-κB activation, as measured by nuclear accumulation of the activated P65 subunit of NF-κB (Fig. 1d).

Animal survival and haemodynamic stability
IκBα overexpression enhanced animal survival (Table 2), and prolonged animal survival (Table 2) compared with vehicle and null transgene groups. There was no difference in survival between the two doses of IκBα. IκBα overexpression enhanced haemodynamic stability, resulting in greater preservation of mean arterial pressure compared with vehicle or null transgene (Table 2).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Vehicle</th>
<th>1 x 10¹⁰ IκBα</th>
<th>5 x 10¹⁰ IκBα</th>
<th>1 x 10¹⁰ Null</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of animals</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>Animal weight (g)</td>
<td>410 (54)</td>
<td>418 (29)</td>
<td>415 (30)</td>
<td>372 (16)</td>
</tr>
<tr>
<td>Arterial pH</td>
<td>7.42 (0.05)</td>
<td>7.42 (0.04)</td>
<td>7.42 (0.05)</td>
<td>7.45 (0.03)</td>
</tr>
<tr>
<td>Arterial Pco₂ (kPa)</td>
<td>4.1 (0.6)</td>
<td>4.6 (0.8)</td>
<td>4.0 (0.5)</td>
<td>3.6 (0.3)</td>
</tr>
<tr>
<td>Arterial P O₂ (kPa)</td>
<td>18.9 (1.3)</td>
<td>18.5 (0.9)</td>
<td>18.8 (0.5)</td>
<td>19.8 (0.6)</td>
</tr>
<tr>
<td>Static compliance (ml mm Hg⁻¹)</td>
<td>0.89 (0.10)</td>
<td>0.96 (0.16)</td>
<td>0.90 (0.12)</td>
<td>0.79 (0.13)</td>
</tr>
<tr>
<td>Arterial bicarbonate (mM)</td>
<td>22.2 (4.6)</td>
<td>24.1 (2.4)</td>
<td>21.8 (1.6)</td>
<td>23.8 (3.2)</td>
</tr>
<tr>
<td>Arterial lactate (mM)</td>
<td>2.3 (1.4)</td>
<td>3.5 (2.0)</td>
<td>2.9 (1.0)</td>
<td>3.4 (1.8)</td>
</tr>
</tbody>
</table>

Table 1 Baseline conditions in animals subjected to VILI. Data are expressed as mean (so)
Fig 1  Overexpression of IkBa reduced NF-kB activity. Delivery of the AAV encoding IkBa resulted in a dose-dependent increase in lung homogenate IkBa FLAG mRNA expression (a) and IkBa protein concentrations, as demonstrated by the representative western blot (b), and by measurement of IkBa in lung homogenates from all animals in each group by ELISA (c). IkBa decreased VILI-induced nuclear accumulation of the activated P65 subunit of NF-kB (d). Vehicle, animals that received intratracheal vehicle alone; IkBa 1×10^{10}, animals that received 1×10^{10} AAV6 particles encoding IkBa; IkBa 5×10^{10}, animals that received 5×10^{10} AAV6 particles encoding IkBa; Null, animals that received 1×10^{10} AAV6 particles encoding a null transgene. ELISA, enzyme-linked immunosorbent assay. *Significantly different from the vehicle group ($P<0.05$, ANOVA).

Table 2  Overexpression of IkBa decreases the severity of VILI. Data are expressed as mean (so) or median (IQR). Final data collected upon completion of the experimental protocol. *Significantly different from the vehicle group

