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Electroporation mediated in-vivo Gene Delivery of the Na⁺/K⁺-ATPase pump Reduced Lung Injury in a mouse model of Lung Contusion

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Abstract

Objective—Lung contusion (LC) is an independent risk factor for Adult Respiratory Distress Syndrome (ARDS). The final common pathway in ARDS involves accumulation of fluid in the alveoli. In this study we demonstrate the application of a potential gene therapy approach by delivering the Na⁺/K⁺-ATPase pump sub-units in a murine model of LC. We hypothesized that restoring the activity of the pump will result in removal of excess alveolar fluid and additionally reduce inflammation.

Methods—Under anesthesia, C57/BL6 mice were struck along the right posterior axillary line 1 cm above the costal margin with a cortical contusion impactor. Immediately afterwards; 100 µg of plasmid DNA coding for the α,β of the Na⁺/K⁺-ATPase pump were instilled into the lungs (LC EP-pump group). Contusion only (LC only) and a sham saline instillation group after contusion were used as controls (LC EP-sham). Using a BTX 830 Electroporator, 8 electrical pulses of 200-V/cm field strength were applied transthoracically. Mice were sacrificed at 24hr, 48hr and 72hr post-delivery. Bronchial alveolar lavage (BAL) was recollected to measure albumin and cytokines by ELISA. Pulmonary compliance was measured and lungs were subject to histopathologic analysis.

Results—Following the electroporation and delivery of genes coding for the α,β subunits of the Na⁺/K⁺-ATPase pump, there was a significant mitigation of acute lung injury as evidenced by reduction in BAL levels of albumin, improved P-V curves and reduced inflammation seen on histology.

Conclusion—Electroporation mediated gene transfer of the subunits of the Na⁺/K⁺-ATPase pump enhanced recovery from acute inflammatory lung injury following LC.

Keywords

Lung contusion; electroporation; gene delivery; Na⁺/K⁺-ATPase pump

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INTRODUCTION

Acute lung injury (ALI) and its more severe form, Acute Respiratory Distress Syndrome (ARDS) are common, devastating clinical syndromes that affect large numbers of patients and have up to 40% mortality with the current standard of care. It is estimated that there are almost 200,000 cases of ALI in the United States every year, leading to 74,500 deaths and 3.6 million hospital days¹. Blunt trauma to the chest and its resulting lung contusion (LC) is a well-documented risk factor for the development of ALI and ARDS². Despite 40 years of research, only moderate advances have been made in the treatment of these syndromes, and the economic burden and mortality still remains unacceptably high³.

The severity of clinical hypoxemia and mortality has a direct correlation with decreases in alveolar fluid clearance (AFC) and increases in the permeability of the alveolar epithelial-endothelial barrier^{4,5}. Permeability injury has been also implicated in disruption of surfactant function causing alveolar collapse; as well as infiltration of plasma proteins and influx of neutrophils into the alveolar space leading to an increase in lung inflammation, perpetuating lung injury^{6,7}. It has been recognized that some of the differences in fluid clearance and barrier function could be regulated at a genetic and epigenetic level rather than the type, time and degree of the initial insult. Thus manipulations of gene activity to reverse these processes are highly desirable⁸.

Vectorial transport of Na^+ appears to be the main mechanism by which alveoli are able to clear edema from the lung. The active component of this transport is the Na^+/K^+ -ATPase trans-membrane pump which is located on the basolateral portion of the alveolar epithelial cells⁹⁻¹¹. By exchanging 3 Na^+ intracellular molecules for 2 K^+ extracellular molecules into the lung interstitium, an osmotic gradient is created that allows water to be reabsorbed from the alveolar airspace. Furthermore it is thought that the Na^+/K^+ -ATPase participates in the formation and maintenance for alveolar tight junctions enhancing the epithelial barrier function^{12,13}. Several groups have shown that up-regulation of the activity of the Na^+/K^+ -ATPase can be achieved by a gene therapy approach^{9,14,15}. As an example adenoviral delivery of the β_1 subunit of the Na^+/K^+ -ATPase pump gene can overexpress and increase the physical amount of functional enzymes at the basolateral membranes in intact rat lungs augmenting the transport of Na^+ across the lung epithelium. This has been found to be protective when given before insult in different models of lung injury (hyperoxia-induced, hypoxia-induced, lipopolysaccharide-induced). We have reported that delivery of naked plasmid DNA using electroporation is a simple and reproducible method for gene transfer into the lungs^{16,17}. However these mechanisms of fluid transport have not been evaluated in unique models of lung injury as exemplified by lung contusion.

