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ORIGINAL ARTICLE

β1-Na^+,K^+-ATPase gene therapy upregulates tight junctions to rescue lipopolysaccharide-induced acute lung injury

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Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are associated with diverse disorders and characterized by disruption of the alveolar-capillary barrier, leakage of edema fluid into the lung, and substantial inflammation leading to acute respiratory failure. Gene therapy is a potentially powerful approach to treat ALI/ARDS through repair of alveolar epithelial function. Herein, we show that delivery of a plasmid expressing β1-subunit of the Na^+,K^+-ATPase (β1-Na^+,K^+-ATPase) alone or in combination with epithelial sodium channel (ENaC) α1-subunit using electroporation not only protected from subsequent lipopolysaccharide (LPS)-mediated lung injury, but also treated injured lungs. However, transfer of α1-subunit of ENaC (α1-ENaC) alone only provided protection benefit rather than treatment benefit although alveolar fluid clearance had been remarkably enhanced. Gene transfer of β1-Na^+,K^+-ATPase, but not α1-ENaC, not only enhanced expression of tight junction protein zona occludins-1 (ZO-1) and occludin both in cultured cells and in mouse lungs, but also reduced pre-existing increase of lung permeability *in vivo*. These results demonstrate that gene transfer of β1-Na^+,K^+-ATPase upregulates tight junction formation and therefore treats lungs with existing injury, whereas delivery of α1-ENaC only maintains pre-existing tight junction but not for generation. This indicates that the restoration of epithelial/endothelial barrier function may provide better treatment of ALI/ARDS.

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INTRODUCTION

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) have important roles for causing acute respiratory failure. Although decreases in mortality have been achieved over the past 15 years, ALI/ARDS continues to have mortality rates between 25 and 40% in the United States, leading up to 74 500 deaths and 3.6 million hospital days every year.[1] ALI/ARDS is initially characterized by disruption of the alveolar-capillary interface and leakage of protein-rich edema fluid into the interstitium and alveolar space, followed by extensive release of inflammatory cytokines and chemokines and neutrophil infiltration.[2] This causes reduced gas exchange, systemic inflammation and multi-organ failure. As such, there is no single gene or mechanism that causes ALI/ARDS, nor is there a single gene that can treat the disease.

It has been previously thought that the best way to control and treat ALI/ARDS is to increase alveolar fluid clearance (AFC). Consequently, researchers have focused on overexpressing or upregulating ion channels in the lung, including the Na^+,K^+-ATPase, the epithelial sodium channel (ENaC) and the CFTR (cystic fibrosis transmembrane regulator).[3–7] In the case of the Na^+,K^+-ATPase, direct overexpression or indirect activation by gene transfer of Na^+,K^+-ATPase subunits or the β_2-adrenergic receptor or CFTR genes, respectively, results in the increased movement of Na^+ ions from the alveolar space into the interstitium and the consequent movement of water to follow.[8] Using electroporation techniques developed in our laboratory to efficiently deliver DNA to the lung without injury,[9–12] we have shown that, indeed, AFC rates can be increased almost twofold after electroporation-mediated transfer of β1-subunit of the Na^+,K^+-ATPase (β1-Na^+,K^+-ATPase) to the lungs of healthy rats.[13] We have also shown that transfer of genes encoding the α-subunit and/or β-subunit of the Na^+,K^+-ATPase using electroporation protects from subsequent endotoxin-induced lung injury in mice, and treats previously injured lungs by upregulating AFC.[14] Although these results are promising, greater efficiency is needed along with clear mechanistic understanding of the approach.

One approach to achieve this goal would be to increase the overall vectoral flow of Na^+ across the alveolar epithelium. Since the Na^+,K^+-ATPase resides in the basolateral membrane of the alveolar epithelium and transports Na^+ from the inside of the cell out to the interstitium, we reasoned that if we could also overexpress a Na^+ channel on the apical side of the cell, we may be able to increase the vectoral flow of Na^+ ions and hence water across from the airspace into the cell and then out into the interstitium and pulmonary circulation. We hypothesized that by taking this approach we may be able to better treat ALI. The ENaC is one of the major Na^+ transporting channels in the apical membrane of alveolar epithelial cells and is made up of three highly homologous subunits, α, β and γ. It has been shown that α-ENaC knockout mice die shortly after birth, primarily from failure to clear their lungs of fluid.[15] Further, overexpression of ENaC subunits in transgenic mice leads to reduced levels of airway surface liquid and a cystic fibrosis-like phenotype. We reasoned, therefore, that its overexpression could improve AFC *in vivo*.

In this study, we explored whether electroporation-mediated transfer of plasmids expressing the Na^+,K^+-ATPase β1-subunit alone or in combination with α1-subunit of ENaC (α1-ENaC) results in better treatment of lungs with pre-existing injury. Our results demonstrated that rates of AFC were not directly correlated with outcome in an injury model. Interestingly, we found that gene transfer of the Na^+,K^+-ATPase β1-subunit, but not α1-ENaC, upregulates tight junction formation to improve alveolar epithelial/endothelial barrier function and thus affects lung permeability, resulting in treatment benefit.

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RESULTS

Overexpression of the ENaC α1-subunit or Na+,K+-ATPase β1-subunit enhances AFC in mouse

Gene transfer of the α1-ENaC or the β1-Na+,K+-ATPase to mouse lungs by electroporation increased mRNA levels in mouse lungs twofold to threefold higher over those in naive lungs whereas transfer of the control pcDNA3 had no effect on their expression (Figures 1a and b). Similar increases in expression at the protein level were also seen (Figures 1c and d). Our previous studies have reported that electroporation-mediated gene transfer of β1-Na+, K+ATPase increased AFC by 74% in the isolated rat lungs.[13] To determine whether AFC can be further increased by expressing transporters at both cell surfaces, plasmids encoding α1-ENaC or β1-Na+,K+-ATPase were delivered to mouse lungs by aspiration and electroporation either individually or in combination. Two days later, AFC was measured in live mice using a modification of the mechanically ventilated intact lung model, which maintains ventilation, oxygenation and serum pH.[3,16] Electroporation of pcDNA3, an empty plasmid, did not increase AFC, compared with naive mice. By contrast, electroporation of α1-ENaC alone or in combination with β1-Na+,K+-ATPase into mouse lungs significantly increased AFC by 95.9 or 119.4%, respectively (Figure 2a). Furthermore, gene transfer of β1-Na+,K+-ATPase alone increased AFC by 43.2%, slightly less than that seen by us previously.[13] Similarly, the inclusion of procaterol (10^{-8} mol l^{-1}), the alveolar epithelial β_2-adrenergic receptor-specific agonist, in the instillation solution also increased AFC by 124.4%. Importantly, the increases in AFC were correlated with expression of α1-ENaC (Figure 2b, $R^2 = 0.7824$) or β1-Na+,K+-ATPase (Figure 2c, $R^2 = 0.5774$) in mouse lungs. These results suggest that electroporation can effectively deliver plasmids expressing the α1-ENaC subunit or the β1-Na+, K+-ATPase subunit, resulting in increased AFC, which is correlated with their expression in mouse lungs.

Gene transfer of the ENaC α1-subunit or Na+,K+-ATPase β1-subunit protects from lipopolysaccharide-induced lung injury

To determine whether co-expression of these sodium transporters provided greater protection from endotoxin-induced lung injury than we have previously reported with the Na+,K+-ATPase alone,[14] individual α1-ENaC and β1-Na+,K+-ATPase expressing plasmids, or a combination of the two, were transferred to mouse lungs by electroporation. After 24 h, lipopolysaccharide (LPS) was delivered intratracheally to induce lung injury. One day later, protection or injury was assessed by measurement of wet-to-dry ratios, histological analysis and protein content, cellularity and primary neutrophil (PMN) infiltration of bronchoalveolar lavage (BAL) fluid (Figure 3).

