CYTOKINE-ION CHANNEL INTERACTIONS IN PULMONARY INFLAMMATION

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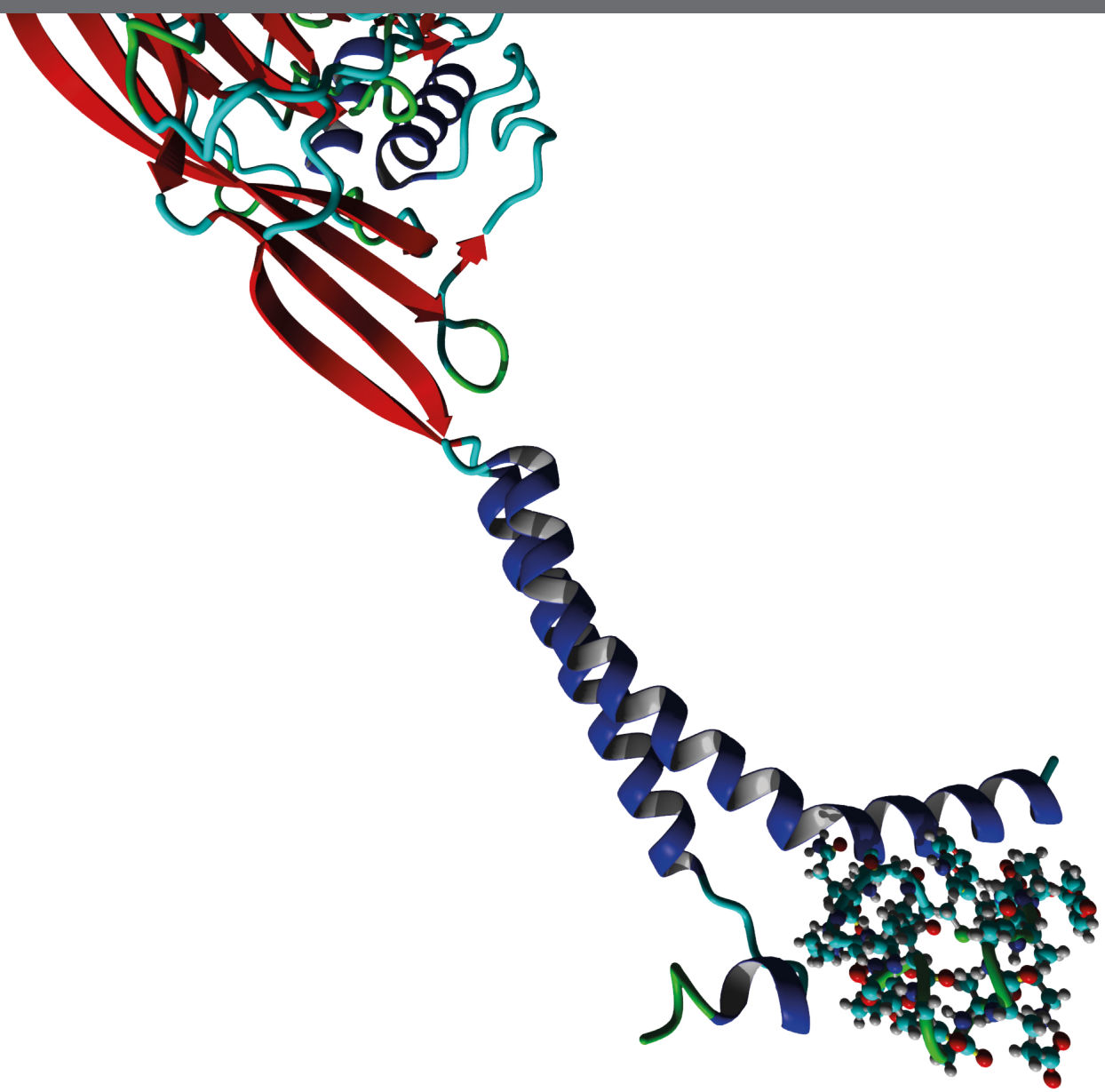

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CYTOKINE-ION CHANNEL INTERACTIONS IN PULMONARY INFLAMMATION

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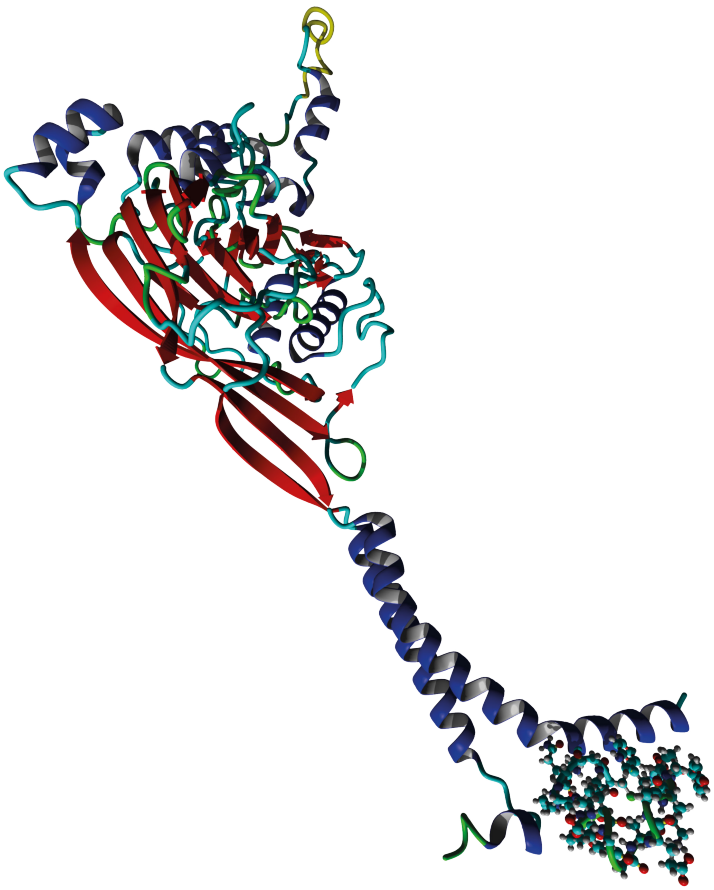

Molecular docking of the TNF-derived TIP peptide to a ASIC1a-based model of the alpha subunit of the epithelial sodium channel (ENaC)
Image: David Kaftan, PhD.
David Kaftan is currently affiliated to Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic.

This Research Topic assembles original contributions and reviews from an international consortium of PIs related to interactions between pro-inflammatory cytokines and ion channels during acute lung injury and chronic heart failure.

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EDITORIAL
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Editorial: Cytokine-Ion Channel Interactions in Pulmonary Inflammation

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Editorial on the Research Topic

Cytokine-Ion Channel Interactions in Pulmonary Inflammation

The remarkable architecture of our airways, with 23 levels of branching from the trachea to the approximately 600 million alveoli in an adult lung, accounts for an alveolar epithelial exchange surface of up to 100 m^2; several 100-fold higher than the surface area of a sphere with a similar volume. These alveoli, together with the surrounding capillaries, form the air-blood barrier, the primary site of gas exchange, allowing O_2 uptake from the alveolar space and CO_2 removal from the blood (1). This complex, but vulnerable system requires that alveoli remain relatively dry and that alveolar-capillary barriers are tight, as assured by tight and adherens junction proteins (2). Ion channels and transporters are crucially involved in keeping the alveolar space dry by promoting alveolar liquid clearance (ALC). Vectorial sodium transport through the apically expressed epithelial sodium channel (ENaC) and the basolateral sodium-potassium pump (Na^+,K^+-ATPase) in type II and I alveolar epithelial cells mediates reabsorption of liquid from the alveolar into the interstitial space, from where it is removed by the lymphatic system Hamacher et al.; Huppert and Matthay; Wynne et al.; Peteranderl et al.; Vadász and Sznajder. Unfortunately, under clinical conditions such as acute lung injury (ALI), ARDS or severe pneumonia, vectorial Na^+ transport is often impaired (3).

In this Research Topic contributed by an international consortium of researchers, several novel aspects of cytokine-ion transporter interactions are reported in the context of lung inflammation and injury. Wynne et al. review how the disturbed balance between pro- and anti-inflammatory cytokines (such as TNF-α, TGF-β1, IL-1β and IFN-γ, and IL-10 and IL-1 receptor antagonists) can shift the system to impaired ALC and to development of pulmonary edema. The authors focus on mechanisms by which pro-inflammatory cytokines affect surface expression, maturation and open probability of ENaC. Apart from impaired ALC, also compromised alveolar-capillary barriers can accompany ARDS, ALI and severe pneumonia. Hamacher et al. provide an overview of recent literature on how inflammation depends on complex and time-dependent co-signaling, interactions between the involved cell types, as well as on cell demise and barrier dysfunction. They reflect on how fluid reabsorption can still function in the presence of impaired alveolar epithelial-capillary barriers and reduced expression of ion transporters, with a special emphasis on ischemia-reperfusion lung injury upon lung transplantation.

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Because the alveolar-epithelial barrier is an integral component of the mammalian innate immune system, the effects of bacterial and viral infections of the distal airways on Na^+ transporters, ALC and integrity of the barrier itself are of high pathophysiological and clinical relevance. In a series of *in vivo* and *in vitro* studies, Brazee et al. demonstrate a central role for the pro-inflammatory protein FXYD5, a tissue-specific regulator of the Na^+,K^+-ATPase expression, which is upregulated in alveolar epithelial cells upon Gram-negative infection, in alveolar barrier damage secondary to lipopolysaccharide treatment. The authors establish that this detrimental effect is primarily mediated by NF-κB-dependent epithelial production of C-C chemokine ligand-2 (CCL2) and subsequent recruitment of monocytes to the lung. A better understanding of the mechanisms by which alveolar epithelial FXYD5 modulates cytokine and chemokine expression may foster the development of new therapies for the treatment of pulmonary inflammation following exposure to various Gram-negative bacteria. Peteranderl et al. expand on how alterations of ion transport function during influenza a virus (IAV) infection serve as an additional feedback loop on the respiratory inflammatory profile, that may further aggravate disease progression. They moreover discuss the recently discovered role of IFN-γ and of lung macrophage-derived TRAIL in Na,K-ATPase function and ALC. The authors critically review recent preclinical studies modulating alveolar–capillary fluid homeostasis and how these data may foster the development of novel therapeutic approaches to improve outcomes in IAV-induced lung injury. Coates et al. demonstrate how pulmonary damage inflicted by the immune response to IAV may be as important to the development of severe lung injury as the cytotoxic effects of the virus itself, especially in children. The authors highlight how activation of the NOD-like receptor protein 3 (NLRP3) inflammasome by the IAV matrix 2 (M2) proton channel and the subsequent secretion of the inflammatory cytokines IL-1β and IL-18 induce alveolar-epithelial damage and pulmonary edema under these conditions. In view of the failure of the IL1 blocking agent anakinra to improve lung injury in juvenile mice with IAV infection, the authors postulate that strategies blunting activation of NLRP3 rather than blocking certain pro-inflammatory cytokines, might be more successful to treat IAV pneumonia and IAV-associated respiratory distress, especially in children.