<table>
<thead>
<tr>
<th>Variable</th>
<th>Vehicle</th>
<th>1×10^{10} IkBa</th>
<th>5×10^{10} IkBa</th>
<th>1×10^{10} Null</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal survival (number, %)</td>
<td>4 (40)</td>
<td>8 (80)*</td>
<td>7 (70)*</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Duration of animal survival (h)</td>
<td>2.7 (1.0)</td>
<td>3.8 (0.4)*</td>
<td>3.6 (0.7)*</td>
<td>2.2 (0.8)</td>
</tr>
<tr>
<td>Final arterial pH</td>
<td>7.25 (0.12)</td>
<td>7.25 (0.11)</td>
<td>7.21 (0.07)</td>
<td>7.15 (0.06)</td>
</tr>
<tr>
<td>Final arterial CO₂ tension (kPa)</td>
<td>5.4 (0.9)</td>
<td>6.3 (1.6)</td>
<td>6.4 (1.6)</td>
<td>7.0 (2.7)</td>
</tr>
<tr>
<td>Final arterial O₂ tension (kPa; FIO₂ = 1.0)</td>
<td>10.8 (10.5, 12.8)</td>
<td>24.9* (12.6, 59.2)</td>
<td>39.1 (12.6, 48.9)</td>
<td>11.2 (7.8, 19.4)</td>
</tr>
<tr>
<td>Final serum bicarbonate (mM)</td>
<td>17.5 (3.5)</td>
<td>19.0 (5.0)</td>
<td>17.6 (3.4)</td>
<td>15.5 (2.3)</td>
</tr>
<tr>
<td>Final lactate (mM)</td>
<td>4.9 (1.4)</td>
<td>4.5 (1.8)</td>
<td>4.2 (3.1)</td>
<td>5.9 (2.3)</td>
</tr>
<tr>
<td>Final mean arterial pressure (mm Hg)</td>
<td>69.5 (14.2)</td>
<td>97.8 (9.1)*</td>
<td>97.5 (10.1)*</td>
<td>75.2 (7.1)</td>
</tr>
</tbody>
</table>
IkB\(_\alpha\) decreased VILI severity
IkB\(_\alpha\) reduced the decrement in arterial P\(_{O2}\) after high-stretch ventilation, resulting in an increased end-protocol arterial P\(_{O2}\) compared with vehicle or null transgene (Table 2). IkB\(_\alpha\) attenuated the increase in alveolar–arterial oxygen gradient induced by VILI (Fig. 2A). IkB\(_\alpha\) overexpression reduced the decrement in lung static compliance (Fig. 2B), and decreased lung permeability as assessed by protein leak into BAL fluid (Fig. 2C) at the higher but not the lower vector dose.

IkB\(_\alpha\) decreased pulmonary inflammation
IkB\(_\alpha\) overexpression suppressed alveolar cellular infiltration, reducing total cell (Table 3) and neutrophil counts (Fig. 2D). IkB\(_\alpha\) overexpression decreased alveolar concentrations of IL-1\(\beta\) and IL-10, but not alveolar TNF-\(\alpha\), IL-6, and CINC-1 (Table 3).

IkB\(_\alpha\) reduced histological injury
IkB\(_\alpha\) reduced VILI-induced histological injury, decreasing alveolar tissue fraction and increasing alveolar airspace fraction (Fig. 3A) compared with vehicle and null transgene. Representative tissue sections from each group demonstrate this protective effect of IkB\(_\alpha\) overexpression (Fig. 3B and D).

Protective lung ventilation
There was no effect of IkB\(_\alpha\) overexpression on indices of physiological function or on the inflammatory response in animals that received non-injurious ventilation (Table 4).

Discussion
Despite advances in mechanical ventilation, stretch injury remains a significant contributor to morbidity and mortality in ARDS. Mechanical ventilation can increase the risk of ARDS.
in critically ill patients that do not already have ARDS,18 and it can worsen pre-existing ARDS.5 Even when protective ventilation strategies are used, the heterogeneous nature of ARDS means that regional lung areas can still be subject to overdistension.3 4 An alternative approach to minimize the impact of VILI is to develop strategies to directly attenuate the response to high lung stretch. Activation of the NF-κB signalling pathway appears to be a key mechanism by which mechanical ventilation-induced cell stretch results in cellular activation, inflammation, and injury.19 We found that pulmonary overexpression of IκBα attenuated VILI, enhanced animal survival, and decreased lung injury. These findings provide novel insights regarding the role of NF-κB in VILI, and suggest that inhibiting NF-κB could have therapeutic potential in the setting of injurious mechanical ventilation.

**NF-κB and acute lung injury**

NF-κB is normally sequestered in the cytoplasm of non-stimulated cells bound to IκB inhibitory proteins. Upon activation by cell stretch, the cytosolic inhibitor IκB complex is inactivated and dissociates, allowing NF-κB to translocate to the nucleus to initiate gene transcription.5 Inhibition of NF-κB is protective in several models of non-septic ARDS, including pulmonary6 and systemic reperfusion, and endotoxaemia.7 NF-κB decoy oligodeoxynucleotides reduce lung injury in mice in the early phases of caecal ligation and puncture-induced sepsis.8 Selective inhibition of vascular endothelial NF-κB activity in endotoxaemic transgenic mice reduced lung inflammation and increased survival, but did not alter bacterial clearance, in septic mice.20 Activation of NF-κB has been implicated in the pathogenesis of stretch-induced injury,20 21 and strategies to block NF-κB, such as systemic pharmacological block3 or NF-κB antibodies,10 reduce VILI severity.

Of concern, non-selective inhibition of NF-κB can constitute a double-edged sword, with potentially deleterious effects. NF-κB promotes cell survival, resolution of inflammation, and enhances repair after injury. Inhibition of NF-κB retards pulmonary17 and intestinal21 epithelial wound healing, of direct relevance to the pathogenesis of VILI. Inhibition of NF-κB reversed the protective effects of hydrogen inhalation on epithelial cell survival and lung function in an in vivo murine VILI model.22 Of particular concern is the risk that NF-κB inhibition can worsen pneumonia-induced ARDS, where immune competence is essential for eradication of the infectious agent. For example, pulmonary overexpression of IκBα decreased the severity of acute *Escherichia coli* pneumonia but worsened prolonged pneumonia.14 Strategies to inhibit NF-κB more selectively may maximize therapeutic benefit while reducing the potential for adverse effects.