The wet-to-dry ratio following administration of LPS was increased with 4.91 ± 0.04, compared with 4.38 ± 0.03 in naive lungs. Lungs electroporated individually with α1-ENaC or β1-Na+, K+-ATPase plasmids, or in combination showed markedly less pulmonary edema, compared with those electroporated with the

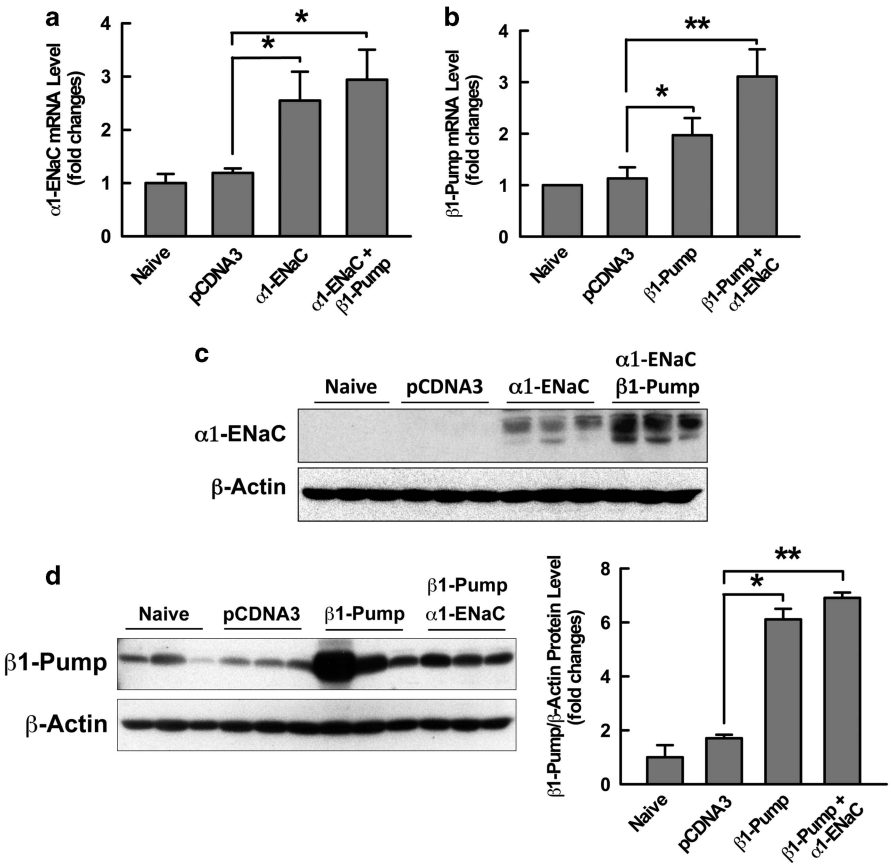

Figure 1. Transgenes are expressed in mouse lungs. Empty plasmid (pcDNA3) or plasmids expressing DDK-tagged α1-ENaC or GFP-tagged β1-Na+,K+-ATPase subunit, either alone or in combination were delivered to the lungs of mice by electroporation. Two days later, expression of DDK-tagged α1-ENaC (~110 kdal; **a**, **c**) and GFP-tagged β1-Na+,K+-ATPase subunits (~55 kdal; **b**, **d**) was assessed in mouse lungs by quantitative PCR and western blots and densitometry relative to β-actin (~43 kdal), respectively. Separate lobes from the lungs from each mouse were used for mRNA and protein isolation. An anti-DDK antibody was used in (**c**), accounting for the lack of signal in naive and pcDNA3 samples. We used six mice per condition in these experiments. Statistical analysis was done by one-way ANOVA (mean ± s.e.m.; $n = 6$). $^*P < 0.05$ or $^{**}P < 0.01$ compared with pCDNA3.

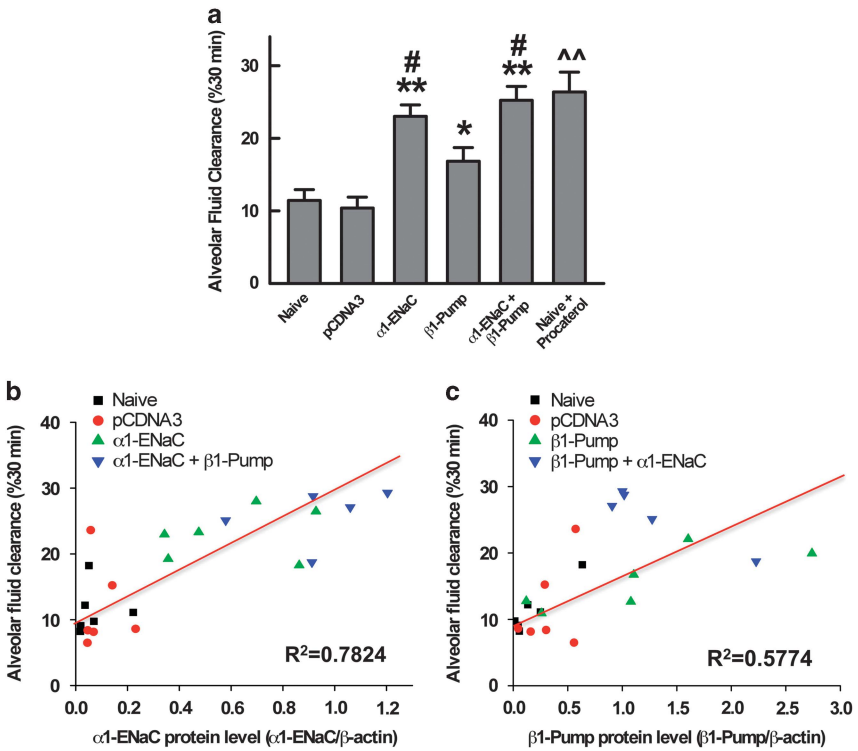

Figure 2. Electroporation-mediated gene transfer of α1-ENaC and β1-Na+,K+-ATPase subunits increases AFC, which is correlated with their expression in mouse lungs. In all, 100 μg of plasmid in 50 μl was administered to mouse lungs by aspiration and electroporation. Two days later, AFC was measured in living mice and is shown as percentage of total instilled volume cleared in 30 min (**a**). Statistical analysis was done by one-way ANOVA (mean ± s.e.m., $n = 6$). $^{\#}P < 0.05$ or $^{\wedge\wedge}P < 0.01$ compared with Naive and $^{*}P < 0.05$ or $^{**}P < 0.01$ compared with pcDNA3. Relationship and correlation analysis of α1-ENaC (**b**) or β1-Na+,K+-ATPase (**c**) protein expression and AFC are shown.

control pcDNA3 plasmid (Figure 3a). Histological evaluation revealed mild edema in mice that received plasmids expressing α1-ENaC or β1-Na+,K+-ATPase alone, or in combination, whereas there was severe edema in mice that received the control pcDNA3 plasmid or LPS alone (Figure 3b). To determine whether electroporation-mediated gene transfer of α1-ENaC or β1-Na+,K+-ATPase, plasmids either individually or in combination reduced neutrophilic inflammation, cells present in the bronchoalveolar space were assessed in BAL fluid by cytospin analysis. Total protein and cell count in BAL fluid were assessed and increased tenfold due to an increase in the number of neutrophils after LPS exposure compared with naive mice. As can be seen, transfer of pcDNA3 before LPS instillation resulted in no change in protein content or cellularity of BAL fluid compared with LPS only mice. By contrast, transfer of α1-ENaC or β1-Na+,K+-ATPase either individually or in combination significantly decreased the protein level and cellularity in BAL fluid (Figures 3c and e). Importantly, these gene transfers markedly reduced PMNs to 19.73 ± 2.70, 22.77 ± 5.64 or 16.47 ± 3.30 ($\times 10^5$/ml), respectively, compared with 46.55 ± 6.09 ($\times 10^5$/ml) of the control pcDNA3 (Figure 3d). Respective cytospin images of these observation are shown in Figure 3f. Collectively, these results demonstrate that electroporation-mediated gene transfer of individual α1-ENaC or β1-Na+,K+-ATPase or in combination can protect against subsequent LPS-induced ALI by reducing pulmonary edema and neutrophil influx. However, in all cases, transfer of both α1-ENaC and β1-Na+,K+-ATPase plasmids together showed no benefit over α1-ENaC alone.