Patients with extensive permeability edema require ventilation strategies. However, ventilation itself may further damage the already injured lungs (4) (ventilator-induced lung injury, VILI), by augmenting inflammation and barrier dysfunction and by reducing ALC. The pro-inflammatory cytokine TNF-α, the generation of which is significantly increased in ARDS patients, plays a crucial role in the pathogenesis of VILI. TNF-α binds to two types of membrane receptors, TNF receptor 1 (TNF-R1), which carries a death domain and thus signals apoptosis, and TNF-R2, which is not a death receptor. TNF-R1 was demonstrated to mediate VILI in mice, whereas TNF-R2 rather plays a protective role (5). Using ventilated as well as spontaneously breathing acid aspiration-induced ALI mouse models, Wilson et al. investigates whether intratracheal or intranasal pretreatment of animals with a TNF-R1 (p55)-targeting domain antibody (dAb) can partially rescue the ALI phenotype. The study establishes that TNFR1-targeting dAb attenuates lung injury and edema formation in both models of acid-induced ALI, with a protection from a single dose lasting up to 24 h. Apart from its TNF receptor binding sites, TNF-α also carries a spatially distinct functional domain, which has lectin-like activity and which can be mimicked by a 17 residue peptide, the TIP peptide (a.k.a. AP301 and Solnatide) (6). The TIP peptide directly binds to the α-subunit of ENaC and as such increases both the surface expression and the open probability of the channel (7), even in the presence of bacterial toxins, such as the pore-forming toxin pneumolysin, the main virulence factor of *Streptococcus pneumoniae*. The TIP peptide in a phase 2a clinical trial in ALI patients significantly improved liquid clearance in a sub-group of patients with a SOFA score > 11 (8). Willam et al. demonstrate that the TIP peptide can activate ENaC channels displaying frameshift mutants of the α-subunit associated with pseudohypoaldosteronism type 1B (PHA-1B), a rare, life-threatening, salt-wasting disease. ENaC-α is however also a subunit of the recently discovered hybrid non-selective cation (NSC) channels in alveolar epithelial cells, together with the acid sensing ion channel 1a (ASIC-1a). Czikora et al. present original data demonstrating that apart from alveolar epithelial cells, also capillary endothelial cells express both active ENaC and NSC channels and that binding of TIP peptide to ENaC-α protects capillary barrier function in pneumolysin-treated human lung microvascular endothelial cells. These data thus indicate that the ENaC-α subunit, apart from playing a crucial role in ALC in the alveolar epithelium, can also strengthen barrier function in the capillary endothelium. Recent studies have moreover shown a protective role of this ENaC subunit, as well as of the β1 subunit of the Na-K-ATPase, in LPS-induced ALI in mice (9, 10). It is important to note that mechanisms impairing barrier function in alveolar epithelial cell monolayers can also negatively affect ENaC expression, at least partially in a transient receptor potential vanilloid 4 (TRPV4)-dependent manner (11).

Gas exchange disturbances secondary to severe pulmonary edema lead to hypoxia and hypercapnia. While O_2 supplementation and mechanical ventilation improve hypoxia in most cases, lung protective ventilation settings (required to limit VILI) often lead to further CO_2 retention. Vadász and Sznajder discuss how hypoxia and hypercapnia by distinct and specific molecular mechanisms impair the function of the Na,K-ATPase and ENaC, and as such blunt ALC and lead to persistence of alveolar edema. They highlight recent discoveries in sensing and signaling events initiated by hypoxia and hypercapnia, which may promote the identification of potential novel therapeutic targets in the treatment of ARDS. Gwoździńska et al. demonstrate the molecular mechanism by which elevated CO_2 levels promote activation of inflammatory signaling pathways. These in turn facilitate phosphorylation, ubiquitination and subsequent endocytosis of ENaC-β, thereby impairing ENaC activity and ALC. Optimal gas exchange requires the integrity of the alveolar-capillary barrier and an effective ALC. As respiratory failure is a consequence of acute barrier disruption in patients with ARDS, several recent studies

have focused on mechanisms that may promote both barrier repair and upregulation of ALC. Huppert and Matthay present an elegant overview demonstrating that mesenchymal stem cells (MSCs) have the capacity to both improve alveolar epithelial barrier integrity and ion channel function, including ENaC, thus improving alveolar fluid balance. As such, MSCs might represent a promising therapeutic candidate for treating ARDS.

It is important to note that while the function of the Na^+ transporters, ENaC and Na^+,K^+-ATPase are critical to maintain an optimal alveolar fluid balance, the function of some other channels expressed in the distal lung epithelium and/or endothelium can foster pathological mechanisms leading to pulmonary edema. Scheraga et al. discuss how the mechano-sensitive cation channel TRPV4 affects cytokine secretion and pulmonary inflammation in asthma, cystic fibrosis, pulmonary fibrosis and ARDS. Whereas, TRPV4 alters mucociliary clearance and epithelial cell pro-inflammatory cytokine/chemokine secretion in CF, in asthma the channel mediates hypotonicity-induced airway hyper-responsiveness, but not release of Th2 cytokines. Moreover, in pulmonary fibrosis, TRPV4 mediates mechano-sensing that drives myofibroblast differentiation and experimental lung fibrosis. Recently, TRPV4 activation was demonstrated to impair ENaC-α subunit expression in alveolar epithelial cells (11). Malczyk et al. review recent data on the deleterious role of the canonical or classical transient receptor potential channel 6 (TRPC6), a Ca^{2+}-permeable non-selective cation channel widely expressed in the lung and vascular tissues, in pulmonary vascular remodeling in idiopathic pulmonary arterial hypertension and in endothelial barrier disruption in ALI. Whereas, TRPC6 activators may be useful to redirect blood flow from non-ventilated regions to oxygen-rich regions of the lungs to avoid life-threatening arterial hypoxemia, TRPC6 inhibitors might represent a valuable therapeutic approach in excessive vascular remodeling or enhanced endothelial permeability.

Patients with heart failure often present with alveolar edema, primarily as a consequence of increased hydrostatic gradients secondary to elevated pulmonary vascular pressures. In contrast to severe pneumonia and ARDS, the alveolar epithelial-capillary barrier remains intact under these conditions. Azzam et al. highlight the role of the Na^+/H^+-exchanger (NHE) in the intracellular pH-dependent induction of pro-inflammatory cytokine generation, as can occur during acutely increased left atrial pressure or during chronic heart failure. Paradoxically, although the ability of the lungs to clear edema is impaired in acutely increased left atrial pressure, in chronic heart failure (CHF) ALC is mostly increased, particularly in compensated CHF. The authors discuss whether pro-inflammatory cytokines have a causal role in CHF pathology or whether they rather represent biomarkers for disease prognosis. They moreover critically review recent clinical trials with anti-inflammatory agents, such as the IL1 blocker anakinra in this context. Weidenfeld and Kuebler review recent data demonstrating that an acute increase in left atrial pressure (a model of acute heart failure) inhibits amiloride-sensitive Na^+-uptake across the alveolar epithelium. They also discuss the concomitant stimulation of Na^+- and Cl^--uptake via the basolaterally-expressed Na^+-K^+-Cl^- cotransporter 1 (NKCC1) and Cl^--secretion into the alveolar space via apically-expressed CFTR under these conditions. This may lead to Cl^--driven alveolar liquid secretion counteracting Na^+-driven ALC, representing an active mechanism that drives formation of alveolar edema. In line with this notion, they demonstrate that inhibition of CFTR and NKCC1 not only blocks active alveolar liquid secretion but, via a feedback loop, also improves ALC and therefore attenuates edema formation. As such, anti-CFTR, anti-NKCC1, anti-NHE and anti-inflammatory therapies may hold promise to improve cardiogenic edema.

In summary, this Research Topic provides the reader with a combination of original and review contributions in order to present an update and an overview of the interactions between pro-inflammatory cytokines and ion transporters regulating alveolar fluid balance with relevance to pulmonary disease states, such as viral and bacterial pneumonia, ischemia-reperfusion-induced lung injury, VILI, ARDS, pulmonary hypertension and acute and chronic heart failure. Although this article series by no means addresses all aspects of this complex matter, these manuscripts may nevertheless foster the development of novel therapies toward alveolar-capillary barrier dysfunction and pulmonary edema.

AUTHOR CONTRIBUTIONS

IV and RL edited this Research Topic and have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Regulation of Lung Epithelial Sodium Channels by Cytokines and Chemokines

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Acute lung injury leading to acute respiratory distress (ARDS) is a global health concern. ARDS patients have significant pulmonary inflammation leading to flooding of the pulmonary alveoli. This prevents normal gas exchange with consequent hypoxemia and causes mortality. A thin fluid layer in the alveoli is normal. The maintenance of this thin layer results from fluid movement out of the pulmonary capillaries into the alveolar interstitium driven by vascular hydrostatic pressure and then through alveolar tight junctions. This is then balanced by fluid reabsorption from the alveolar space mediated by transepithelial salt and water transport through alveolar cells. Reabsorption is a two-step process: first, sodium enters *via* sodium-permeable channels in the apical membranes of alveolar type 1 and 2 cells followed by active extrusion of sodium into the interstitium by the basolateral Na^+, K^+-ATPase. Anions follow the cationic charge gradient and water follows the salt-induced osmotic gradient. The proximate cause of alveolar flooding is the result of a failure to reabsorb sufficient salt and water or a failure of the tight junctions to prevent excessive movement of fluid from the interstitium to alveolar lumen. Cytokine- and chemokine-induced inflammation can have a particularly profound effect on lung sodium transport since they can alter both ion channel and barrier function. Cytokines and chemokines affect alveolar amiloride-sensitive epithelial sodium channels (ENaCs), which play a crucial role in sodium transport and fluid reabsorption in the lung. This review discusses the regulation of ENaC *via* local and systemic cytokines during inflammatory disease and the effect on lung fluid balance.

Keywords: lung, sodium channels, epithelial sodium channel, cytokines, physiology, inflammation, acute lung injury, acute respiratory distress syndrome

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INTRODUCTION

The maintenance of a thin fluid layer on the surface of the alveolar epithelium is critical for respiration. Two primary mechanisms regulate this fluid layer: Starling's forces and active sodium (Na^+) transport. Starling's forces determine the movement of water from intravascular to extravascular or interstitial spaces caused by hydrostatic and oncotic pressures. An increase in pulmonary vascular pressure accounts for the increased alveolar flooding seen in cardiogenic pulmonary edema. However, the other regulator of the thickness of the alveolar fluid layer is the active transport of Na^+, followed by potential-driven anion movement through cystic fibrosis transmembrane

conductance regulator, and the aquaporin-mediated transport of water. The epithelial sodium channel (ENaC) is critical in the maintenance of the epithelial fluid layer. This review focuses on the primary physiological mechanisms required to maintain and regulate this layer and is an overview of the pathophysiological mechanisms of cytokine-mediated ENaC regulation in the lung (**Figure 1**).

PULMONARY PHYSIOLOGY

The primary function of the airways is exchange of gases; thus, both the anatomy and physiology of the lung have evolved to distribute gases efficiently. The diffusion of gases is facilitated in the alveoli by the large total surface area, coupled with thin, yet strong and elastic membranes (1). Human lungs are composed of a series of branched tubes, where conducting airways lead to the terminal respiratory units that are in close proximity to the vasculature (2–4).