**Selective NF-κB inhibition decreases VILI**

IκBα overexpression attenuated the stretch–induced depletion of cellular IκBα, and reduced nuclear translocation of the activated NF-κB heterodimer. IκBα overexpression reduced lung
injury severity as evidenced by an attenuation of the decrement in systemic oxygenation, reduction in lung permeability, reduction in the extent of histological lung injury, and reduced alveolar neutrophil infiltration. Both doses of IκBα attenuated the lung injury, a finding likely explained by the fact that both doses increased IκBα concentrations to levels greater than that seen in animals that received protective ventilation. Our findings are consistent with that of You and colleagues who found that pre-treatment with the pharmacological NF-κB inhibitor pyrrolidine dithiocarbamate attenuated injury associated with one-lung ventilation in rabbit. In contrast, another study of NF-κB inhibition in VILI did not demonstrate benefit; however, these investigators used a rodent high-stretch ventilation protocol of just 60 min, which was not associated with a decrease in oxygenation or structural evidence of lung injury.

**NF-κB inhibition: mechanisms of action**

Activation of NF-κB plays a key role in lung neutrophil recruitment in pre-clinical VILI models. IκBα overexpression suppressed alveolar neutrophil infiltration and reduced injury severity, suggesting that this might be a key protective mechanism. In the clinical setting, the finding that NF-κB activation occurs in neutrophils with VILI while decreased neutrophil NF-κB activation is associated with a less severe clinical course, emphasizing the therapeutic potential of NF-κB inhibition in patients with ARDS.

IκBα overexpression produced a modest alteration in the cytokine profile induced by VILI. While IκBα decreased alveolar concentrations of IL-1β and IL-10, it did not significantly reduce alveolar TNF-α, IL-6, or CINC-1. The effect of IκBα on alveolar IL-10 concentrations might reflect inhibition of macrophage IL-10 production, which is in part NF-κB mediated. Our findings, while similar qualitatively, are less pronounced compared with these reported in other studies, where inhibition of NF-κB potently suppressed lung pro-inflammatory cytokine release in the isolated lung. This might be explained, at least in part, by the non-recirculating nature of lung perfusion in the isolated lung model which likely results in mediators not being metabolized. Secondly, the degree of lung stretch applied in isolated perfused models tends to be greater than that possible in *in vivo* models.

**Limitations**

A number of limitations need to be considered. First, high airway pressures, beyond those seen clinically, were used to cause a severe stretch-induced injury in these studies. This model is intended to reflect the gross over-distension that regions of the lungs of patients with ARDS experience. Ventilation with a peak inspiratory pressure as high as 45 cm H2O is commonly used in pre-clinical studies to induce VILI. In terms of clinical relevance, there is strong evidence that regional lung areas are subject to gross over-distension in ARDS patients. A second limitation is the fact that the model chosen was an isolated high-stretch model. While high tidal volume ventilation can directly cause ARDS, VILI is generally seen in the context of other disease processes. However, we wished to specifically examine the potential for NF-κB inhibition to attenuate VILI. Thirdly, where animals met the prespecified termination criteria with regard to the severity of injury, final data were collected and the experiment was terminated. As a result, animals that received the IκBα transgene survived longer, and therefore underwent injurious ventilation for longer periods, potentially reducing the effect size. Fourthly, there are limitations to the gene overexpression approach. Expression of AAV-delivered transgenes is relatively slow, which requires that animals be pre-treated with the AAV vector. Our aim was to demonstrate proof-of-principle that intrapulmonary gene therapy with the IκBα gene could reduce VILI severity. For clinical translation, this would have relevance for clinical situations where the insult is predictable, for example, in patients undergoing one-lung anaesthesia for

---

**Table 4** Overexpression of IκBα does not alter lung function in animals subjected to protective lung ventilation. Data are expressed as mean (so) or median (IQR). Final data collected upon completion of the experimental protocol. BAL, bronchoalveolar lavage