Gene transfer of the Na+,K+-ATPase β1-subunit, but not the ENaC α1-subunit, treats lungs with existing LPS-induced lung injury

To determine whether the same approach could be used to rescue previously injured lungs, target genes were delivered into injured lungs by electroporation. Mouse lungs were injured by intratracheal administration of LPS (5 mg kg^{-1}) and, 1 day later, plasmids expressing α1-ENaC or β1-Na+,K+-ATPase were electroporated to the lungs either individually or in combination. Two days later, injury was assessed by measurement of wet-to-dry ratios, histological analysis and BAL protein levels and cellularity. Contrary to the protection experiment, little difference in lung injury was seen after gene transfer of α1-ENaC alone compared with LPS only or LPS treated with control pcDNA3 plasmid mice. To ensure that the lack of outcome improvements following transfer of the α1-ENaC was not simply due to lack of expression from the plasmid, we analyzed the levels of ENaC mRNA and found that gene transfer resulted in a 2.5- to 3-fold increase in its expression, suggesting that this possibility cannot account for the result (Supplementary Figure S1). By contrast, gene transfer of β1-Na+,K+-ATPase alone or combination with α1-ENaC resulted in significant reductions in lung injury compared with pcDNA3, as measured by wet-to-dry ratios (Figure 4a, 4.74 ± 0.03 or 4.77 ± 0.03 vs 4.89 ± 0.02, respectively). Histologically, Figure 4b shows evidence of reduced edema and inflammation in mice that received gene transfer of β1-Na+,K+-ATPase alone or in combination with α1-ENaC compared with LPS-challenged mice alone or LPS-challenged mice that received the empty plasmid pcDNA3. Transfer of β1-Na+,K+-ATPase alone or in combination with α1-ENaC also reduced total protein levels in BAL fluid by 32.9 or 38.1%, respectively, compared with LPS-injured mice receiving pcDNA3 (Figure 4e). Similarly, total cells and PMNs in BAL fluid from those mice were significantly reduced after electroporation of β1-Na+,K+-ATPase alone or in combination with α1-ENaC. Although transfer of α1-ENaC alone slightly reduced both total protein and cell numbers in BAL fluid, they were not statistically significant (Figures 4d and e). To determine whether this reduced lung inflammation was accompanied by decreased production of cytokines and chemokines, a 23-panel multiplex immunoassay

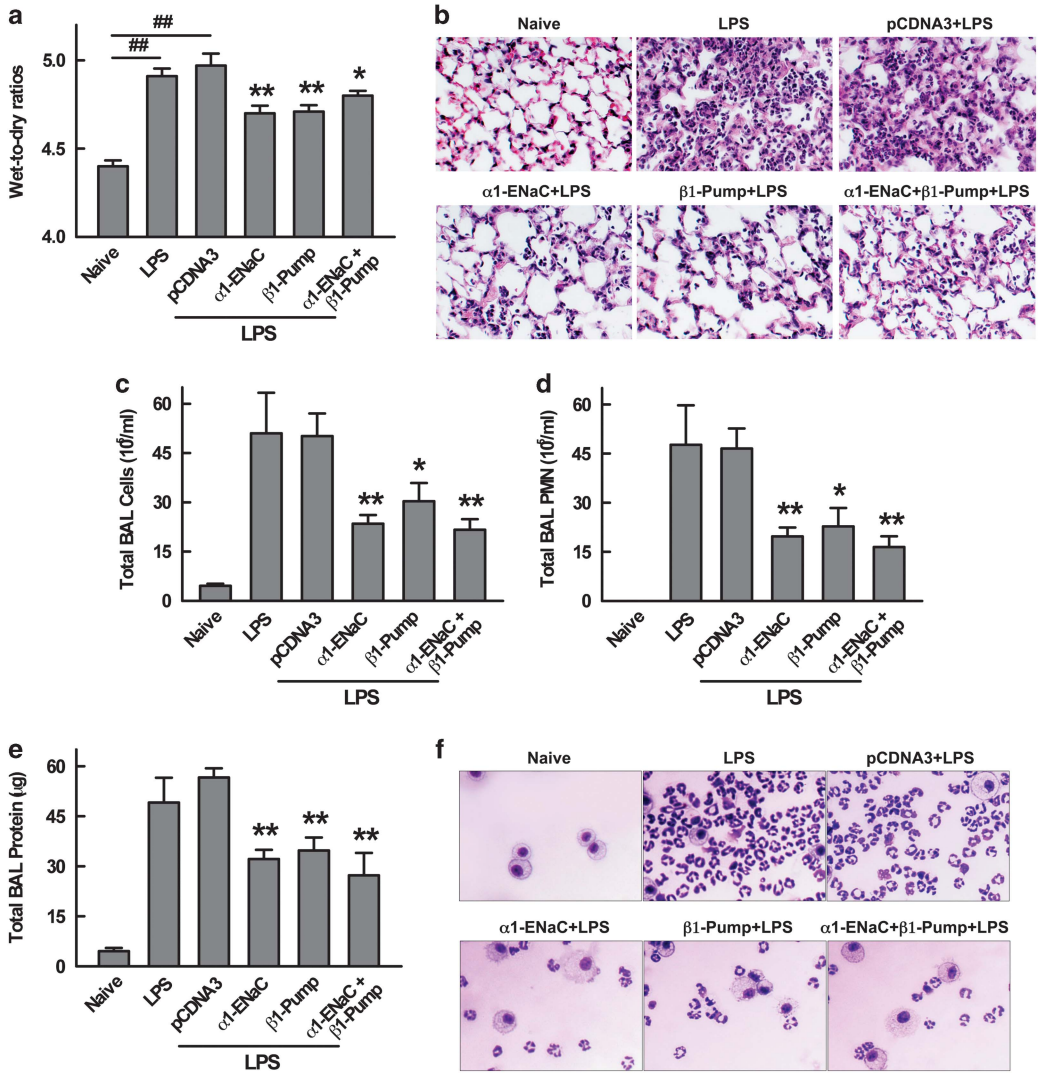

Figure 3. Electroporation-mediated gene transfer of α1-ENaC or β1-Na$^+$,K$^+$-ATPase can protect from subsequent LPS-induced lung injury. In all, 100 μg of plasmid in 50 μl was administered to mice by aspiration and electroporation. One day later, LPS (5 mg kg^{-1}) was administered to the lungs and 24 h after this, lungs were removed for gravimetric analysis. (**a**) Wet-to-dry ratios are shown as mean ± s.e.m. ($n = 7$–9). (**b**) Hematoxylin and eosin staining was used to compare the histological features. BAL fluid was collected from lungs and analyzed for cellularity (**c**), total PMNs (**d**), protein levels (**e**) and representative pictures show PMN infiltration (**f**). Statistical analysis was done by one-way ANOVA. $^{\#\#}P < 0.01$ compared with naive and $^{*}P < 0.05$ or $^{**}P < 0.01$ compared with pCDNA3.

was performed in BAL fluid. No difference was observed between any condition in LPS-injured mice (Supplementary Table S1). Taken together, these results demonstrate that electroporation-mediated transfer of individual β1-Na$^+$,K$^+$-ATPase or in combination with α1-ENaC can reduce the severity of injury in previously damaged lungs, but the underlying mechanism is not related to anti-inflammatory effects of overexpressed β1-Na$^+$,K$^+$-ATPase.