The primary respiratory units, or alveoli, are composed of a single, polarized, epithelial cell layer that separates a gas-filled compartment and the pulmonary circulation (5). The two predominant cell types in this cell barrier are the squamous type I (AT1) and cuboidal type 2 (AT2) cells. The majority of the alveolar surface area consists of AT1 cells: the remainder of the area (≈2–5%) is AT2 cells. Both cell types contribute to alveolar fluid transport (6–10). These cells are responsible for Na^+ transport from the apical to basolateral surface and maintenance of a thin layer of isotonic fluid on the alveolar surface. The AT2 cells have an additional function: they are also responsible for the secretion of surfactant, which is necessary to lower the surface tension at the interface of air and water and increase lung compliance. Overall, this anatomical structure and physiology ensures that the alveolar spaces remain open for gas exchange.

Paradigm for Fluid Transport: Role of the ENaC

Regulation of the fluid interface occurs primarily through regulating Na^+ uptake *via* ENaC in both AT1 and AT2 cells. After ENaC-mediated entry of Na^+ across the apical membrane, Na^+ leaves the cell across the basolateral membrane *via* the Na^+–K^+ ATPase and enters the interstitium where it is in equilibrium with vascular Na^+. Some investigators have suggested that regulation of the ATPase also plays a role in controlling trans-epithelial Na^+ transport (11–15); however, we will not consider ATPase regulation in this review. The paradigm in which vectorial Na^+ transport is considered a primary drive for fluid transport from the alveolar surface has been established by numerous studies where pharmacological inhibitors of apical Na^+ channels have been shown to reduce the rate at which fluid is cleared (16–21).

Regulation of ENaC in the Airway

Epithelial sodium channel is composed of three homologous subunits, such as α, β, and γ. Together, these subunits assemble in the endoplasmic reticulum and traffic to the apical membrane and are highly selective for Na^+ (22). Using ENaCα-subunit knock-out mice, investigators first showed the importance of

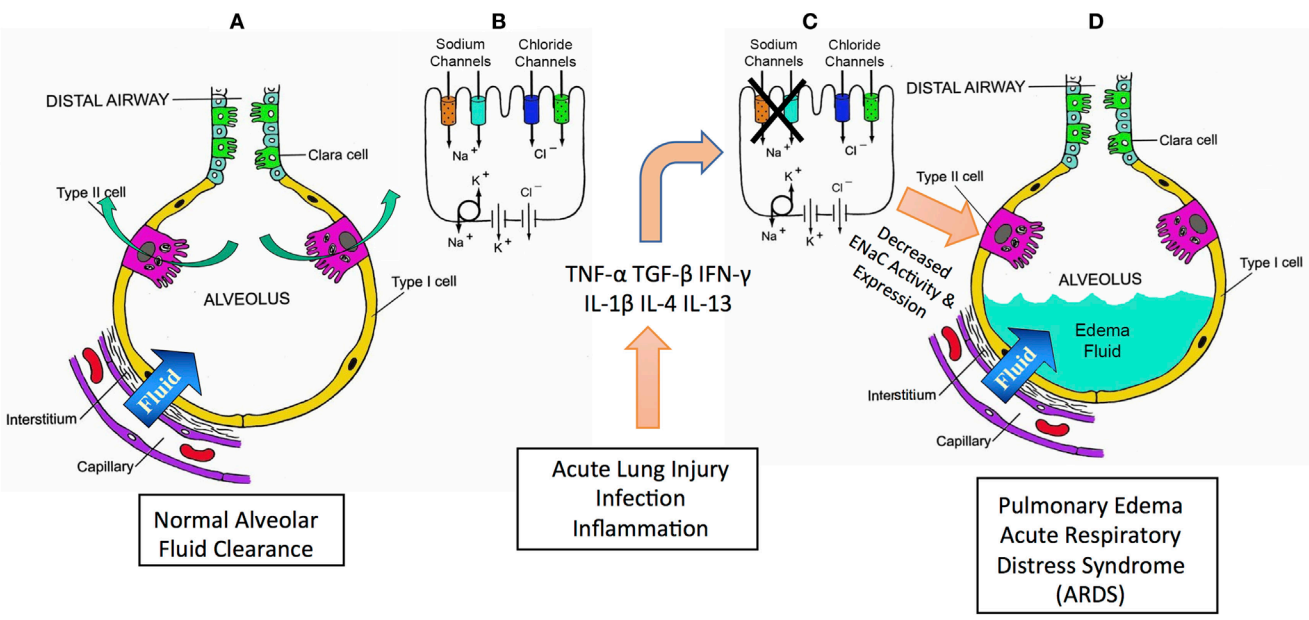

FIGURE 1 | Summary schematic for role of cytokines in mediating pulmonary edema and acute respiratory distress syndrome (ARDS). Acute lung injury (ALI), ARDS, and pneumonia are all pathologies characterized by lung edema and alveolar flooding. Pneumonia mortality is typically caused by flooding of the pulmonary alveoli, preventing normal gas exchange and consequent hypoxemia. Airways normally have a critically regulated fluid layer essential for normal gas exchange and removal of foreign particulates from the airway **(A)**. Maintaining this fluid layer in the alveoli depends critically on sodium reabsorption mediated by epithelial sodium channels (ENaCs) and CFTR chloride channels **(B)**. During ALI, sepsis, inflammation or infection, inflammatory cytokines are produced that inhibit ENaC **(C)**. A decrease in ENaC reabsorption allows fluid to accumulate in the alveoli causing alveolar flood in loss of normal gas exchange and consequent hypoxemia **(D)**.

ENaCα for proper lung function: neonates lacking ENaCα died within 40 h of birth (23). The α subunit is the ionophoric component of the heteromultimer and is required for the expression and assembly of functional ENaCs at the apical membrane. The importance of ENaC to normal lung function is underscored by the phenotype of several monogenetic disorders that affect ENaC. Patients with pseudohypoaldosteronism (PHA), a condition resulting from ENaC partial loss-of-function, were found to have twice the volume of airway surface liquid compared normal levels (24). Mice lacking the ubiquitin ligase, NEDD4-2, had increased levels of ENaC expression and increased ENaC-mediated current in AT2 cells (25). Additionally, overexpression of ENaCβ in an ENaCβ transgenic mouse model leads to airway dehydration and mucous obstruction, comparable to many features observed in cystic fibrosis (CF) (26). Together, these studies highlight the importance of proper ENaC expression and regulation for the airways.

Understanding the regulation of ENaC is significant for understanding lung fluid balance, as ENaC dysregulation is the source of pathological lung edema. In recent years, probably because monogenetic disorders often alter ENaC trafficking, much of the focus has examined how regulation of the number of channels at the apical membrane of alveolar epithelial cells can alter Na^+ transport. However, since ENaC is an ion channel, regulating how much of the time the channel spends open (the open probability, P_o) is also important. Both Liddle's syndrome and PHA type 1 (PHA 1) are conventionally described as changes in channel density (an increase and decrease, respectively); however, examination of single ENaCs in these two syndromes shows that ENaC P_o also changes. There are an observed increase in channel activity in Liddle's (27) and a decrease in activity in PHA I (28). Steroid hormones increase Na^+ transport and are often thought to do so by increasing subunit transcription and translation. Although Frindt and Palmer (29) have shown in Na^+-transporting epithelial tissue that this is indeed true, the increase in subunit density accounts for less than 25% of the increase in trans-epithelial Na^+ current implying that the remaining 75% is due to an increase in single channel P_o. Single channel recordings show that acute application of steroids dramatically increases single channel P_o (30–32).

Kleyman, Hughey, and their co-workers have shown that the α and γ subunits of ENaC must be proteolytically cleaved to be active loops (33–37). Some investigators have suggested that such cleavage might be a mechanism by which ENaC in the apical membrane could be regulated. In fact, proteolysis does appear to be required for ENaC to have any appreciable activity, and may be required for it to reach the membrane. As such, proteolysis appears to be, more or less, an all-or-none phenomenon: channels that are uncleaved are capable of little if any activity. However, under conditions of normal Na^+ transport most channels are cleaved. Under these conditions, cleaved channels are capable of a wide range of activity by changing their P_o.

Changes in membrane ENaC can occur by changing the rate of insertion into the membrane after transcription and translation (38). However, in any time frame less than 24 h, ENaC in the membrane is altered by recycling from intracellular pools into the membrane (22) or internalization of ENaC into recycling or degradative pools. Removal of ENaC occurs primarily *via* the ubiquitin ligase, NEDD 4-2, which targets ENaC for removal and proteosomal degradation (39, 40).

Therefore, in this review, we address both the regulation of P_o in cleaved channels and change in membrane channel protein density.

The regulation of ENaC occurs *via* multiple, redundant systems to ensure that Na^+ transport is not limited. ENaC is regulated by a many agents including transmitters interacting with G-protein-coupled receptors (GPCRs), circulating hormones, cytokines and chemokines, and reactive oxygen and nitrogen species. The regulation of ENaC *via* hormones and GPCRs is not a primary focus of this review, but we briefly review ENaC activation and regulation *via* steroids since their actions often interact with the activities of cytokines and chemokines.

In the lung, the glucocorticoid receptor (GR) is the primary receptor for corticosteroids (41–43). Once activated, the GR activates response elements inducing the transcription of signaling kinases, such as the serum- and glucocorticoid-regulated kinase 1 (30, 44, 45). Ligand-mediated activation of the GR *via* corticosteroids is used clinically as an anti-inflammatory treatment. The positive effects of corticosteroid therapy lie in the ability of the GR to bind to and inhibit nuclear factor kappa-light-chain-enhancer of activated B cells (Nf-κB) (46–48). Nf-κB is an important mediator of cytokine signaling. This transcription factor increases cyclooxygenase 2-induced prostaglandin production, as well as increases other proinflammatory factors (49). Corticosteroids reduce inflammation propagated *via* Nf-κB-mediated mechanisms but may not affect inflammation mediated by other signaling pathways. Indeed, GR activation may actually augment some downstream signaling pathways, such as those mediated through Smad proteins (50). This distinction is important because of the many heterogeneous pathways activated by each cytokine (51–54).

PULMONARY PATHOPHYSIOLOGY

Regulation of the air/water interface is crucial for gas exchange, as the amount of the alveolar fluid layer must be precise. With injury, the inability of the lungs to clear this fluid can lead to pulmonary edema. Increased fluid accumulation can result from compromised ENaC function or when there is an asymmetrical hydrostatic force from the vasculature, pushing fluid from capillaries into the alveolar space (e.g., pulmonary hypertension) (5). In addition, tight junctions that maintain structural integrity and a tight epithelial layer can be disrupted, resulting in increased permeability. In fact, high levels of pulmonary inflammation causing airway tight junction damage that compromise alveolar barrier function is a primary cause of epithelial injury (55). And lastly, many proinflammatory and noxious factors cause changes in Na^+ transport. Dysfunction in any single factor can lead to a dysregulation of the alveolar fluid (5). Because of the increased morbidity and mortality associated with alveolar fluid accumulation, understanding the mechanisms that regulate these factors are vital.