We hypothesized that gene delivery through electroporation can be applied to animal models of trauma-induced lung injury including lung contusion. Additionally we postulated that delivery of Na^+/K^+ -ATPase pump subunits following LC would mitigate lung injury. As described below, the delivery of genes for Na^+/K^+ -ATPase using electroporation, following LC, showed significant improvement in lung injury and inflammation. This methodology could represent a new treatment strategy for trauma related ALI/ARDS.

MATERIALS AND METHODS

Induction of Isolated Lung Contusion

All protocols and procedures received the approval from the institutional University Committee on Use and Care of Animals (UCUCA) at the University of Michigan (Ann Arbor, MI). Male, C57/B6 (Harlan Laboratories), 26-30g (8-12 weeks old) were given an intra-peritoneal injection of ketamine/xylazine mixture. Lung contusion was induced

adapting methods describe by Hoth *et al* (2007)¹⁸. This model represents a survivable, non-lethal model of LC and these animals recover without any additional need for ventilatory support (thus eliminating the possibility of ventilator-induced lung injury). Briefly, after induction of anesthesia the mouse was placed in left lateral decubitus position. The hair of the right chest wall was clipped off. The right chest was struck 1 cm above the costal margin along the posterior axillary line using an electrical cortical contusion impactor (Custom Design and Fabrications Co., Richmond, Virginia) with a piston velocity of 5.8 m/sec adjusted to a depth of 10 mm. This allows the transmission of energy equivalent to 152 Joules/m², creating a well-defined area of lung contusion on the right side (Figure 1). No reported deaths were found during this portion of the procedure. Additionally nine uninjured mice were used as naive controls to obtained baseline values.

Study groups

After induction of lung contusion, animals were divided in three groups: Lung Contusion Only (LC); Lung Contusion with Electroporation of α_2 and β_1 subunits of the Na⁺/K⁺-ATPase pump (LC-EP-pump) and finally Lung Contusion with Electroporation of 100 mM NaCl only (LC-EP-sham). Three different time points (24, 48 and 72 hours) representing various attributes of acute inflammation were selected for each group (n=9).

Gene Delivery by Electroporation

Within several minutes after lung contusion under the same anesthetic setting, a needle tracheotomy was performed to introduce 100 micrograms of plasmid DNA suspended in a 100 mM NaCl solution into the lungs. Spontaneous breaths allowed dispersion of DNA into both lungs. After assuring adequate ventilation, using a BTX 830 Electroporator (Harvard Apparatus, Holliston, MA) 8 pulses of 200 V/cm field strength were applied across the thorax with 10×20 mm TEN electrodes placed under each forelimb (with K-Y Jelly to ensure conductance). Each pulse was 10 ms duration and separated by 1 second. The amount of energy generated corresponds to 0.3 Joules/kg, a level well below the dosage used for defibrillation. After electroporation of DNA or sham instillation, animals were recovered over a heated pad; the cervical incision was sutured closed and the animals were returned to the vivarium.

Plasmids encoding α_2 and β_1 subunits of the Na⁺/K⁺-ATPase

Two individual plasmids were used together, one containing the gene for the α_2 catalytic subunit of the Na⁺/K⁺-ATPase pump and a second containing the β_1 regulatory subunit of the same pump (Generously provided by David Dean, PhD, University of Rochester, NY). Gene expression was driven by a cytomegalovirus immediate early promoter/enhancer (CMVie) placed in front of the each expression cassette (ORF). An SV40 DNA targeting sequence (DTS) was placed behind the ORF, to allow ubiquitous cell expression. The CMV promoter is known to drive expression for up to 7 days, with a peak expression in the first 48 hours^{17,19}. These plasmids were propagated in *E. coli* electro-competent cells (Invitrogen, Carlsbad, CA) and were purified using Qiagen Gigaprep kits (Qiagen, Chatsworth, CA). The DNA was resuspended in 10 mM Tris, pH 8.0, 1 mM ethylenediaminetetraacetic acid, and 140 mM NaCl. In separate experiments, a third plasmid containing a GFP- β_1 subunit of the Na⁺/K⁺-ATPase was used for co-localization studies in lung epithelium.