Gene transfer of the Na$^+$,K$^+$-ATPase β1-subunit, but not the ENaC α1-subunit, increases tight junction protein zona occludins and occludin expression in both healthy and injured mice

Although decreased edema fluid was predicted following delivery of either sodium transporter, the decrease in infiltrating neutrophils following delivery of the β1-Na$^+$,K$^+$-ATPase suggested that additional mechanisms were involved. Previous studies have reported that Na$^+$,K$^+$-ATPase activity is involved in the regulation of tight junction structure and function.[17] To determine whether tight junction formation was associated with the effects of gene transfer of β1-Na$^+$,K$^+$-ATPase on LPS-induced ALI, expression of tight junction proteins zona occludins-1 (ZO-1) and occludin was measured in both healthy and LPS-injured lungs. As shown in Figure 5a, delivery of a plasmid expressing α1-ENaC had no effect in healthy animals on either ZO-1 or occludin expression, nor did delivery of the control plasmid pcDNA3. By contrast, delivery of a plasmid expressing β1-Na$^+$,K$^+$-ATPase alone or in combination with α1-ENaC significantly enhanced both ZO-1 and occludin expression in healthy animals by threefold to fourfold.

We next measured ZO-1 and occludin expression in LPS-injured lungs in our protection experiments. ZO-1 and occludin expression was decreased twofold after transfer of pcDNA3 before LPS administration, compared with their expression in uninjured naive lungs (Figure 5b). Similarly, expression of ZO-1 and occludin was decreased twofold to threefold in mice following treatment with LPS alone (Supplementary Figure S2). Lungs electroporated with β1-Na$^+$,K$^+$-ATPase alone, or in combination with α1-ENaC before LPS injury led to a significant increase in both ZO-1 and occludin expression. Transfer of α1-ENaC alone resulted in occludin expression similar to the levels of naive animals and had no effect on ZO-1 expression when delivered before LPS administration (Figure 5b).

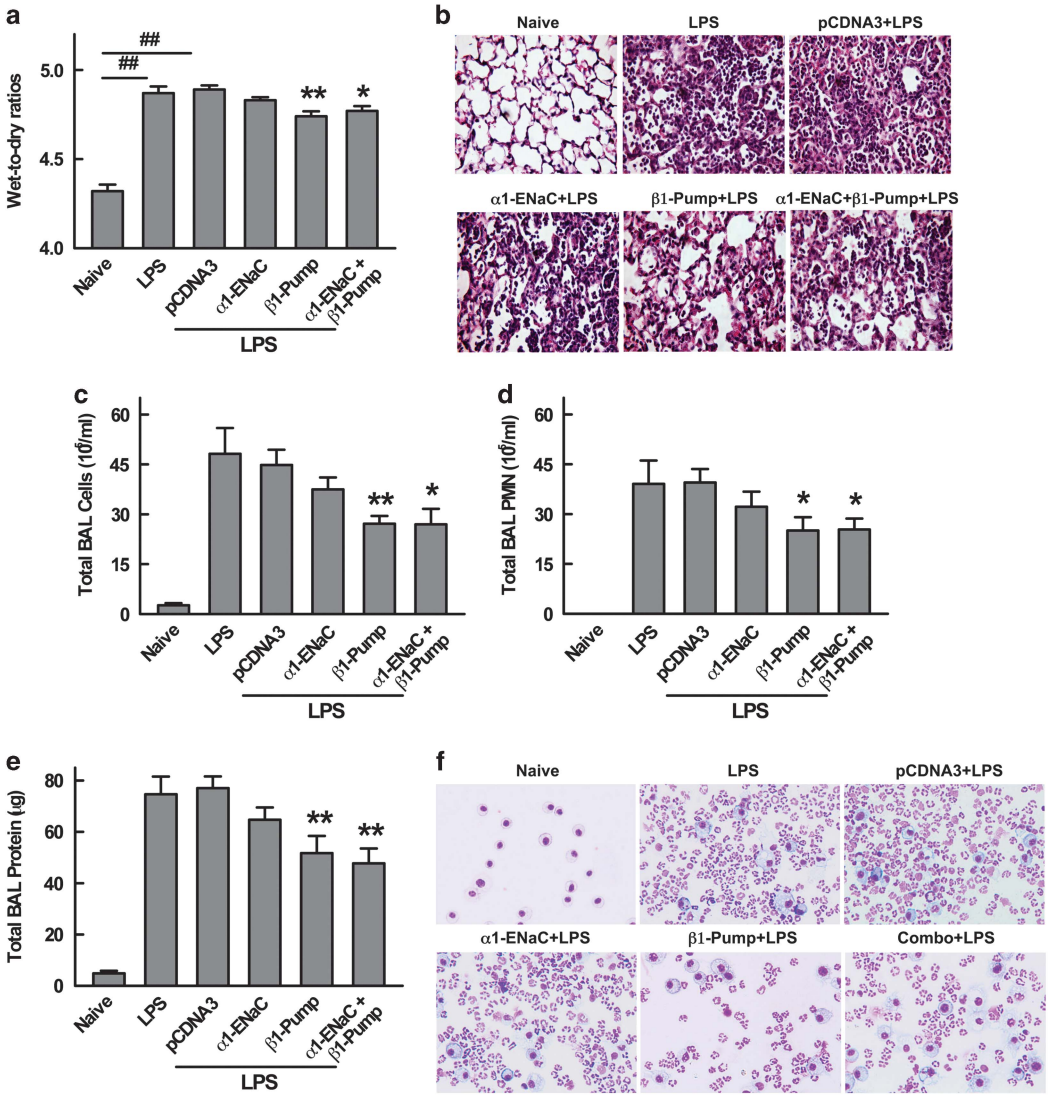

Figure 4. Electroporation-mediated gene transfer of β1-Na^+,K^+-ATPase, but not α1-ENaC, can treat LPS-injured lungs. LPS (5 mg kg^{-1}) was intratracheally administered to mice and 1 day later, 100 μg of plasmid in 50 μl was delivered to the lungs by electroporation. After 2 days, lungs were removed for gravimetric analysis. (**a**) Wet-to-dry ratios are shown as mean ± s.e.m. ($n = 6$). (**b**) Hematoxylin and eosin staining was used to compare the histological features. All figures are at original magnification × 200. (**c**) Cellularity, (**d**) total PMNs and (**e**) protein levels are measured in BAL fluid, and (**f**) representative pictures show PMN infiltration. Statistical analysis was done by one-way ANOVA. $^{\#\#}P < 0.01$ compared with naive and $^{*}P < 0.05$ or $^{**}P < 0.01$ compared with pCDNA3.

We also evaluated tight junctions in treatment groups that were injured before gene delivery. Figure 5c shows that ZO-1 and occludin expression was markedly reduced after delivery of pcDNA3 following LPS administration, compared with their levels in uninjured naive lungs, which are not received any experimental procedure. Delivery of plasmids expressing the β1-Na^+,K^+-ATPase alone or in combination with α1-ENaC to previously injured lungs significantly enhanced occludin expression twofold compared with pcDNA3, but had no effect on ZO-1 expression. Moreover, neither ZO-1 nor occludin expression was increased in mice following gene transfer of α1-ENaC alone after LPS administration.