<table>
<thead>
<tr>
<th>Variable</th>
<th>Vehicle</th>
<th>1 × 10^10 IκBα</th>
<th>5 × 10^10 IκBα</th>
<th>1 × 10^10 Null</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal survival (number, %)</td>
<td>3 (100)</td>
<td>3 (100)</td>
<td>3 (100)</td>
<td>3 (100)</td>
</tr>
<tr>
<td>Duration of animal survival (h)</td>
<td>4 (0)</td>
<td>4 (0)</td>
<td>4 (0)</td>
<td>4 (0)</td>
</tr>
<tr>
<td>Final arterial pH</td>
<td>7.36 (0.01)</td>
<td>7.39 (0.02)</td>
<td>7.38 (0.02)</td>
<td>7.36 (0.11)</td>
</tr>
<tr>
<td>Final arterial CO₂ tension (kPa)</td>
<td>5.0 (0.3)</td>
<td>4.1 (0.3)</td>
<td>4.6 (0.6)</td>
<td>3.8 (0.5)</td>
</tr>
<tr>
<td>Final arterial O₂ tension (kPa; FV0 = 1.0)</td>
<td>69.6 (5.0)</td>
<td>67.7 (5.5)</td>
<td>69.8 (1.3)</td>
<td>65.3 (4.9)</td>
</tr>
<tr>
<td>Final serum bicarbonate (mM)</td>
<td>20.3 (1.7)</td>
<td>20.5 (1.3)</td>
<td>21.2 (1.7)</td>
<td>18.0 (2.3)</td>
</tr>
<tr>
<td>Final lactate (mM)</td>
<td>3.8 (0.5)</td>
<td>4.3 (0.4)</td>
<td>4.0 (0.7)</td>
<td>5.6 (1.2)</td>
</tr>
<tr>
<td>Static compliance (ml mm Hg⁻¹)</td>
<td>0.84 (0.03)</td>
<td>0.85 (0.05)</td>
<td>0.91 (0.08)</td>
<td>0.91 (0.07)</td>
</tr>
<tr>
<td>Final mean arterial pressure (mm Hg)</td>
<td>93.0 (5.6)</td>
<td>87.0 (7.6)</td>
<td>84.7 (6.7)</td>
<td>91.0 (3.5)</td>
</tr>
<tr>
<td>Wet-dry weight ratio</td>
<td>4.26 (0.90)</td>
<td>5.39 (0.61)</td>
<td>4.76 (0.74)</td>
<td>4.41 (0.52)</td>
</tr>
<tr>
<td>BAL neutrophils (× 10⁴ ml⁻¹)</td>
<td>96.5 (29.7)</td>
<td>53.2 (10.1)</td>
<td>62.0 (17.6)</td>
<td>50.1 (10.7)</td>
</tr>
<tr>
<td>BAL neutrophils (%)</td>
<td>5 (4.3)</td>
<td>4.7 (3.2)</td>
<td>5.2 (3.4)</td>
<td>2.0 (1.0)</td>
</tr>
<tr>
<td>BAL neutrophils (× 10³ ml⁻¹)</td>
<td>4.8 (4.4)</td>
<td>2.6 (1.9)</td>
<td>2.8 (1.0)</td>
<td>1.0 (0.4)</td>
</tr>
</tbody>
</table>
thoracic surgery. The low toxicity of the AAV vector makes it a good choice for a ‘preventive’ therapy. We did not find any evidence that the AAV vector produced injury in these studies; nevertheless, the potential exists for these vectors to produce adverse effects. Lastly, the numbers of animals used in the studies of protective ventilation, while low, were sufficient to demonstrate an absence of direct effect of IκBα overexpression or vector administration.

Conclusions

Pulmonary overexpression of IκBα enhanced animal survival, decreasing the severity of the decrement in lung function, while also modulating the inflammatory response to high-stretch ventilation. These findings provide novel insights regarding the effects of NF-κB in VILI, and suggest that inhibiting pulmonary NF-κB could have therapeutic potential in the setting of injurious mechanical ventilation.

Supplementary material

Supplementary material is available at British Journal of Anaesthesia online.

Authors’ contributions

J.G.L., M.H., and D.O.T. designed the project. M.H., G.F.C., and C.M. performed the experiments. D.O.T., M.C., and J.D. prepared the AAV vectors. B.A. carried out cytokine analysis. J.G.L. and M.H. analysed the data. M.H. and J.G.L. drafted the manuscript and are guarantors of the paper.

Declaration of interest

None declared.

Funding

This study was funded by the Health Research Board, Ireland (RP-2008-193 and HRA _POR-2011-1061), and by the European Research Council (ERC-2007-StG 207777). J.D. is a postdoctoral fellow with the Health Research Board, Ireland. D.O.T. is a Research Fellow with the European Research Council.

References

2 Zambon M, Vincent JL. Mortality rates for patients with acute lung injury/ARDS have decreased over time. Chest 2008; 133: 1120–7


28 Held HD, Boettcher S, Hamann L, Uhlig S. Ventilation-induced chemokine and cytokine release is associated with activation of nuclear factor-kappaB and is blocked by steroids. *Am J Respir Crit Care Med* 2001; **163**: 711–6


*Handling editor: H. C. Hemmings*