Immune Responses in the Airway

During inflammation most cells are capable of secreting a variety of small molecular weight proteins, called cytokines and

chemokines, which communicate the inflammatory signals. In the airway, resident immune cells are mostly the alveolar macrophages; however, during infection or inflammation, other mononuclear and granular immune cells infiltrate (56). Several studies have proposed a role for the airway epithelium in propagating the immune response, especially as a "first responder" since the airway is the first to sense viral and bacterial pathogens as they enter the body. This layer can be an active participant in the immune response, producing a variety of cytokines and chemokines, as well as exclusive epithelial-derived cytokines (55, 57, 58).

Direct interaction with pathogens, such as influenza, reduces ENaC activity (59). However, other evidence suggests that some of the more chronic effects of pathogens may be *via* noxae-stimulated chronic cytokine production (60–62). Additionally, inflammatory activation of the airway epithelium can result in local nitric oxide (NO) production, most likely *via* increased cytokine production, further reducing ENaC activity and fluid transport (59, 63–67). Cytokines frequently increase local levels of reactive oxygen species (ROS) as well. Interestingly, ROS has been shown to activate ENaC at relatively low concentrations but to inhibit ENaC at higher concentrations often associated with massive pathogen-induced cytokine production (40, 68, 69). The overall redox environment of the alveoli is crucial and can rapidly change, often driven by high levels of Rac1-NADPH oxidase activity in AT1 cells (67, 70).

CYTOKINE-MEDIATED REGULATION OF ENaC

Some of the earliest studies revealed a correlation between large and sustained proinflammatory cytokine increases in bronchioalveolar lavage (BAL) fluid and an unfavorable outcome in acute respiratory distress syndrome (ARDS) (71). Overall, increased cytokine levels from lung injury can quickly lead to the accumulation of alveolar fluid, edema, and then acute respiratory distress. Thus, proinflammatory cytokines and chemokines produce a feed forward cycle decreasing lung Na^+ transporter expression, as well as activity.

Regulation of ENaC *via* Tumor Necrosis Factor (TNF)-α

The TNF super family comprises 19 members and was originally named for its role in apoptosis (53). The best-studied member of this family is TNF-α, which plays a role in propagating the immune response and secretion of other cytokines. TNF-α was implicated in the pathogenesis of pulmonary edema, and increased levels were observed in patients with ARDS (72, 73). Monocytes and macrophages produce significant TNF-α, but it is also produced by alveolar epithelial cells following lipopolysaccharide stimulation (74).

Although TNF-α can bind to two different receptors that are linked to separate signaling pathways, much of the work in the airway has focused on TNF receptor 1. The effect that TNF-α elicits on ENaC function, and alveolar liquid clearance, seems to be critically dependent upon receptor activation or receptor-independent mechanisms and has been shown using both *in vitro* and *in vivo* models (75–78).

Tumor necrosis factor receptor 1-mediated activation of NF-κB increases cytokine (IL-1, IL-8, IL-6) and chemokine production. It also increases the expression of adhesion molecules including selectins, vascular cell adhesion molecules, and intercellular adhesion molecule (ICAM)-1 (53, 79, 80). In freshly isolated AT2 cells, TNF-α decreased α- and γ-ENaC mRNA and protein levels and reduced amiloride-sensitive trans-epithelial current (75, 78).

Tumor necrosis factor-α also plays an especially important role in endothelial activation, as well as disturbing the epithelial tight junction barrier. Disruption of the tight junctions not only leads to respiratory distress and increased exudate but also may reduce alveolar fluid clearance as well (81). TNF-α reduces the expression of tight junction proteins, including the claudins and zonula occludens protein 1, thus increasing alveolar permeability (55, 82). Consequently, TNF-α has a critical and multi-faceted role in the development of ARDS. TNF-α not only regulates Na^+ and water clearance but also disrupts tight junction barriers and endothelial integrity and contributes to a pro-inflammatory environment.

Interestingly, TNF-α contains not only a receptor-binding domain but also a lectin-like domain (referred to as a TIP domain) that is spatially distinct from the receptor-binding site (83, 84). TNF-α produces an opposite response when there is binding of the lectin-like domain, or TIP, to certain oligosaccharides at high concentrations of TNF-α. This process increases Na^+ uptake in AT2 cells and may account for the differential responses to TNF-α (85–87). *In vivo*, a peptide analog of TIP increased clearance in a murine flooded-lung model (85). Czikora and colleagues also demonstrated that this TIP domain directly binds to and, then, activates ENaC (83). This implies an endogenous mechanism to limit the effects of high TNF-α concentrations. Use of this may become a novel method in counteracting reduced alveolar clearance.

Regulation of ENaC *via* Transforming Growth Factor (TGF)-β1

Transforming growth factor-β1 is a pathogenic cytokine, which has been implicated in the early phase of acute lung injury (ALI) prior to ARDS (72, 88). TGF-β1 levels were increased in ARDS patients compared to healthy controls (89). Furthermore, active TGF-β1 levels were more than doubled in the epithelial lining fluid from ARDS patients (90). As mentioned earlier, corticosteroids are a common tool to reduce inflammation and aid in lung clearance. Interestingly, TGF-β actually reduces the ability to produce multiple steroids, possibly leading to the inability for self-healing and furthering inflammatory damage, in addition to the activation of multiple Smad pathways (50, 52). Some of these pathways may be insensitive to corticosteroid treatment. However, there is still much to learn regarding which Smad-mediated pathways are downstream of TGF-β signaling during ALI and ARDS.

Other studies have specifically have explored the role of TGF-β in alveolar flooding. Using a bleomycin-induced lung injury model, TGF-β1-inducible genes were dramatically

increased as early as 2 days, suggesting that TGF-β1 may precede alveolar flooding (91). Of interest, TGF-β may actually remain latent locally, covalently attached to a latency-associated peptide (LAP); pulmonary epithelial cells can activate and cause dissociation of TGF-β from LAP (92–94). One member of the integrin family, αvβ6, was recently shown to be a ligand for LAP (93). αvβ6 is expressed normally at lower levels, yet increased significantly with injury revealing a novel mechanism for rapid and local TGF-β activation (95). TGF-β is also redox sensitive, and *in vitro* models of increased ROS *via* ionizing radiation revealed another mechanism for TGF-β activation (96). Together, these studies show multiple, redundant possibilities for systemic and paracrine TGF-β activation during lung injury.

One of the first studies to directly implicate TGF-β1 in regulating ENaC was by Frank and colleagues. They showed that TGF-β1 reduced amiloride-sensitive Na^+ transport in lung epithelial cells. Additionally, TGF-β1 reduced αENaC mRNA and protein expression *via* an ERK1/2 pathway in a model of ALI, thus promoting alveolar edema (97). *In vivo* studies then showed that TGF-β1 reduces vectorial Na^+ and water transport and that this process occurs independently from increases in epithelial permeability (97, 98). Interestingly, TGF-β was also found to have an integral role in ENaC trafficking. Peters and colleagues were the first to demonstrate this acute regulation of ENaC in the lung; they found that TGF-β induces ENaC internalization *via* interaction with ENaCβ (99). In summary, TGF-β has been implicated in multiple mechanisms reducing ENaC expression and apical localization, thus contributing to the pathophysiology of ARDS and pulmonary edema (100).

Regulation of ENaC by Interferon-γ

The interferons (IFN) are a family of proteins originally classified by their ability to reduce viral replication. This family consists of both Type I and Type II IFNs; INF-γ is the only member of the Type II IFN family and is structurally unrelated to the other IFNs. During inflammation, INF-γ is secreted by multiple immune cells, but mostly by T lymphocytes. INF-γ increases ICAM-1 levels and increases NO production *via* inducible nitric oxide synthase. Little is known about the role of INF-γ in ENaC regulation; however, studies using human bronchial epithelial cells (BECs) showed that INF-γ treatment significantly reduced trans-epithelial Na^+ transport in normal human BECs (101).

Regulation of ENaC by the Interleukins: IL-1β, IL-4, and IL-13

ENaC Regulation by IL-1β

Several interleukins are correlated with the early stages of ALI; however, the best studied is IL-1β. This cytokine plays a diverse role in the pathogenesis of ALI and ARDS. IL-1β levels are increased in the BAL fluid, as well as the pulmonary edema fluid, of patients with ALI (102–106). IL-1β levels are higher in the pulmonary lavage fluids compared to serum suggesting that there is a local, pulmonary source for IL-1β similar to that of TGF-β (71, 104). An earlier study by Pugin and colleagues suggested that of the cytokines present in the BAL fluid, IL-1β is the most biologically active, and others have suggested that the source may be from early-infiltrating neutrophils (103, 107). BAL fluids from ARDS patients applied to AT2 cells increased ICAM-1 expression, while IL-1 inhibition reduced the increase in ICAM-1 (103).

IL-1β also seems to have significant effects on endothelial leakage and permeability. *In vitro*, IL-1β treatment significantly increased microvascular permeability (108). Several studies have also demonstrated that when given intratracheally, IL-1β increased endothelial permeability and lung leak (108–111). More recently, IL-1β has been shown to directly affect ENaC expression. Incubation with IL-1β reduced ENaC mRNA protein expression, possibly through promoter inhibition and a p38 MAPK-dependent mechanism. Additionally, IL-1β application reduced apical ENaC protein and amiloride-sensitive trans-epithelial current and Na^+ flux (112).

Other studies have tried to reverse IL-1β effects. *In vitro* modeling suggests that the reduction of IL-1β, *via* suppressor of cytokine signaling-1, can rescue the IL-1β-mediated suppression of ENaCs (113). When investigating patients with ALI, those who had an increased activation of the stress protein response (SPR) positively correlated with preserved alveolar clearance rates (114). Thus, activation of this SPR during immune-related injury may ameliorate effects of IL-1β, if used as a "preconditioning" agent (115).

ENaC Regulation by IL-4 and IL-13

Classically, increases in IL-4 and IL-13 are associated with an increased goblet-cell hyperplasia and mucous secretion. These cytokines are implicated in allergic airway diseases and CF and contribute to reduced ciliary movement reducing the ability to clear the airways. These related cytokines frequently share signaling cascades and receptor subunits, such as the IL-4 receptor (116). However, studies in airway epithelial cells from human bronchi suggest that these cytokines may also alter ion transport. IL-4 significantly reduced ENaC subunits γ and β; interestingly, αENaC levels were not altered. IL-4 and IL-13 treatments reduced amiloride-sensitive short circuit current (using an Ussing chamber), which was reversed with an IL-4 receptor antagonist (116). Although these studies were investigating allergic diseases, one could infer a similar involvement in a variety of other inflammatory conditions where there are increased IL-4/IL-13 levels and reduced ENaC function.

CONCLUSION: BALANCING THE INFLAMMATORY MILIEU

Delineating the role of pro-inflammatory cytokines is important for the understanding of alveolar flooding and ALI; however, the lack of anti-inflammatory cytokines also plays a crucial role in mediating the "balance" necessary for regulating the epithelial fluid lining. Studies have shown that there is an increased mortality when there are reduced levels of "anti-inflammatory" cytokines, such as IL-10 and the IL-1 receptor antagonists (117). Much work is needed to understand the diverse and redundant roles of cytokines in disease progression. Nonetheless,

pro-inflammatory cytokines seem to reduce the total expression, apical localization, and activity of ENaC in the lungs *via* multiple mechanisms (**Figure 1**). Given the prominent role for ENaC in maintaining alveolar fluid levels, understanding how inflammatory cytokines regulate ENaC will allow for the development of therapies to treat these complex diseases.