Lung Mechanics and Pressure Volume Measurements

On the day of harvest, euthanasia was performed by exsanguination After induction of anesthesia, through a midline laparotomy blood was collected from the IVC. Exsanguination was performed by transection both IVC and Aorta. Without opening the chest, a 22-gauge metallic cannula was inserted into the trachea through a midline cervical incision. Animals

were then connected to a SCIREQ® Flexivent (Montreal, Canada). Immediate postmortem ventilation was performed with parameters that are considered standard in a mouse: Tidal volume of 10 ml/kg, Respiratory Rate of 150 and positive end expiratory pressure (PEEP) of 2 cm H₂O. The SCIREQ® Flexivent ventilator introduces force-oscillation with a computer controlled graphite piston and measures pressures and volumes in different sites along the circuit. Different type of breaths called Perturbations were used and allowed calculation of quasi-static pressure volume (P-V) curves, compliance (C_{est}), elastance (E_{est}), maximal volumes and hysteresis. The validity of these measurements has been described elsewhere and is comparable to other mechanical ventilator systems²⁰. We have previously noticed that the contribution of the chest wall in the total compliance of the respiratory system is minimal in these animals and these measurements were carried out with the chest wall closed.

BAL collection

After P-V measurements, with the metal cannula still in place, BAL was performed by injecting 2 × 600 µl of ice cold phosphate buffered saline (PBS). Both recovered aliquots were pooled together and centrifuged at 1500g for 3 min at 4°C. Supernatant was then frozen for subsequent albumin and cytokine analyses. Cell pellet was re-suspended in 4 mL of PBS and white blood cells differential counts were performed using Flow cytometry with gates created with antibodies against GR1 for activated neutrophils (PMN); F4/80+CD11b for activated macrophages (aMØ) and CD11c+MHC-II for lung dendritic cells (D).

Albumin concentrations in BAL

Albumin concentrations in BAL were measured by enzyme-linked immuno-sorbent assay (ELISA) with a polyclonal rabbit anti-mouse albumin antibody and detected by colorimetry using horseradish peroxidase labeled goat anti-rabbit immunoglobulin G (BD Biosciences Pharmingen, San Diego, CA). Mouse albumin (Sigma, St. Louis, MO) was used as a standard.

Cytokines and chemokines

Soluble concentrations of BAL IL-1β, IL-6, IL-10, KC, MIP-2, MCP-1 and MCP-5 were measured using commercially available mice cytokine ELISA kits from R&D Systems (Minneapolis, MN) and performed in triplicate.

Histopathology

For this purpose two animals were sacrificed for each time point of injury. After euthanasia by abdominal exsanguination, trachea was cannulated and the chest cavity was opened through midline sternotomy. The right heart ventricle was injected with 5 ml of ice cold PBS flushing the lung circulation free of blood. Tissue was then fixed with 1% formalin drained into the lung by gravity, processed and stained with hematoxylin and eosin (H-E staining) for examination.

Additional animals were electroporated under same conditions as described above with the exception of using a plasmid coding for a Green Fluorescent Protein (GFP) -β₁ subunit of the Na⁺/K⁺-ATPase fusion protein. After sacrifice, the lungs were harvested and preserved in OCT media. Frozen sections in the areas of lung contusion were prepared at 5 µm thickness and mounted directly with ProLong® Gold with DAPI (Invitrogen, Carlsbad, CA). No fixing reagents were used, as paraformaldehyde and formaldehyde become auto-fluorescent in the presence of surfactant. An Olympus epifluorescent microscope from the University of Michigan Imaging Core was used to obtain images of GFP fluorescence and DAPI nuclear counter stain.

Statistical analyses

Data are expressed as mean $\pm$ SEM for a minimum of 9 animals per group. One-way analysis of variance (ANOVA) with Bonferroni *post hoc* test was used for all intergroup pairwise comparisons. Statistical significance was considered when a *p* value of 0.05 was obtained. Values were calculated and graphics were generated using GraphPad Prism 5.0 (La Jolla, CA).