Gene transfer of the Na^+,K^+-ATPase β1-subunit, but not the ENaC α1-subunit, improves LPS-injured lung permeability in mice

To further demonstrate whether β1-Na^+,K^+-ATPase regulation of tight junctions contributed to its treatment of LPS-induced ALI, we measured lung permeability by Evans Blue dye (EBD) leakage from blood into airways. Pulmonary leakage in response to LPS was increased threefold to fourfold due to endothelial and/or epithelial barrier disruption compared with naive mice (Figure 6). Transfer of the control plasmid pcDNA3 after LPS instillation resulted in no change in pulmonary leakage, nor did transfer of the α1-ENaC plasmid. As we expected, gene transfer of β1-Na^+,K^+-ATPase markedly reduced previously LPS-induced pulmonary leakage to 0.131 ± 0.013, compared with 0.254 ± 0.014 of the empty plasmid pcDNA3. Collectively, these results suggest that gene transfer of β1-Na^+,K^+-ATPase, but not α1-ENaC, has a critical role in inhibiting pulmonary leakage and thus functionally enhances the endothelial and/or epithelial barrier(s).

Expression and localization of ZO-1 and occludin is enhanced in β1-Na^+,K^+-ATPase-overexpressed 16HBE14o- and human pulmonary artery endothelial cells

To establish the effects of β1-Na^+,K^+-ATPase gene transfer on tight junction formation, we used 16HBE14o- human bronchial epithelial cells as a lung epithelial cell model and primary human pulmonary artery endothelial cells (HPAEC) as a lung endothelial cell model, as relatively few cell lines express tight junction proteins under culture

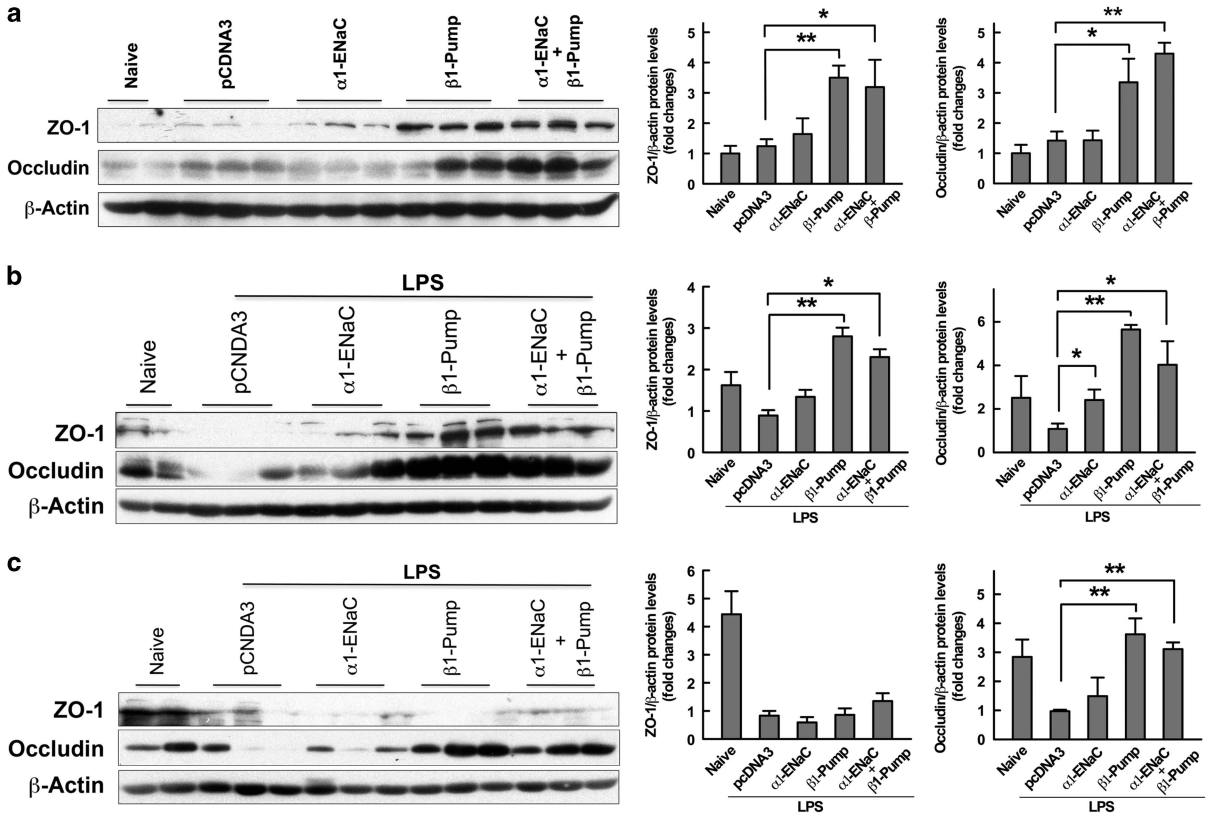

Figure 5. Gene transfer of β1-Na^+,K^+-ATPase subunit enhances tight junction protein ZO-1 and occludin expression in mouse lungs. ZO-1 (~225 kdal) and occludin (~65 kdal) expression was measured in mouse lungs by western blot and densitometry relative to β-actin (~43 kdal) after gene transfer of α1-ENaC and β1-Na^+,K^+-ATPase subunits without LPS injury (**a**) or with LPS-induced ALI after (**b**) or before (**c**) gene delivery. Statistical analysis was done by one-way ANOVA (mean ± s.e.m.; $n = 5$-6). $^*P < 0.05$ or $^{**}P < 0.01$ compared with pCDNA3.

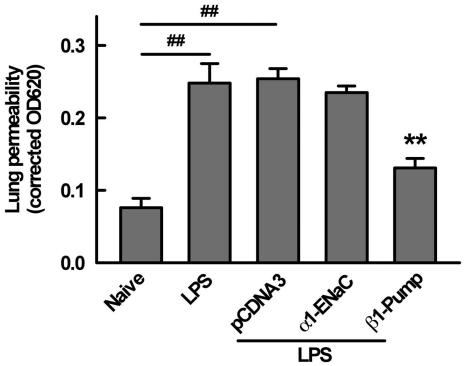

Figure 6. Electroporation-mediated gene transfer of β1-Na^+,K^+-ATPase, but not α1-ENaC, attenuates LPS-induced pulmonary leakage. Pulmonary permeability was measured by EBD leakage from blood to airways. EBD (30 mg kg^{-1}) was administrated by tail-vein injection 47 h after gene transfer. One hour later, lungs were perfused to remove EBD in the vasculature. Extracted EBD from the perfused lungs was quantified by measuring at 620 nm and 740 nm and shown as mean ± s.e.m. ($n = 7$–11). Statistical analysis was done by one-way ANOVA. $^{\#\#}P < 0.01$ compared with naive and $^{**}P < 0.01$ compared with pCDNA3.

conditions. Cells were transfected with plasmids expressing GFP-tagged β1-Na^+,K^+-ATPase or DDK-tagged α1-ENaC, respectively, and analyzed 2 days later. As shown in Figures 7a and b, western blot analysis showed that exogenous α1-ENaC and β1-Na^+,K^+-ATPase were expressed in both cell lines. Further, overexpression of the β1-Na^+,K^+-ATPase but not α1-ENaC led to twofold or threefold increases in ZO-1 and occludin protein levels in both 16HBE14o- and HPAEC, compared with the control plasmid.

Next, we carried out qPCR in transfected 16HBE14o- cells and HPAEC to determine whether the increased ZO-1 and occludin expression seen following β1-Na^+,K^+-ATPase gene transfer was regulated at the transcriptional level. As shown in Figure 7c, increased ZO-1 and occludin mRNA levels were observed after cells transfection with β1-Na^+,K^+-ATPase in 16HBE14o- cells by 3.2- and 1.5-fold compared with pcDNA3, respectively. Similar results were seen in HPAEC (Figure 7d). These results suggest that mRNA expression of ZO-1 and occludin is upregulated by β1-Na^+,K^+-ATPase in both lung epithelial and endothelial cells.