AUTHOR CONTRIBUTIONS

BW and DE conceived and wrote the manuscript. LZ, VL, H-PM, and RH edited and approved the manuscript.

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Alveolar Fluid Clearance in Pathologically Relevant Conditions: *In Vitro* and *In Vivo* Models of Acute Respiratory Distress Syndrome

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Critically ill patients with respiratory failure from acute respiratory distress syndrome (ARDS) have reduced ability to clear alveolar edema fluid. This reduction in alveolar fluid clearance (AFC) contributes to the morbidity and mortality in ARDS. Thus, it is important to understand why AFC is reduced in ARDS in order to design targeted therapies. In this review, we highlight experiments that have advanced our understanding of ARDS pathogenesis, with particular reference to the alveolar epithelium. First, we review how vectorial ion transport drives the clearance of alveolar edema fluid in the uninjured lung. Next, we describe how alveolar edema fluid is less effectively cleared in lungs affected by ARDS and describe selected *in vitro* and *in vivo* experiments that have elucidated some of the molecular mechanisms responsible for the reduced AFC. Finally, we describe one potential therapy that targets this pathway: bone marrow-derived mesenchymal stem (stromal) cells (MSCs). Based on preclinical studies, MSCs enhance AFC and promote the resolution of pulmonary edema and thus may offer a promising cell-based therapy for ARDS.

Keywords: acute respiratory distress syndrome, alveolar fluid clearance, mesenchymal stem (stromal) cells, pulmonary edema, vectorial ion transport

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INTRODUCTION

Pulmonary edema is the abnormal accumulation of fluid in the interstitium and air spaces of the lungs, which leads to impaired gas exchange and respiratory failure. Pulmonary edema can develop from increased pulmonary vascular pressure from left heart failure (cardiogenic pulmonary edema) (1) or from lung parenchymal damage from increased endothelial and epithelial permeability (non-cardiogenic pulmonary edema) (2). In both cases, the mechanism for the resolution of alveolar edema is the same: active ion transport across the alveolar epithelium creates an osmotic gradient that drives alveolar fluid clearance (AFC) (3). In the presence of acute lung endothelial and epithelial injury, there is complexity in describing the forces responsible for lung fluid clearance, meaning removal of edema from the lung itself. Net AFC does depend on an intact epithelial barrier that can transport ions from the apical to the basolateral surface and create a mini-osmotic gradient for alveolar fluid absorption. If transvascular fluid flux is increased across lung endothelium from increased pressure or increased permeability, then the rate of AFC will be reduced. Also, net lung fluid clearance will be less. Lung lymphatics do remove edema fluid in either hydrostatic or increased

permeability lung edema, but they cannot entirely compensate for an increase in transvascular fluid flux or impaired AFC.

Acute respiratory distress syndrome (ARDS) is a syndrome of acute respiratory failure caused by non-cardiogenic pulmonary edema. The most common cause of ARDS is bacterial or viral pneumonia (4). Sepsis due to non-pulmonary sources, trauma, aspiration, pancreatitis, transfusion reactions, and drug reactions can also lead to ARDS (4). Criteria for the diagnosis of ARDS have changed over time, but the current definition includes arterial hypoxemia with PaO_2/FiO_2 ratio less than 300 mmHg, bilateral radiographic opacities, without evidence of that is not fully explained by cardiac failure or fluid overload (5). The mortality of ARDS is approximately 25–40% (6), and treatment remains primarily supportive with lung protective ventilation and a fluid conservative strategy (7).

Because ARDS has a broad clinical phenotype, it has been challenging to translate cell and animal studies to pharmacologic therapies that reduce human morbidity and mortality. Nonetheless, *in vitro* and *in vivo* studies have produced important insights about the pathogenesis of this condition, paving the way for targeted therapeutics. This review will focus on: (1) mechanisms that mediate the clearance of pulmonary edema in the uninjured lung, (2) why AFC is reduced in ARDS, resulting in the accumulation of pulmonary edema fluid, and (3) one potential treatment for ARDS with a cell-based therapy that may accelerate the rate of AFC.

PULMONARY EDEMA FLUID CLEARANCE IN THE UNINJURED LUNG

Before discussing AFC in ARDS, it is first important to review how pulmonary edema fluid is cleared in the uninjured lung. In the uninjured lung, vectorial ion transport across the alveolar epithelial cells creates an osmotic gradient that drives fluid from the airspaces into the lung interstitum (**Figure 1**). It was initially thought that alveolar epithelial type II cells were the primary cell responsible for vectorial ion transport, but subsequent studies demonstrated an important role for type I cells as well (8). The transport of sodium ions is the most important driver for the generation of the osmotic gradient: sodium is transported through the sodium channel (ENaC) on the apical surface and then by the Na/K ATPase on the basolateral surface into the lung microcirculation (9, 10). Knockout of the alpha-subunit of ENaC in mice resulted in the inability to remove lung fluid at birth with subsequent respiratory failure and death (9). In addition, non-selective cation channels, cyclic nucleotide-gated channels, and the cystic fibrosis transmembrane conductance regulator chloride channel also contribute to the creation of the osmotic gradient (3, 11). Aquaporins facilitate the movement of water across the epithelial surface, but are not required for fluid transport (12).

This system of active ion-driven alveolar fluid reabsorption is the primary mechanism that removes alveolar edema fluid under both physiologic and pathological conditions (9, 13, 14). However, in the setting of ARDS, the capacity to remove alveolar edema fluid is reduced, which is termed impaired AFC. A reduction in the rate of AFC in ARDS correlates with decreased survival (15, 16). Therefore, it is critical to better understand why AFC is reduced in ARDS to better understand the pathogenesis of this condition.

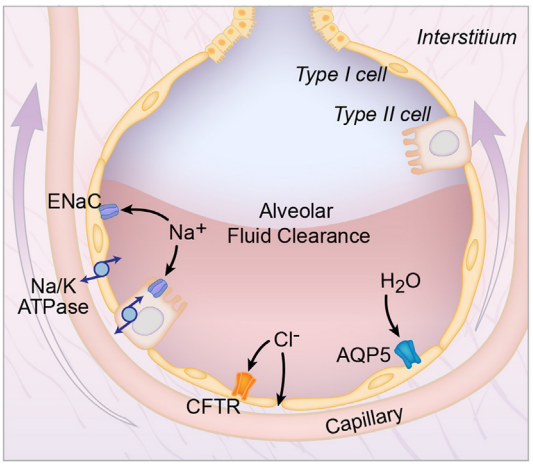

FIGURE 1 | Alveolar fluid clearance pathways. Shown are the interstitial, capillary, and alveolar compartments, with pulmonary edema fluid in the alveolus. Both type I (yellow) and type II (orange) alveolar cells are involved in transepithelial ion transport. Sodium (Na^+) is transported across the apical side of the type I and type II cells through the epithelial sodium channel (ENaC), and then across the basolateral side *via* the sodium/potassium ATPase pump (Na/K-ATPase). Chloride (Cl^-) is transported *via* the cystic fibrosis transmembrane conductance regulator (CFTR) channel or by a paracellular route. Additional cation channels also transport ions across the alveolar epithelium (not shown). This vectorial ion transport creates an osmotic gradient that drives the clearance of fluid. Specifically, water (H_2O) moves down the osmotic gradient through aquaporin channels, such as aquaporin 5 (AQP5) or *via* an intracellular route (not shown).

PULMONARY EDEMA FLUID CLEARANCE IN ARDS

Multiple mechanisms explain why AFC is reduced in ARDS. First, both hypoxia and hypercapnia impair AFC. ENaC transcription and trafficking is downregulated and Na/K-ATPase functions less efficiently under states of low oxygen or high carbon dioxide, in part, because reactive oxygen species trigger endocytosis and cell necrosis (17–19). Therefore, supplemental oxygen and correction of hypercapnia can enhance the resolution of alveolar edema (17).

Second, biomechanical stress can reduce AFC. High tidal volumes and elevated airway pressures injure the alveolar epithelium, inducing cell death and inflammation, which reduces AFC (20). If pulmonary hydrostatic pressures are elevated, the rate of AFC is also reduced. These findings help explain the success of lung protective ventilation strategies and conservative fluid strategies in reducing the morbidity and mortality of ARDS (21, 22).

Third, ARDS pulmonary edema fluid contains high levels of pro-inflammatory cytokines including IL-1β, IL-8, TNFα, and TGFβ1 (23–25). Under controlled conditions, this inflammatory response is important for pathogen clearance. However, when excessive levels of cytokines are present, they instead can cause

alveolar injury and decreased AFC (26–29). Moreover, once the epithelial barrier is breeched and alveolar fluid is released, components of the alveolar fluid may be recognized to induce downstream inflammatory and immune responses (30). There are no current therapies that directly target this pathway in the treatment of ARDS, although lung protective ventilation itself reduces pro-inflammatory cytokines such as IL-6 and IL-8 (4, 31). The remainder of this review will summarize our current understanding of alveolar ion transport in ARDS based on pathologically relevant *in vitro* and *in vivo* models, and discuss one potential new therapy that targets this pathway.

In 2006, Fang et al. developed a model of a polarized human alveolar type II cell that facilitated *in vitro* studies of AFC (32) (**Figure 2**). Using this model, Lee et al. found that transepithelial fluid transport is less effective in the presence of ARDS edema fluid and found that there are increased levels of cytokines and decreased levels of ion transport proteins in the presence of ARDS edema fluid compared to a plasma control (33). These data support the hypothesis that cytokine expression is increased in alveolar epithelium during ARDS, resulting in a decreased expression of alveolar ion channels and accumulation of alveolar edema fluid. In addition, the inflammatory edema fluid can also cause alveolar cell injury and necrosis, resulting in altered epithelial tight junctions (34, 35). The loss of tight junctions can negate the osmotic gradient, offsetting the effects of vectorial ion transport.

Subsequent *in vivo* studies expanded upon these findings and demonstrated that net AFC was reduced under clinically relevant pathologic conditions in animal models. In sheep, live *Pseudomonas aeruginosa* decreased AFC at both 4 and 24 h, an effect, which was associated with decreased AFC (36). In a mouse model of influenza pneumonia, the authors demonstrated that there was decreased AFC due to inhibition of the ENaC epithelial sodium channel (37). These and other studies confirmed that AFC is reduced in lung injury through decreased efficiency of alveolar ion channels as well as altered permeability of the normally tight alveolar epithelium.