RESULTS

1. Lung Electroporation can be performed safely in mice with LC

A total of 92 mice were subjected to lung contusion followed by tracheotomy, tracheal instillation and 8 pulse electroporation at field strengths of 200 V/cm. Of these only 7 died (7.6% mortality) and their deaths were directly attributed to hypoventilation during fluid delivery into the trachea. Most of these deaths occurred during the initial 20 animals and in a phase when the laboratory personnel were being trained with this technique. Our ability significantly improved as the experiment went on without any additional mortality. Prior experiments performed on naive animals showed that electroporation alone or electroporation with DNA did not show any inflammation in the alveoli as assessed by H-E staining¹⁷.

2. Transgene expression of the GFP- β_1 subunit of the Na⁺/K⁺-ATPase was present in areas of LC

In order to study the distribution of DNA and to see if it can effectively target injured lung parenchyma after LC, a plasmid containing a GFP- β_1 subunit of the Na⁺/K⁺-ATPase was electroporated and lungs were harvested at 48 h post-injury. Injured animals that were electroporated with vehicle saline delivery with no plasmid DNA (*p*-sham) were used as controls (Figure 1A). Frozen sections were selected from areas of grossly contused lung. As seen in Figure 1B, fluorescent morphologic analysis revealed that Alveolar Epithelial Cells (AEC) type 1 (AT-1) and type 2 (AT-2), as well as alveolar Macrophages (aM ϕ) showed expression of the GFP- β_1 subunit, thus confirming that plasmid DNA and its electroporation mediated expression was able to target areas of damaged lung.

3. Electroporation of Na⁺/K⁺-ATPase pump genes improved and altered lung inflammation following LC

At harvest, gross morphologic changes indicating areas involved with LC were observed. Injured lung segments were fixed for permanent sectioning and processed for H-E staining. As seen in Figure 1C, well expanded alveoli with the presence of the occasional alveolar Macrophage characterized the normal lung. Lung contusion (LC-only) produces a diffuse alveolar hemorrhage with influx of inflammatory cells and obliteration of airspaces (Figure 1D). The addition of tracheotomy with saline injection into the lung and no therapeutic gene delivery provoked an even more severe inflammatory response (LC + EP-sham) (Figure 1E). The delivery of the Na⁺/K⁺-ATPase genes, using electroporation, showed a dramatic decrease in the areas of alveolar inflammation, an increase in the number of expanded alveoli and an increased numbers of white blood cells in the airspace as early as in 24 h (Figure 1F). The predominant cell type appeared to be PMNs; however their viability and functional state were not assessed.

4. Delivery of the Na⁺/K⁺-ATPase gene improved lung injury as evidenced by improved compliance and reduced permeability after lung contusion

Unilateral lung contusion (LC-only) can significantly decrease both lung compliance and lung hysteresis as seen on quasi-static pressure volume curves obtained from a Flexivent ®

SCIREQ ventilator. Electroporation of the Na^+/K^+ -ATPase gene improved compliance curves and hysteresis of the respiratory system at all the time points (24 h, 48 h and 72 h) (Figure 2A, B and C, respectively). TLC-30 (another parameter of lung compliance quantifying the volume that lungs can accommodate at 30 cm H_2O of pressure) showed significant improvement as compared to LC and LC-EP-sham groups at 48 hours (Figure 3).

In normal circumstances, albumin and other serum proteins are not present in broncho-alveolar fluid. However, the disruption of the alveolar epithelial barrier will allow albumin to leak into the airspaces and can be used a surrogate of permeability injury. As seen in Figure 4, the delivery of genes encoding the Na^+/K^+ -ATPase diminished the amount of albumin recovered in BAL fluid suggesting an improvement in barrier function at all-time points. On the other hand LC-only and LC-EP-sham had levels that were several orders of magnitude above naive control, peaking at 48 h with a significantly slower rate of resolution.