To determine the localization of the increased ZO-1 and occludin, 16HBE14o- cells were plated at low confluence after transfection. Two days later, immunofluorescence staining was performed to determine fusion protein expression (green) and tight junction formation (red) using anti-ZO-1 or occludin antibodies. Confocal microscopy showed that after transfection with β1-Na^+,K^+-ATPase, cells began to form tight junctions, revealing a chicken wire pattern of staining for ZO-1 (Supplementary Figure S3a) or occludin (Figure 7e). Similar findings were observed for HPAEC (Supplementary Figure S3b). By contrast, there was no chicken wire pattern after transfection with α1-ENaC or pcDNA3. Collectively, overexpression of β1-Na^+,K^+-ATPase, but not α1-ENaC, in both 16HBE14o- and HPAEC cells promotes tight junction formation.

DISCUSSION

In the present study, we tested whether electroporation-mediated gene transfer of the α1-ENaC and/or the β1-Na^+,K^+-ATPase to mouse lungs can be used to protect from and treat LPS-induced

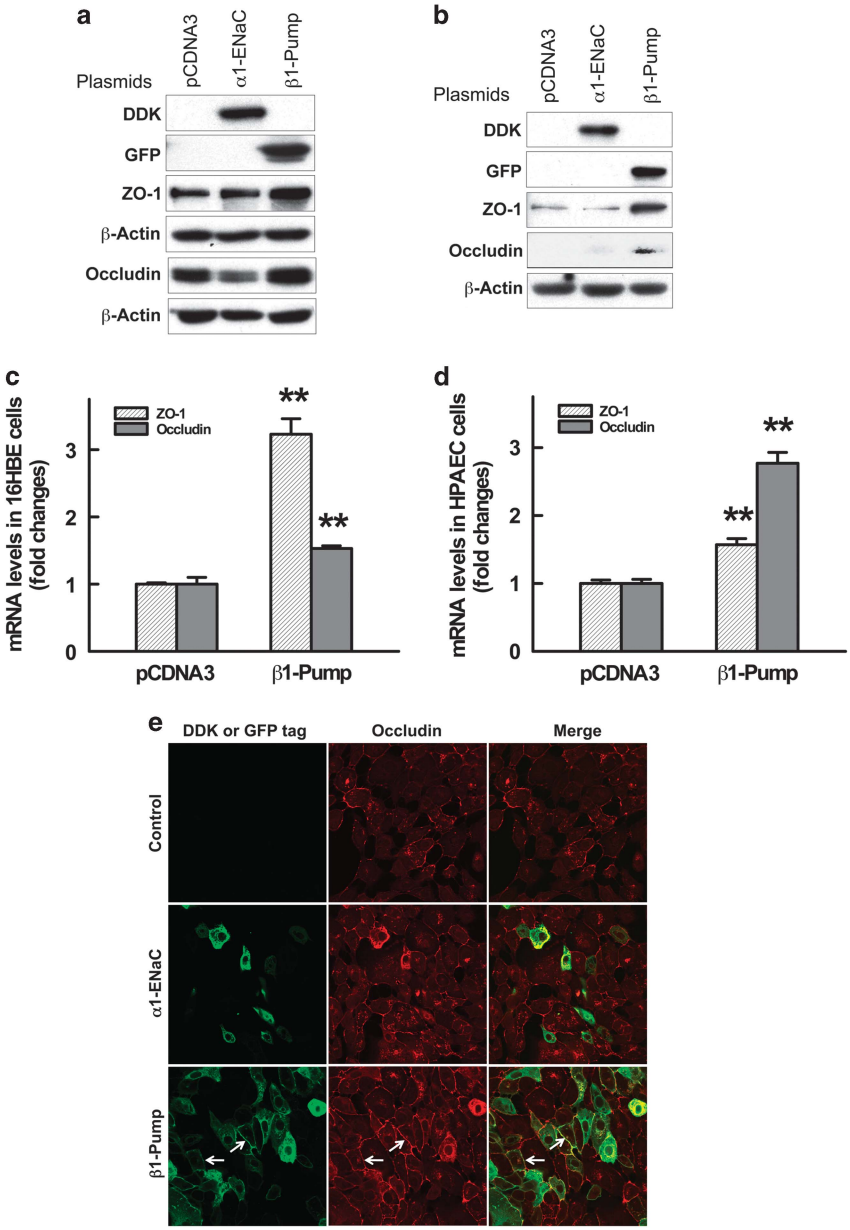

Figure 7. Overexpression of β1-Na^+,K^+-ATPase, but not α1-ENaC, increases expression and mRNA levels of tight junction proteins ZO-1 and occludin in 16HBE14o- (**a**, **c**) and HPAEC (**b**, **d**) cells, 24 h after transfection. All proteins detected on western blots migrated as predicted based on their reported molecular weights; DDK-tagged α-ENaC (~110 kdal), GFP-tagged β1-Na^+,K^+-ATPase (~80 kdal), ZO-1 (~225 kdal), Occludin (~65 kdal) and β-actin (~43 kdal). Statistical analysis was done by one-way ANOVA (mean ± s.d.; $n = 3$). $^{**}P < 0.01$ compared with pCDNA3. Immunofluorescence staining of occludin (**e**) was followed after 16HBE14o- cells transfected with β1-Na^+,K^+-ATPase or α1-ENaC for 48 h, and representative photographs were taken using confocal microscope at ×400 magnification. Arrows show tight junction formation.

lung injury. Although transfer of α1-ENaC provided protection from subsequent injury by increasing AFC, it showed no ability to treat previously injured lungs. By contrast, transfer of β1-Na^+,K^+-ATPase, while improving AFC less than 50% of that seen following α1-ENaC gene transfer, provided not only protection from subsequent LPS-induced lung injury, but also reduced injury in previously injured lungs by improving AFC and alveolar barrier function through upregulation of tight junction complexes. Taken together, our data indicate that while improving AFC by gene transfer with ion channels may partially protect from subsequent lung injury, restoring or strengthening epithelial and/or endothelial barrier function is likely critical to treat already existing ALI/ARDS.

Previous research has demonstrated that overexpression of ENaC or the Na^+,K^+-ATPase could enhance AFC to clear pulmonary edema, and thus provide protection from lung injury induced by ventilation or hyperoxia.[18–20] These studies suggested that the target gene that could produce the greatest increase in AFC in healthy lungs would also provide the greatest protection from and treatment of lung injury. Although numerous studies have reported that transfer of many genes, including ENaC,[21] Na^+, K^+-ATPase subunits,[22] β2-adrenergic receptor[23] and Keratinocyte growth factor,[24] by either viral vectors or non-viral vectors, can protect from subsequent lung injury, none have directly correlated the degree of AFC with the ability to protect lungs from injury. On the basis of our current results, the ability to clear alveolar fluid alone does not correlate with the ability to protect and treat the injured lung. Indeed, it also has been shown by multiple groups that β_2-agonists (such as procaterol or albuterol) can upregulate

activity of the Na^+,K^+-ATPase in cultured cells and AFC in mouse models by either direct stimulation of existing pools of the Na^+,K^+-ATPase or by promoting increased redistribution of the transporters to the cell surface.[25,26] However, in the case of albuterol, several clinical trials found no benefit for patients with ARDS.[27,28] Thus, other mechanisms in addition to improved AFC, namely improved epithelial barrier function, might be involved following gene transfer of α1-ENaC or β1-Na^+,K^+-ATPase.