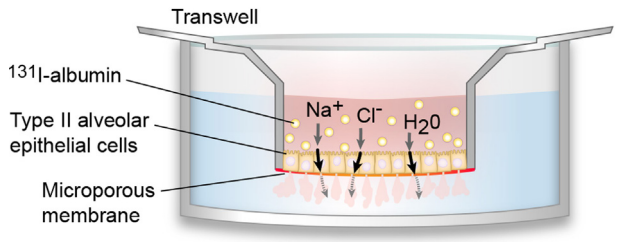

FIGURE 2 | *In vitro* model of polarized human alveolar type II epithelial cells. In 2006, Fang et al. developed an *in vitro* model of the polarized human alveolar epithelial surface, which has been used in multiple subsequent studies of alveolar fluid clearance (AFC) (32). To create this model, type II alveolar epithelial cells are isolated from human donor lungs and cultured on a collagen-I coated 24-well plate where they formed tight monolayers. Pulmonary edema fluid (pink), which contains water (H_2O), sodium ions (Na^+), chloride ions (Cl^-), as well as other ions and proteins, is mixed with ^{131}I-albumin (yellow circles) and introduced to the apical compartment. Pulmonary edema fluid is able to cross the alveolar cell monolayer, but ^{131}I-albumin cannot cross, so it is possible to calculate AFC by measuring the change in ^{131}I-albumin concentration between the apical and basal compartments.

MESENCHYMAL STEM (STROMAL) CELLS (MSCs) AS A PROMISING THERAPY FOR ARDS

The laboratory-based investigations described above significantly advanced our understanding of ARDS pathophysiology, paving the way for targeted molecular therapies to improve the clinical treatment of patients with ARDS. MSCs are one such promising new cell-based therapy, which we will discuss in this section.

Mesenchymal stem (stromal) cells are bone marrow-derived cells that can differentiate *in vitro* into chondrocytes, osteoblasts, and adipocytes, although they do not have true stem cell properties *in vivo* (38). MSCs secrete paracrine factors that can decrease inflammation and enhance endothelial and epithelial repair (39). Several groups are studying the therapeutic potential of MSCs in sepsis (40, 41), diabetes (42), myocardial infarction (43), hepatic failure (44), and acute renal failure (45, 46). It was hypothesized that MSCs might also be beneficial in the treatment of ARDS.

To test this hypothesis, several groups studied whether MSCs could reduce the severity of lung, kidney, and brain injury in preclinical models (47). In 2007, Gupta et al. reported that treatment with MSCs improved survival and reduced pulmonary edema in *Escherichia coli* endotoxin-induced lung injury in mice (48). Subsequent studies showed that MSCs attenuated lung injury in mice and in *ex vivo* human lungs injured with live bacteria (49, 50). MSCs also enhance bacterial clearance and improve survival in mouse and rat models of sepsis (41, 51), and they have beneficial effects in ventilator-induced acute lung injury (52). Based on this preclinical data, phase 1 and 2 clinical trials are currently testing MSCs as a therapy for ARDS (53).

Given the potential therapeutic benefit of MSCs in the treatment of ARDS, it is important to understand their mechanism of action and several possible mechanisms have been implicated to date. In 2010, Fang et al. used SiRNA knockdown of paracrine soluble factors in the setting of MSC treatment *in vitro* in cultured human type 2 cells and found that angiopoietin-1 secretion was partially responsible for the beneficial effect of MSCs (54). Subsequent studies suggested that interleukin-1 receptor antagonist and growth factors such as keratinocyte growth (KGF) factor may also be involved in this process (52). KGF can upregulate AFC in *ex vivo* human lungs injured by endotoxin (55). A 2015 study demonstrated that lipoxin A4, a pro-resolving lipid mediator, is upregulated in the presence of MSCs, suggesting that this molecule may be important for MSC-mediated resolution of lung injury (56). Other studies indicated that the therapeutic effects of MSCs may also mediate the release of microvesicles, which are involved in cell–cell communication (57) or due to mitochondrial transfer (58). Thus, several mechanisms may explain MSC-mediated resolution of lung injury, and further studies are needed to fully characterize this process.

DISCUSSION

The importance of alveolar ion transport in AFC has been established, but further work is needed to better characterize this process. For example, type I alveolar cells participate in apical–basolateral fluid transport (8, 59), but there is no suitable cell culture model for type I cells so the physiology of these cells is not as well understood. In addition, sodium transport has been well characterized, but the contribution of transport of other ions is not as clear. Future experiments are needed to clarify the role of type I versus type II alveolar cells and the roles of additional ion channels in alveolar fluid transport.

The *in vitro* and *in vivo* models of ARDS have enhanced our understanding of ARDS pathophysiology. Not only are these models useful for the study of ARDS but measurements of AFC and paracellular permeability can also be used to better understand other pulmonary conditions. For example, Chan et al. (60) compared the extent to which avian influenza A (H5N1) virus and seasonal influenza A (H1N1) virus impair AFC and protein permeability using the transwell model first used in the ARDS models (60). The authors found that avian influenza A (H5N1) virus causes a more severe reduction in alveolar protein transport than the seasonal influenza A (H1N1) virus, mimicking its greater clinical severity. Future work can use these models to better understand how AFC is affected in other pulmonary pathologies as well.

Preliminary preclinical experiments have suggested that MSCs promote the resolution of alveolar edema fluid. It is possible that MSCs act directly on alveolar ion channels *via* cell–cell interactions or indirectly *via* paracrine factors; future experiments are needed to clarify their mechanism of action in the lung. If MSCs indeed promote AFC, they may serve as a promising cell-based therapy for ARDS.

SUMMARY

In this review, we have discussed how vectorial ion channels in alveolar epithelium generate an osmotic gradient that drives AFC in both physiologic and pathologic conditions. Both AFC and paracellular permeability can be measured using *in vitro* and *in vivo* models of ARDS, and these studies indicate that vectorial ion transport is less effective in injured lungs than in uninjured lungs. Recent studies suggest that MSCs interact with alveolar epithelium and ion channels to increase AFC and thus may serve as a promising treatment for ARDS.

AUTHOR CONTRIBUTIONS

LH and MM composed this mini-review manuscript together. LH is the first author.

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Gas Exchange Disturbances Regulate Alveolar Fluid Clearance during Acute Lung Injury

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Disruption of the alveolar–capillary barrier and accumulation of pulmonary edema, if not resolved, result in poor alveolar gas exchange leading to hypoxia and hypercapnia, which are hallmarks of acute lung injury and the acute respiratory distress syndrome (ARDS). Alveolar fluid clearance (AFC) is a major function of the alveolar epithelium and is mediated by the concerted action of apically-located Na^+ channels [epithelial Na^+ channel (ENaC)] and the basolateral Na,K-ATPase driving vectorial Na^+ transport. Importantly, those patients with ARDS who cannot clear alveolar edema efficiently have worse outcomes. While hypoxia can be improved in most cases by O_2 supplementation and mechanical ventilation, the use of lung protective ventilation settings can lead to further CO_2 retention. Whether the increase in CO_2 concentrations has deleterious or beneficial effects have been a topic of significant controversy. Of note, both low O_2 and elevated CO_2 levels are sensed by the alveolar epithelium and by distinct and specific molecular mechanisms impair the function of the Na,K-ATPase and ENaC thereby inhibiting AFC and leading to persistence of alveolar edema. This review discusses recent discoveries on the sensing and signaling events initiated by hypoxia and hypercapnia and the relevance of these results in identification of potential novel therapeutic targets in the treatment of ARDS.

Keywords: hypoxia, hypercapnia, alveolar fluid clearance, Na,K-ATPase, epithelial Na^+ channel, acute respiratory distress syndrome, acute lung injury

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A major function of the alveolar epithelium is to maintain alveolar fluid balance resulting in minimal epithelial lining fluid, thus providing optimal gas exchange (1). However, during acute lung injury (ALI) and the clinical acute respiratory distress syndrome (ARDS) the alveolar–capillary barrier fails, which leads to flooding of the alveolar space and causes a severe impairment of gas exchange (2). It is well established that clearance of alveolar edema is markedly impaired in most patients with ARDS and that this impairment is associated with worse outcomes (3). Thus, removal of the excess alveolar fluid is of significant clinical importance. The primary mechanism driving fluid reabsorption from the alveolar space is the active vectorial flux of sodium from the airspaces into the lung interstitium and the pulmonary circulation (1). Sodium, in exchange to potassium, is pumped out of the alveolar epithelial cells (AEC) basolaterally by the Na,K-ATPase, whereas Na^+ enters the cells apically through the amiloride-sensitive and -insensitive epithelial Na^+ channel (ENaC) (1, 4). This vectorial sodium transport process creates an osmotic gradient that drives clearance of fluid from the alveolar space (1). Failure of the alveolar–capillary barrier function leads to alveolar edema and thus to alveolar hypoventilation, resulting in hypoxemia and often elevated CO_2 concentrations

in the blood (hypercapnia) in patients with ARDS (1, 4). This is of particular importance, as several studies have shown that these conditions are not only consequences of alveolar edema but also further exacerbate alveolar fluid dysbalance by promoting formation and inhibiting reabsorption of the edema fluid (5–7). In this review, we will focus on the mechanisms by which hypoxia and hypercapnia impair alveolar fluid clearance (AFC), concentrating on the regulation of the Na,K-ATPase and ENaC in the context of ALI.

ROLE OF HYPOXIA IN INFLAMMATION AND ALVEOLAR FLUID BALANCE IN ALI

Adaptation to hypoxia is critically important for cellular survival as oxygen is required for ATP synthesis in the mitochondria by oxidative phosphorylation (8). During hypoxia, production of ATP is reduced by inhibition of the electron transport chain. In order to reduce energy consumption, protein translation is down-regulated and various processes with high energy demand are inhibited (8). In the context of ALI/ARDS, alveolar hypoxia and systemic hypoxemia occur as the inflamed/injured alveolar–capillary barrier fails. It is generally accepted that hypoxia is intimately coupled to inflammatory states in various organs (9). For example, inflammatory hypoxia, a manifestation of locally increased metabolism and reduced oxygen supply, may drive and further exacerbate inflammatory bowel diseases, such as ulcerative colitis or Crohn's disease (10). While hypoxia *per se* can be an inflammatory stimulus, which up-regulates inflammatory cytokine levels, stabilization of hypoxia-inducible factor (HIF)-1α and activation of adenosine A_{2A} receptor-mediated mechanisms secondary to hypoxia may have significant anti-inflammatory effects in the lung (11, 12). Other than regulating inflammation, it is well established that hypoxia impairs alveolar fluid balance. The first preclinical studies over 15 years ago addressing the effects of hypoxia in intact rat lungs suggested that the impaired fluid balance upon exposing animals to low O_2 levels was due to an inhibition of transepithelial sodium transport processes (5, 13). Importantly, these negative effects of hypoxia on AFC can also be observed in humans and prophylactic administration of salmeterol, a β_2-adrenergic receptor agonist, prevents lung edema in subjects who are susceptible to high-altitude pulmonary edema, probably due to up-regulation of the Na,K-ATPase and/or ENaC (14).