5. Delivery of the genes for the Na^+/K^+ -ATPase modified expression of cytokines and cellular subpopulations in BAL recovered after LC

In order to determine the predominant inflammatory profile in our model of unilateral LC, BAL levels of different cytokines were assessed. Similar to the findings in the rat^{6,21}, unilateral LC in mice was characterized by production of early response cytokines (IL-1 β ; IL-6, KC [a.k.a. CXCL-1] as well elevation of several anti-inflammatory cytokines (IL-10, MCP-1, MCP-5) (Figure 5 **White bars**). The electroporation of the Na^+/K^+ -ATPase gene did produce significant elevations in the concentrations of IL-6, MCP-1, and MCP-5 at 24 and 48 h as compared to uninjured controls and other experimental groups (Figure 5 **Black Bars**). This type of cytokine expression is thought to be regulatory for both migration and differentiation of the monocytes/macrophages lineage in the lung. To evaluate PMN related chemokines, BAL KC (CXCL-1) and MIP-2 were also measured. Levels of MIP-2 remained unchanged following electroporation (Figure 5F). Peak concentrations of KC were observed in all groups at 24 h; however in the LC-EP-pump group its level was slightly reduced as compared to LC-only and LC-EP-sham (Figure 5G).

Analyses of BAL cellularity revealed that the activated alveolar Macrophages with a F4/80 phenotype (aM ϕ) were decreased significantly after LC when compared to naive control (hatched line) (Figure 6A). The electroporation of a saline sham was not different from baseline whereas the electroporation of the pump/exchanger gene increased the number of aM ϕ s in the alveoli. Electroporation of the Na^+/K^+ -ATPase pump did show a neutrophilic influx into the BAL with a rapid decline of this population to baseline values by 72 h (Figure 6B). Finally, Lung Dendritic (D) antigen-presenting cells (MHC-II⁺) were significantly higher in the pump electroporation groups at 48 h and 72 h when compared with contusion only (LC-only) and sham groups (Figure 6C).

DISCUSSION

Thoracic trauma is involved in nearly a third of all trauma admissions to the hospital and often includes significant LC. LC is an independent risk factor for the development of acute lung injury (ALI), acute respiratory distress syndrome (ARDS) and ventilator-associated pneumonia (VAP). These conditions have a particularly high mortality and morbidity rate despite advanced modes of respiratory support and intensive care. In the current manuscript we explore the use of a Gene Therapy approach to enhance the resolution of LC induced lung injury by overexpressing the subunits of the Na^+/K^+ -ATPase pump, an important component in the water and salt homeostasis of the lung. In this proof of concept manuscript, we report that the gene delivery of these pump genes through electroporation following LC is safe and feasible. Additionally, we demonstrate that there is reduction in

lung injury and alteration of inflammation following electroporation of the pump genes into the lungs.

Suboptimal alveolar fluid clearance has been implied as an important risk factor in the mortality of patients who have developed ALI/ARDS^{5,22}. The alveolar epithelium is responsible for fluid removal from the airspaces. In order to accomplish this it utilizes multiple ion transporters: the amiloride sensitive epithelial Na⁺ channels (ENaC), the Na⁺/K⁺-transporting adenosine triphosphatase (Na⁺/K⁺-ATPase), the cystic fibrosis transmembrane conductance regulator (CFTR), several aquaporin channels (AQ-1, AQ-5) as well as several K⁺ channels located on both alveolar type 1 (AT-1) and type 2 cells (AT-2). Of these elements the rate-limiting step is located at the energy dependent Na⁺ transport performed by the Na⁺/K⁺-ATPase pump. Several strategies have been tested to increase the alveolar fluid clearance by manipulating pump function. Catecholamines (dopamine and isoproterenol) have been shown to affect the amount and activity of the Na⁺/K⁺-ATPase subunits on the alveolar basolateral membranes^{22,23}. However, this effect can be exhausted by tachyphylaxis. Alternatively, viral and non-viral gene delivery overexpression of the Na⁺/K⁺-ATPase has been tested to augment alveolar fluid clearance (AFC) by *de novo* synthesis of the pump. Factor *et al* (1998) and others have shown that transfer of the α_2 subunit and β_1 subunit of the Na⁺/K⁺-ATPase, using recombinant adenoviruses, significantly increase AFC in the rat lung^{9,23-26}. This was achieved in both intact lungs as well as injured lungs using models of hyperoxia and ventilator induced lung injuries. Furthermore, the overexpression of the β_2 -adrenergic receptor by the same group resulted in increases in both ENaC and Na⁺/K⁺-ATPase expression. Additionally, other groups have shown similar results with the use of lenti-viruses encoding just the β_1 subunit of the Na⁺/K⁺-ATPase pump¹⁴. However, these studies have drawn criticism from the fact that experimentally measured AFC could only be performed 7 days after gene delivery. This would not be practical in the treatment of trauma-induced lung injuries where the required therapeutic window is within the first few hours after insult.