Owing to the presence of the ENaC and the Na^+,K^+-ATPase in both alveolar epithelial type I (AT1) and type II (AT2) cells, AFC is driven by sodium transport entering the alveolar epithelial cell via ENaC on the apical surface and then being pumped out by the Na^+,K^+-ATPase within the basolateral surface into the lung interstitium and the pulmonary circulation.[8] This flow of water out of the lung depends on intact epithelial and endothelial barriers.[1] In both cell layers, the barrier regulates the movement of ion and macromolecules across cell layers and is regulated by intercellular junctional complexes defined as tight junctions.[29] Tight junctions consist of transmembrane proteins, including occludin, claudins and junctional adhesion molecule families, and peripheral membrane proteins linking the transmembrane proteins and the cytoskeleton, such as ZOs.[30] According to these insights into the mechanisms of ALI/ARDS, treatments of improving alveolar epithelial function might be one of therapeutic strategies to reduce the mortality of patients with ALI/ARDS.

Several studies have reported that the Na^+ ion channel activity of the Na^+,K^+-ATPase has a role in tight junction formation and function in drosophila,[31] zebrafish,[32] during mouse embryo preimplantation[33] and in MDCK epithelial cells.[34] However, all of these studies have relied on either K^+ depletion or treatment with ouabain, an inhibitor, widely presumed to be uniquely specific for the Na^+,K^+-ATPase rather than direct overexpression of the Na^+,K^+-ATPase itself. Rajasekaran *et al.*[35] observed that inhibition of Na^+,K^+-ATPase by K^+ depletion as well as ouabain treatment resulted in occludin hyperphosphorylation and increased tight junction permeability although the inhibition of the Na^+,K^+-ATPase did not influence tight junction protein expression (ZO-1 or occludin) in a differentiated human pancreatic epithelial cell line. Further, the mechanism by which ouabain inhibition of the Na^+,K^+-ATPase regulates tight junction function appears different in different cell types, although the primary changes detected are in occludin hyperphosphorylation.[36,37] Although the sole target of ouabain is largely presumed to be the Na^+,K^+-ATPase, it and other cardiac glycosides also inhibit other enzymes.[38] Moreover, endogenous ouabain is produced in the adrenal cortex and hypothalamus and is hypothesized to bind to cellular receptor(s) and modulate responses independent of Na^+,K^+-ATPase regulation and activity. Studies from Cereijido and colleagues demonstrate that ouabain at concentrations as low as 10 nM, which does not inhibit Na^+,K^+-ATPase and the K^+ balance, acts as a regulator of tight junction function by decreasing tight junction permeability and modulating the tight junction protein expression including claudins 1, 2 and 4 in MDCK epithelial cells, suggesting alternative, non-Na^+,K^+-ATPase-mediated mechanisms due to ouabain signaling.[39] It is possible that the Na^+,K^+-ATPase β-subunit has signaling functions as well as enzymatic activity. Rajasekaran *et al.*[40] demonstrated that the forced expression of E-cadherin and the Na^+,K^+-ATPase β1-subunit, but not either alone, was needed for induction of a polarized phenotype and tight junction formation in mutant MDCK cells that normally displayed reduced levels of E-cadherin and the Na^+,K^+-ATPase β-subunit, implying a link between the Na^+,K^+-ATPase β-subunit and tight junction expression. However, as there was no detectable increase in the levels of β1-Na^+,K^+-ATPase or E-cadherin after co-transfection, the mechanism and possible link is less clear.

Several studies have also demonstrated that the Na^+,K^+-ATPase can localize to adherens junctions.[41–43] Although this appears to be mediated through the cytoplasmic domain of the α-subunit, the β-subunit also has a role in intercellular adhesion as well.[44] It has been demonstrated that the extracellular domain of the β subunit interacts with the same domain of the protein in an adjacent cell, which may contribute to paracellular permeability.[41–43,45,46] The region of the protein needed for these interactions appears to lie within residues 198–207 and shows some heterogeneity between species. Because of this, the interactions are species specific, with the rat protein binding to the rat, but not to the dog protein.[43] The mouse, human and rat proteins differ by only one or two amino acids in this region, so it is possible that these β1-β1 interactions may also be of benefit in the increased barrier function found in the current study.

Our results clearly show that gene transfer and expression of β1-Na^+,K^+-ATPase alone in mouse lungs and in cultured cells leads to increased levels of tight junction proteins. The increased abundance of ZO-1 and occludin is due to the specific expression of the β1-subunit and not to the transfection process or other subunits of the Na^+,K^+-ATPase since neither expression of α1-ENaC, an empty plasmid, nor the α1-subunit of the Na^+,K^+-ATPase (Lin and Dean, unpublished) resulted in any increase in tight junction proteins in cells or animals. How expression of the β1-subunit of the Na^+,K^+-ATPase leads to increased tight junction protein levels is unclear, but our results suggest that at least some of the increase is at the transcriptional level. In our experiments in healthy mice and those with LPS-induced ALI, it is also unclear whether the increase in ZO-1 and occludin occurs in alveolar epithelial cells or in endothelial cells. Clearly, improving barrier function in both cell types would be beneficial to treat the injured lung. Indeed, a 2011 study using human lungs not used for transplant demonstrated that levels of claudin-4, a major tight junction protein, directly correlated with alveolar fluid transport in the lungs.[47] Since the integrity of the alveolar-capillary barrier is crucial to the health of the lung, for fluid balance, and for productive gas exchange, the regulation of both AFC and the barrier itself by gene transfer of the β1-subunit of the Na^+,K^+-ATPase suggests that this may be an attractive approach to treat ALI/ARDS.

Gene therapy may be a powerful approach to treat many acute and chronic pulmonary diseases. To date, a number of viral and non-viral vector systems have been used to deliver transgenes into the lung to treat ALI/ARDS.[48] However, with the presence of barriers to lung gene transfer, such as pulmonary architecture, the innate immune system and immune activation, it is more difficult and less effective to deliver genes into the parenchyma, resulting in delayed use of gene therapy in clinical medicine.[48] Recent research from our laboratory and others has demonstrated that electroporation can be used to efficiently deliver DNA to various tissues, including the lung, with high-level gene expression and without damage.[10–12] Indeed, electroporation has several advantages including the use of easily and cheaply produced plasmids and the ease and simplicity of administration, leading to safe, efficient and reproducible transgene expression in both healthy and injured lungs. Moreover, in a preclinical large animal model of sepsis and ischemia-reperfusion induced ALI/ARDS using pigs, we have seen that transfer of plasmids expressing the same subunits of the Na^+,K^+-ATPase and ENaC as used here to lungs of animals with existing lung injury can improve multiple measures of clinical outcome, including lung compliance, lung oxygenation, pulmonary edema, lung inflammation, lung histology and survival.[49] In these experiments, a sixfold increase in the Na^+,K^+-ATPase β1-subunit was detected, suggesting that similar levels of overexpression could provide benefit in patients. Given that electroporation strategies are clinically approved for drug delivery in the European Union and that several Phase I and Phase II clinical gene therapy trials have shown success in the skin for melanoma, this approach shows clear clinical promise.[50,51]

MATERIALS AND METHODS

Plasmids

The plasmid pcDNA3 was obtained from Promega (Madison, WI, USA). pCMV-SCNN1A expresses a Myc-DDK-tagged human ENaC α1-subunit from Origene (Rockville, MD, USA), and pCMV-Na^+,K^+-ATPase β1 expresses a GFP-tagged rat Na^+,K^+-ATPase β1-subunit as described previously.[13] Plasmids were purified using Qiagen Giga-prep kits (Qiagen, Chatsworth, CA, USA) and suspended in 10 mM Tris-HCl (pH 8.0), 1 mM ethylenediaminetetraacetic acid and 140 mM NaCl.