EFFECTS OF SHORT-TERM HYPOXIA ON ALVEOLAR EPITHELIAL Na^+ TRANSPORT

The molecular mechanisms by which hypoxia down-regulates Na^+ transporters depend on the duration of exposure to low O_2 levels and have been studied in various AEC lines. Severe hypoxia leads to rapid (within minutes) endocytosis of the Na,K-ATPase molecules from the plasma membrane (PM) into intracellular pools, thereby decreasing activity of the enzyme (15). It appears that in the first hour of hypoxic exposure this trafficking event is solely responsible for the hypoxia-induced impairment of Na,K-ATPase function as the total cellular abundance of the transporter remains unchanged, excluding the possibility of accelerated degradation of the transporter upon short-term hypoxia. In line with this notion, the endocytosis of the Na,K-ATPase upon hypoxia is promptly reversible upon reoxygenation (15). Furthermore, it has been reported that the effects of hypoxia on the Na,K-ATPase are mediated by mitochondrial reactive oxygen species as in ρ^0-A549 cells, which are incapable of mitochondrial respiration, and thus unable to generate mitochondrial ROS, hypoxia does not alter the cell surface stability of the Na,K-ATPase (15, 16). Release of mitochondrial ROS upon hypoxic exposure initiates Ca^{2+} release from the endoplasmic reticulum (ER) and redistribution of the calcium sensor STIM1 to the ER PM junctions, thereby resulting in calcium entry through Ca^{2+} release-activated Ca^{2+} channels, which in turn activates Ca^{2+}/calmodulin-dependent kinase kinase (CAMKK)-β, a well-known inducer of the metabolic sensor AMP-activated protein kinase (AMPK) (17). Of note, AMPK is a major regulator of cellular energy balance and activation of the kinase leads to inhibition of processes that require high energy (18); thus, playing a central role in the adaptation to hypoxia. As the Na,K-ATPase accounts for ~30–80% of the energy expenditure of cells (8), rapid down-regulation of the transporter driven by AMPK appears to be key in this adaptation process. Once activated, AMPK-α1 directly phosphorylates protein kinase C (PKC)-ζ at the Thr410 residue (19). This is of relevance as phosphorylation of PKC-ζ at Thr410 drives translocation of the protein kinase to the PM where it phosphorylates the Na,K-ATPase at Ser18. It is well documented that phosphorylation of this serine residue promotes endocytosis of the Na^+ pump from the PM (15). In parallel, upon hypoxic exposure mitochondrial ROS activate RhoA, a member of the Rho GTPase family and its downstream effector, the Rho-associated serine/threonine kinase (ROCK), a central regulator of filamentous actin reorganization, which has been implicated in the control of endocytosis (20, 21). Thus, in the alveolar epithelium the mitochondria serve as hypoxia sensors and release of mitochondrial ROS initiates a rapid and highly specific signaling cascade that leads to endocytosis of the Na,K-ATPase from the PM and thereby alveolar epithelial dysfunction (**Figure 1**).

Moreover, reactive oxygen and nitrogen species (RONS) have also been implicated in the down-regulation of ENaC (22). Recently, two Tyr residues located in the extracellular loop of the α-subunit of ENaC, Tyr279 and 283, have been identified as potential targets of oxidation by RONS (23). Various additional ion channels have been shown to be modulated (mainly down-regulated) by ROS and RONS by regulation of channel transcription, direct oxidation, nitration or nitrosylation of channels, and via interference with signaling patterns regulating activity, trafficking or expression of channels (24).

EFFECTS OF SUSTAINED HYPOXIA ON THE Na,K-ATPase AND ENaC

Sustained hypoxia down-regulates sodium transporter function in the alveolar epithelium by at least two independent mechanisms. As discussed above, during cellular adaptation to hypoxia protein translation is down-regulated to reduce energy

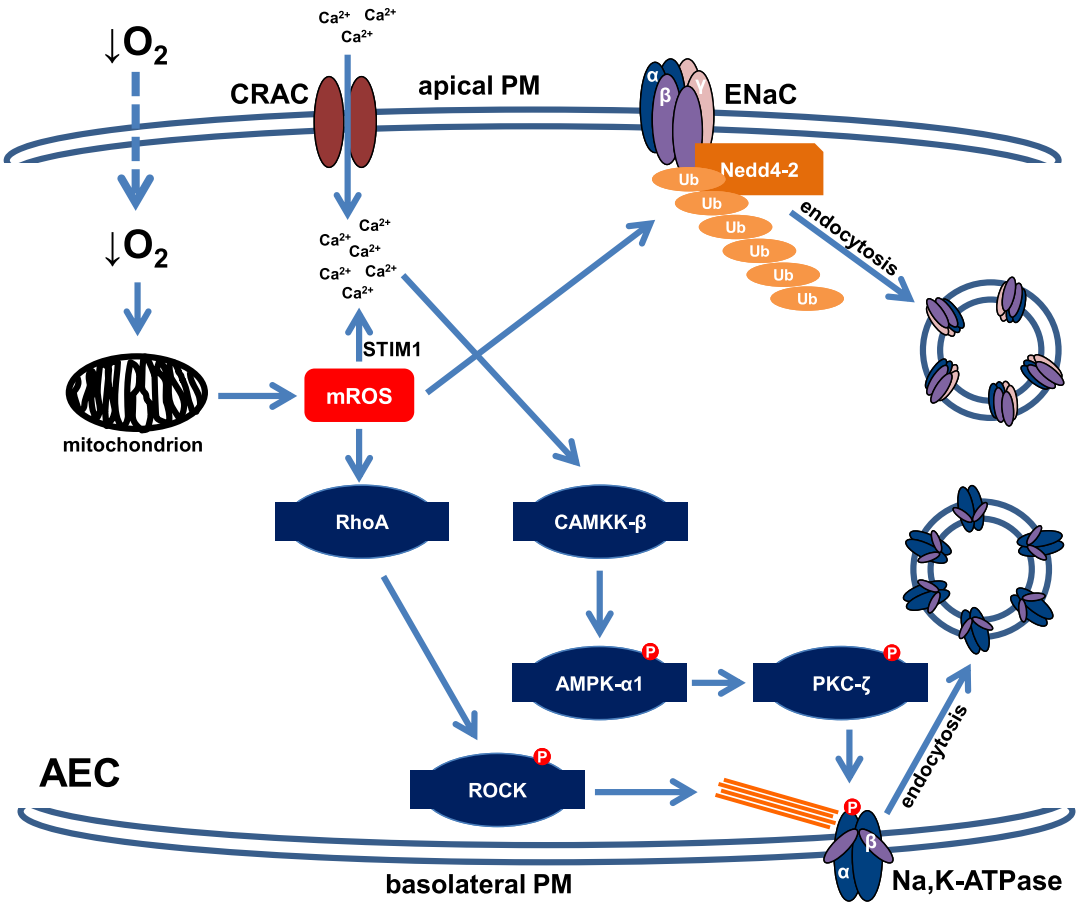

FIGURE 1 | Schematic depiction of the signaling cascades impairing cell surface expression of the Na,K-ATPase and epithelial Na^+ channel (ENaC) upon acute hypoxia. In alveolar epithelial cells (AEC), hypoxia is sensed by mitochondria, which in response release mROS. Increased mROS concentrations lead to Ca^{2+} entry through Ca^{2+} release-activated Ca^{2+} (CRAC) channels by activation of STIM1. Elevated intracellular Ca^{2+} levels result in activation of Ca^{2+}/calmodulin-dependent kinase kinase (CAMKK)-β, which in turn phosphorylates and activates AMP-activated protein kinase (AMPK). Subsequently, AMPK promotes translocation of protein kinase C (PKC)-ζ to the plasma membrane (PM) where it phosphorylates the Na,K-ATPase α-subunit, thereby promoting endocytosis of the transporter. Hypoxia-induced endocytosis of the Na,K-ATPase also requires filamentous actin reorganization, which is mediated by mROS-induced activation of RhoA and ROCK. In parallel, increased mROS levels activate the E3 ubiquitin ligase Nedd4-2, which conjugates ubiquitin molecules to the ENaC β-subunit, thereby leading to endocytosis of the ENaC complex. This down-regulation of both Na,K-ATPase and ENaC cell surface expression results in impaired alveolar fluid clearance during hypoxia.

consumption (8). Indeed, several reports documented that upon long-term hypoxia both mRNA and total protein levels of the Na,K-ATPase and ENaC are decreased (25). A second, and more specific mechanism is the ubiquitination and directed degradation of the transporters. Ubiquitination is a post-translational modification during which ubiquitin molecules are conjugated (mostly but not exclusively) to specific lysine residues of target proteins, thereby controlling stability, function, and localization of the target (26, 27). Regarding the regulation of Na,K-ATPase upon prolonged hypoxic exposure, it has been documented that degradation of the enzyme occurs first (after approximately 2 h) in the PM, whereas exposing AEC to severe hypoxia for up to 24 h results in degradation of the Na,K-ATPase in intracellular pools (16). Considering that the Na,K-ATPase accounts for a significant proportion of the energy expenditure of cells, as mentioned above, it appears logical that as an adaptive mechanism to hypoxia the active Na,K-ATPase molecules (located at the PM) will be removed from the surface and degraded more rapidly than degradation of the inactive molecules (located in the intracellular pools) occurs to reduce cellular energy consumption and thus promote survival (8). A subsequent study established that four Lys residues (Lys16, 17, 19, and 20) surrounding the PKC-ζ phosphorylation site (Ser18) at the N-terminus of the Na,K-ATPase α-subunit are required for ubiquitin conjugation (28). Of note, phosphorylation of the Na^+ pump by PKC-ζ at Ser18 is necessary for ubiquitination, perhaps by increasing affinity of ubiquitin to the phosphorylated target, highlighting the possibility of cross-talk between phosphorylation and ubiquitination (28). The E3 ubiquitin ligase of the Na,K-ATPase remains to be identified. Although the E3 ubiquitin ligase, von Hippel Lindau protein has been implicated in the degradation of the Na,K-ATPase upon hypoxia, it has also been shown that this E3 ligase does

not directly target the Na^+ pump (29). Further research on the E3 ligase targeting the Na,K-ATPase will be of particular importance, as that molecule may represent a highly specific druggable target of impaired AFC upon gas exchange disturbances.

Ubiquitination also plays a central role in the down-regulation of ENaC upon sustained hypoxia. It is well established that the E3 ubiquitin ligase, Nedd4-2 plays a pivotal role in the regulation of ENaC cell surface stability by directly targeting α-, β-, or γ-ENaC depending on the stimulus leading to endocytosis and/or degradation of the channel (30). It has been reported that upon hypoxic exposure for 24 h in mice carrying a truncation of the C-terminus of β-ENaC (homozygous β-Liddle mouse strain), thus preventing interactions with Nedd4-2, amiloride-sensitive AFC remains normal, whereas in wild-type mice AFC is decreased by approximately 70%. Furthermore, a marked reduction in the amiloride-sensitive apical Na^+ current upon hypoxia can be fully prevented by inhibition of the proteasome and by the ROS scavenger *N*-acetyl-cysteine (31), suggesting that the ubiquitin–proteasome system is critically required for the hypoxia-driven down-regulation of ENaC and further confirming the central role of ROS in the hypoxic impairment of alveolar epithelial Na^+ transport processes.