Non-viral modes of gene delivery have met with limited success. Liposome mediated delivery of α -subunits and β -subunits of the Na⁺/K⁺-ATPase were protective when used with a model of thiourea-induced pulmonary edema in mice¹⁵. Plasmid-liposome complexes were shown to have very little inflammatory response. The same approach was used to provide the gene for Keratinocyte Growth Factor (KGF) a potent AT-2 mitogen and upstream regulator of the Na⁺/K⁺-ATPase activity²⁷. By increasing the levels of KGF, these investigators were able to protect the lung from oleic acid induced lung injury. However, the protective effects were modest when compared to adenoviral delivery of the gene or by providing the recombinant protein directly into the lungs.

We have previously shown that the delivery of naked plasmid DNA using electroporation, is a simple and reproducible method for gene transfer to the lungs^{16,17,28}. The use of a very short lived electrical field creates pores in the cell membrane that allow naked plasmid DNA to enter the cell. Delivery of DNA into living animal lungs achieved high expression levels comparable to adenoviral vectors and did not induce pulmonary inflammation. In a model of LPS-induced lung injury, electroporation of α -subunit and/or β -subunit increased AFC both in a protective strategy mode (gene EP before endotoxin) as well as in a rescue strategy mode (gene EP after endotoxin)²⁸. Additionally, not only the AFC was improved, but the number of inflammatory cells recovered in the BAL, were decreased in the treated animals.

In the current study we demonstrate that electroporation of the Na⁺/K⁺-ATPase subunit genes immediately after unilateral LC mitigated the degree of lung injury as measured by quasi-static pressure-volume curves, albumin concentrations in BAL and histology. The maximum positive effect was observed between 24-48 h, corresponding to the peak of

transgene expression whenever a CMV promoter is utilized^{16,17}. The precise mechanism of gene silencing is still not fully known, however processes such as DNA methylation and chromatin remodeling seem to be involved and are important as a host defense mechanisms against viral replication⁸. Prolonged expression can be achieved by substituting the viral promoters by mammalian sequences such as the polyubiquitin C (Ubc) promoter²⁹.

In regards to permeability injury, several lines of evidence point out that the Na^+/K^+ -ATPase -pump is involved in formation, distribution and maintenance of epithelial tight junction and is additionally essential for epithelial polarization. Indeed, ouabain treatment, a potent and specific inhibitor of the Na^+/K^+ -ATPase can disrupt the distribution of ZO-1 and Occludin proteins affecting the formation of Tight Junctions (TJ) and epithelial permeability^{12,30}. The exact mechanism is still unknown but it is thought to involve the trafficking, assembly and positioning of TJ associated proteins within the apical and basolateral domains of the epithelial cell membrane¹³. Whatever the mechanism(s) involved, the increase in permeability of albumin and other macromolecules is integral to the physiopathology of ALI/ARDS. The complex interactions of extravasated serum proteins and surfactant in alveolar airspaces are poorly understood. In experiments with rats with LC, we have reported a strong correlation between the degree of alveolar permeability injury and surfactant inactivation³¹. It is well known that the inactivation of surfactant can lead to more areas of atelectrauma, thereby worsening lung inflammation and mechanics.

Finally, it is difficult to explain why the electroporation of the Na^+/K^+ -ATPase pump into lung epithelia would induce changes in the cellular subpopulations. An attractive hypothesis is that the overexpression of the Na^+/K^+ -ATPase may directly modify the activity of resident alveolar macrophages and induce differentiation of blood circulating monocytes into new macrophages through an MCP-1 and (its related murine analog) MCP-5 pathway. Both these chemokines were significantly elevated in the Na^+/K^+ -ATPase pump electroporated lungs. It has been reported that alveolar macrophages play a critical role in the pathogenesis and subsequent resolution of inflammation after LC^{7,32}.