Cell culture and transfection

16HBE14o- human bronchial epithelial cells were cultured in Dulbecco's modified Eagle's medium (DMEM with high glucose, Cellgro, Manassas, VA, USA) supplemented with 10 mmol l^{-1} HEPES, 10% FBS and glutamine. HPAEC primary human pulmonary artery endothelial cells were cultured in endothelial basal medium 2 (EBM2) with bullet kit additives (Lonza, Walkersville, MD, USA) and 10% FBS. 16HBE14o- cells in suspension were transfected by electroporation (260 V, 500 μF); and adherent HPAEC were transfected with lipofectamine 2000 (Invitrogen, Grand Island, NY, USA) and assayed for expression 48 h later.

In vivo gene transfer and induction of ALI

Male C57BL/6 mice (9–11 weeks) were anesthetized with isoflurane and 100 μg of each plasmid encoding the α1-ENaC and/or β1-Na,K-ATPase was delivered in 50 μl of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA and 140 mM NaCl to mouse lungs by aspiration. Eight 10-ms square wave pulses at field strength of 200 V cm^{-1} were immediately applied using cutaneous electrophysiology electrodes (Medtronic, Redmond, WA, USA) placed on the mouse chest with an ECM830 electroporator (BTX, Harvard Apparatus, Holliston, MA, USA). All LPS-challenged mice received 5 mg kg^{-1} of LPS (*Escherichia coli* O55:B5, 15 000 000 endotoxin units/mg protein; Sigma-Aldrich, St Louis, MO, USA) in 50 μl of phosphate-buffered saline (PBS) by aspiration after (protection) or before (treatment) gene transfer ($n = 5$–9 mice/group). All experimental procedures were performed according to institutional guidelines for the care and use of laboratory animals in an American Association for the Accreditation of Laboratory Animal Care-approved facility.

Western blot analysis and quantitative qPCR

Western blot, mRNA extraction and qPCR analysis were performed as previously described.[52] Briefly, lung tissues or cells were solubilized in lysis buffer containing protease inhibitor. Thirty micrograms of total protein was loaded on 10% SDS-PAGE, transferred onto PVDF membrane and probed with primary antibodies against DDK tag (Origene, Rockville, MD, USA), β1-Na^+,K^+-ATPase (Millipore, Billerica, MA, USA), ZO-1, occludin (Invitrogen) or β-actin (Sigma-Aldrich). Data were analyzed using the NIH Image J software. Total RNA was isolated from lung tissues or cells using an RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's instructions. RNA was subjected to reverse transcription using a Reverse Transcription System (Promega) following the manufacturer's instructions. Real-time PCR amplifications were performed using SYBR Green (Bio-Rad, Hercules, CA, USA). The relative quantities of mRNAs were obtained by the $2^{-\Delta}(^{\Delta}Ct)$ method and normalized with glyceraldehyde-3-phosphate dehydrogenase housekeeping gene.[52] The primer sequences are shown in Supplementary Table S2.

Measurement of AFC in live mice

The method used in this study was performed in live mice as previously described.[16] Briefly, mice maintained at a body temperature of 37 °C were anesthetized with diazepam (5 mg kg^{-1}, i.p.) and pentobarbital (50 mg kg^{-1}, i.p. given 10 min after diazepam). The trachea was cannulated with a 5-mm, 20-gauge angiocath (Becton-Dickenson, Sandy, UT, USA), and the catheter was connected to a small animal ventilator (Harvard Apparatus, Holliston, MA, USA) before paralysis with pancuronium bromide (0.04 mg, i.p.). Mice were ventilated with 100% oxygen and a tidal volume of 10 ml kg^{-1} at a frequency of 160 breaths per minute. Three hundred milliliters of an isosmolar (324 mOsm), 0.9% NaCl solution containing 5% acid-free Evans Blue-labeled bovine serum albumin (0.15 mg ml^{-1}, Sigma) was instilled into the endotracheal catheter over 10 s followed by 200 μl of air to position the fluid in the alveolar space. Mice were kept supine, inclined to 30°, and ventilated for 30 min, after which the chest was opened to produce bilateral pneumothoraces to facilitate aspiration of fluid from the tracheal catheter. Protein concentration in the aspirate was assessed using a Bradford assay (Bio-Rad Laboratories); and AFC was calculated using the following equation: $AFC = 1 - (C_0/C_{30})$, where C_0 is the protein concentration of the instillate before instillation and C_{30} is the protein concentration of the sample obtained at the end of 30 min of mechanical ventilation. Clearance is expressed as a percentage of total instilled volume cleared/30 min. Procaterol (a specific β_2AR agonist, 10^{-8} M) was administered in the instillate as a positive control.

Measurement of wet-to-dry ratios

The effect of LPS-induced ALI on total lung water content was determined at 24 or 72 h after instillation of LPS. Mice were exsanguinated via laceration of left renal artery and vein, and then lungs were excised and surface liquid was blotted away. Wet lung weight was assessed and a stable dry weight was obtained after lungs were placed in a hybridization oven at 70 °C for 72 h.

BAL analysis

BAL was performed as described previously.[14] Briefly, two separate 0.7 ml aliquots of sterile PBS were instilled into mouse lungs for lavaging. The fluid was placed on ice for immediate processing, and the total number of cells in the lavage was counted using a hemocytometer. Total protein was measured using a Bradford assay (Bio-Rad), and cells from BAL were stained with Diff-Quik (Siemens, Newark, DE, USA) after cytospin.

Histological analysis

Lungs were inflated with 20 ml kg^{-1} 10% (vol/vol) buffered formalin immediately after mice were killed and used for paraffin-embedded sections. Sections (5 μm) were stained with hematoxylin and eosin, blinded and reviewed for analysis of inflammatory response and pathological changes in the lung.

Pulmonary permeability analysis

Pulmonary permeability was measured by EBD leakage from blood into airways.[53,54] Mice ($n = 7$–11) were challenged by intratracheal administration of LPS and, 1 day later, plasmids expressing α1-ENaC or β1-Na^+,K^+-ATPase alone were electroporated to the lungs. EBD (30 mg kg^{-1}, Sigma) was administrated by tail-vein injection 47 h after gene transfer. One hour later, lungs were perfused with 5 ml of sterile PBS to remove EBD in the vasculature and then removed, photographed and dried at 60 °C. Twenty-four hours later, EBD was extracted in formamide (Fisher Scientific, Pittsburgh, PA, USA) at 37 °C for 24 h and quantified by measuring spectrophotometrically at 620 and 740 nm, correcting by using the formula $E_{620}(EBD) = E_{620} - (1.426 \times E_{740} + 0.030)$.[55]

Immunofluorescence staining

Following transfection, 16HBE14o- cells were plated in MatTek dishes (MatTek Corporation, Ashland, MA, USA) and stained by immunofluorescence after 2 days in culture as previously described.[40] Cells were fixed in ice-cold methanol at −20 °C for 30 min, incubated with Alexa Fluor 488-conjugated rabbit antibody against DYKDDDDK (Cell Signaling, Danvers, MA, USA) or FITC-conjugated goat antibody against GFP (Abcam, Cambridge, MA, USA) for 1 h at room temperature, washed four times in PBS, and then incubated with Alexa Fluor 594-conjugated mouse antibody against ZO-1 or occludin (Invitrogen) for 1 h at room temperature. Cells were visualized with a fluoview laser scanning confocal microscope (FV1000-IX81, Olympus, Center Valley, PA, USA).

Statistical analysis

Quantitative results are expressed as mean ± s.e.m. for *in vivo* studies and mean ± s.d. for *in vitro* experiments. The data were evaluated statistically with one-way ANOVA and *P*-values < 0.05 were considered as statistically significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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