Gene Therapy has been traditionally viewed as an approach to modify and correct permanent defects of the genome. However, the present manuscript describes a method where a short term transgene expression can potentially reverse an important pathological process related to trauma. We are currently evaluating a similar therapeutic strategy in additional models of severe lung injury such as LC +gastric aspiration and LC with super added pneumonia. As our understanding of the different molecular mechanisms of ALI, ARDS and sepsis improves, short term gene therapy is likely to be an important therapeutic strategy. With electroporation, we now have a technique that would allow the use of other molecular targets in studying the biology of ALI/ARDS.

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Figure 1. Electroporation of the Na^+/K^+ -ATPase genes improved and altered lung inflammation after lung contusion

Forty eight hours after injury and electroporation of (A) saline sham with no plasmid and (B) a plasmid containing the gene for the GFP- β_1 of the Na^+/K^+ -ATPase pump (**p-GFP-pump**), lungs were harvested, OCT-frozen sections were performed and directly mounted with antifading agent containing DAPI nuclear counter stain. GFP expression can be seen in alveolar type 1-cells (**triangular end arrows**) alveolar type 2-cells (**diamond end arrows**) and macrophages (**double arrow**). As early as 24 hours post-injury the LC EP pump group (F) showed a histologic improvement with increased areas of expanded alveoli and diminished inflammation as compared to other groups (LC-only and LC EP-sham, D and E, respectively). An uninjured control (C) is shown for comparison purposes. Bar represents 100 μm

Figure 2. Electroporation of the Na^+/K^+ -ATPase pump improves Pressure-Volume curves and hysteresis following LC injury

After LC and delivery of genes containing the Na^+/K^+ -ATPase pump, mice were euthanized and immediate postmortem ventilation was performed with a Flexivent[®] SCIREQ ventilator. Group that was electroporated with the functional plasmid (LC EP-pump) showed improvement of quasistatic P-V curves close to normal at 48 h. ($n=9 \pm \text{SEM}$). Curves were created from over 4000 observed data points.

Figure 3. Electroporation of the Na^+/K^+ -ATPase pump improves lung compliance by TLC-30 measurement

Total Lung Capacity (TLC-30) is a sensitive indicator of lung injury. LC EP-pump groups (black bars) showed better volumes of inflation at 30 cm H_2O inspiratory pressure than their controls indicative of better compliance. ($n=9 \pm \text{SEM}$) One way ANOVA * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$ vs Naive (hatched line). # $p < 0.01$ vs LC-only

Figure 4. Delivery of the Na^+/K^+ -ATPase genes reduced permeability injury after lung contusion
 Albumin recovered in BAL fluid is marker of permeability injury. Delivery of the Na^+/K^+ -ATPase genes by electroporation after injury (black bars) significantly reduced levels of albumin in comparison to controls, almost to naïve background values at 72 h (hatched line). (n=9 ± SEM) One way ANOVA * $p < 0.05$ vs Naïve (hatched line). # $p < 0.05$ vs LC-only.

Figure 5. BAL Cytokine profile after LC and gene delivery of the Na⁺/K⁺-ATPase genes
 ELISA of cell free supernatant post LC injury was performed to quantify cytokine levels (A. IL-1 β ; B. IL-6; C. IL-10; D. MCP-1; E. MCP-5; F. MIP-2; G. KC; respectively) in BAL. Increased levels of IL-6, MCP-1 and MCP-5 levels at early time points were found in LC EP pump groups, which are important regulators of monocytes/macrophages migration and differentiation in the lung. (n=9 $\pm$ SEM) One way ANOVA * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$ vs Naïve (hatched line). # $p < 0.05$; $p < 0.01$; ### $p < 0.001$ vs LC-only. See text for details.

Figure 6. Flow cytometry of cellular subpopulations after electroporation and delivery of the Na^+/K^+ -ATPase genes

After LC injury BAL was obtained and flow cytometry was used to determined different subpopulations of cells at 24, 48 and 72 h. LC EP pump showed increased numbers of Macrophages and Dendritic cells at early time points, suggesting their involvement in the resolution of LC. (n= (n=9 $\pm$ SEM) One way ANOVA * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$ vs Naïve (hatched line). # $p < 0.05$; $p < 0.01$; ### $p < 0.001$ vs LC-only.