ROLE OF HYPERCAPNIA IN INFLAMMATION AND ALVEOLAR FLUID BALANCE IN ALI

While in most patients with ARDS hypoxia can be corrected by the use of mechanical ventilation with elevated inspired fractions of oxygen, hypercapnia often persists in part due to the low tidal volume ventilation strategy, which is required to minimize further ventilator-induced lung injury (32). While "protective" mechanical ventilation with low tidal volumes is clearly beneficial (33), the effects of hypercapnia in the context of lung injury remain a topic of intense debate. Several studies suggested that hypercapnia is tolerable or even beneficial whereas others documented that various aspects of the hypercapnic effects on alveolar epithelial function are deleterious, leading to the terms of permissive, therapeutic, and non-permissive hypercapnia, respectively (6, 34). It is very well established that excessive inflammation plays a central role in the pathogenesis ARDS (35). Moreover, respiratory acidosis (a decrease in the pH of the blood secondary to hypercapnia) has several anti-inflammatory properties, such as reduction of pro-inflammatory cytokines, impairment of neutrophil function, and inhibition of generation of free radicals (36). Thus, it appears logical that hypercapnia (or the associated acidosis) may be beneficial in the context of ARDS. In contrast, a recent secondary analysis of three large prospective non-interventional clinical studies recruiting mechanically ventilated patients with moderate and severe ARDS in over 900 ICUs from 40 countries documented that hypercapnia is independently associated with a markedly higher ICU mortality (37), which is further supported by another study in which hypercapnic acidosis in the first 24 h after ICU admission was associated with higher hospital mortality (38). There are several factors that may lead to worse outcomes of hypercapnic patients with ARDS. Although, and as discussed above, the hypercapnia-associated acidosis may exhibit early anti-inflammatory effects; recently, it has become increasingly evident that hypercapnia impairs innate immunity, thereby potentially increasing susceptibility of patients with ARDS to bacterial infections (39, 40). Furthermore, recent studies established that hypercapnia, independently of changes in pH, impairs alveolar epithelial fluid balance by inhibiting AFC, and thus resolution of pulmonary edema (41–43). As it is well documented that clearance of the excess, protein-rich alveolar edema in patients with ARDS is critical for survival, this aspect is of clinical relevance.

EFFECTS OF ACUTE HYPERCAPNIA ON THE Na,K-ATPase AND ENaC

Because elevated CO_2 levels impair AFC within minutes, it has been hypothesized that much like variations in oxygen concentration, levels of CO_2 may be sensed by the alveolar epithelium (41, 43–45). It was described several decades ago that excitable cells, such as specialized brainstem neurons or the glomus cells of the carotid body serve as central and peripheral chemoreceptors of CO_2 and depolarize upon hypercapnia (46). In contrast, only recently it became evident that elevated CO_2 levels also initiate specific signaling patterns in non-excitable cell types, such as the alveolar epithelium, independently of intra- or extracellular pH, carbonic anhydrases, or ROS (41, 42). Most recently, the hemichannel connexin 26 has been implicated in CO_2 sensing (47). Interestingly, the high CO_2-induced signaling leads to a rapid down-regulation of the Na,K-ATPase activity, thereby inhibiting AFC, one of the major functions of the alveolar epithelium (41, 42). This hypercapnia-induced signaling pattern has been dissected in the past years and we now know that elevated CO_2 levels increase intracellular Ca^{2+} concentrations within seconds leading to activation of CAMKK-β, which stimulates the metabolic sensor AMPK. Similarly to the effects of hypoxic exposure, the hypercapnia-induced activation of AMPK leads to translocation of PKC-ζ to the PM, where the kinase phosphorylates the Na,K-ATPase α-subunit, thereby promoting endocytosis of the transporter from the PM (41, 43). The endocytosis of the Na,K-ATPase also requires activation of the c-Jun N-terminal kinase (JNK), which is similarly to PKC-ζ also downstream of AMPK in the CO_2-induced signaling cascade (48). Upon hypercapnia, activated JNK phosphorylates the scaffolding protein LMO7b at the Ser1295 residue, which enables interaction of the scaffolding protein with the Na,K-ATPase at the PM of AEC, thereby promoting endocytosis and thus inhibition of the transporter (49). Of note, the requirement of JNK in the hypercapnia-induced inhibition of the Na,K-ATPase was not only shown in mice, rats, and human cells but also in *Drosophila melanogaster*, suggesting that at least some elements of the CO_2-induced signaling pattern are evolutionarily conserved (48). Interestingly, this pathway overlaps with that of initiated by acute hypoxia; however, the effects of hypercapnia are independent of mitochondrial ROS. Furthermore, unlike in hypoxia the source of Ca^{2+} upon hypercapnic exposure of the alveolar epithelium remains unknown and the regulation of the endocytic machinery appears to be different in hypoxia and

hypercapnia, where activation of RhoA and ROCK as opposed to JNK and LMO7b are required, respectively (**Figure 2**). Moreover, some effects of hypercapnia and hypoxia are opposing, as elevated CO_2 levels inhibit the HIF-driven adaptation mechanisms to hypoxia (50). Recently, an alternative and AMPK-independent pathway has also been identified in the elevated CO_2-induced down-regulation of the Na,K-ATPase. It has been reported that hypercapnia also activates the recently identified metabolic sensor CO_2/HCO_3^- responsive soluble adenylyl cyclase (CO_2/HCO_3-sAC), which by producing cAMP in specific microdomains in the proximity of the PM led to activation of protein kinase A (PKA) type Iα that phosphorylated the actin cytoskeleton component α-adducin at Ser726, thereby promoting endocytosis of the Na,K-ATPase (51). This novel pathway is AMPK-independent as AMPK phosphorylation upon hypercapnia also occurs in the presence of an siRNA against CO_2/HCO_3-sAC, similarly to PKA activation in AEC after AMPK silencing, suggesting that both pathways are required for the hypercapnia-induced down-regulation of Na,K-ATPase cell surface stability.

Most recently, the molecular mechanism impairing ENaC cell surface stability in AEC upon acute hypercapnic exposure has been described (52). Upon hypercapnia, extracellular signal-regulated kinase (ERK), which has been previously identified in the CO_2-induced signaling pattern as an activator of AMPK (53), directly phosphorylates the ENaC β-subunit at Thr615. Moreover, JNK, which is activated by AMPK upon activation of the latter kinase by ERK, phosphorylates Nedd4-2 at Thr899, thereby increasing the activity of the E3 ubiquitin ligase (52). These phosphorylation events promote the interaction of β-ENaC and Nedd4-2 and lead to polyubiquitination

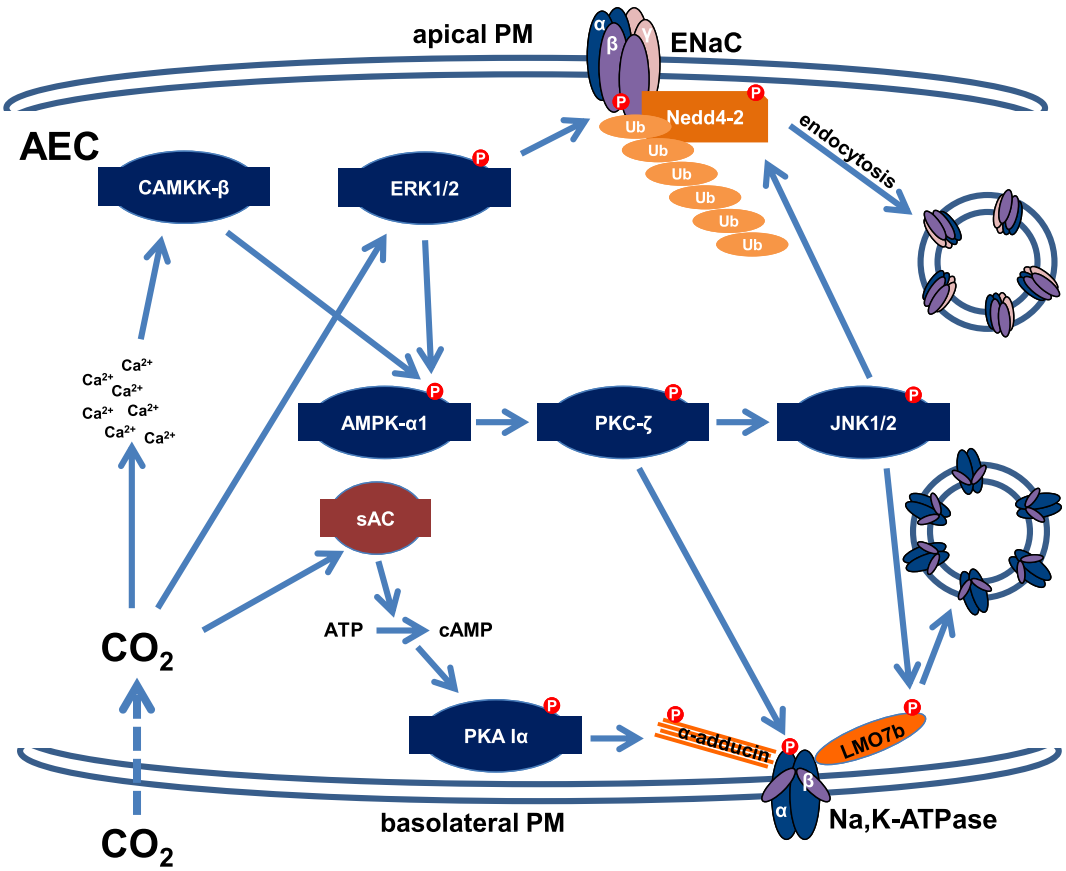

FIGURE 2 | Schematic representation of the signaling signatures down-regulating the Na,K-ATPase and epithelial Na^+ channel (ENaC) upon acute hypercapnia. Hypercapnia leads to phosphorylation and subsequent endocytosis of Na,K-ATPase by an AMP-activated protein kinase (AMPK)-dependent and an AMPK-independent mechanism. An acute elevation in CO_2 levels in alveolar epithelial cells (AEC) leads to an increased intracellular Ca^{2+} concentration by a yet unidentified mechanism. A subsequent activation of the Ca^{2+}/calmodulin-dependent kinase kinase (CAMKK)-β/AMPK-α/protein kinase C (PKC)-ζ signaling cascade results in phosphorylation of the Na,K-ATPase α-subunit. PKC-ζ also activates c-Jun N-terminal kinase (JNK), which phosphorylates the scaffolding protein LMO7b, thereby promoting endocytosis of the Na,K-ATPase. Furthermore, elevated CO_2 is sensed by the sAC, which in turn activates protein kinase A (PKA) type Iα by cAMP in microdomains at close proximity of the basolateral membrane, resulting in phosphorylation of α-adducin, which is required for the rearrangement of the actin cytoskeleton necessary for endocytosis. Moreover, CO_2 activates extracellular signal-regulated kinase (ERK), which is also required for AMPK stimulation. ERK phosphorylates the ENaC β-subunit, thereby attracting the E3 ubiquitin ligase Nedd4-2, which is phosphorylated and activated by JNK upon hypercapnic exposure, leading to polyubiquitination of β-ENaC, and a reduction of ENaC abundance at the apical PM. Collectively, these mechanisms impair the function of both the Na,K-ATPase and ENaC and are responsible for the hypercapnia-induced inhibition of alveolar edema clearance.

of β-ENaC and subsequent endocytosis of the ENaC complex, thereby reducing cell surface stability of the channel.

EFFECTS OF SUSTAINED HYPERCAPNIA ON ALVEOLAR EPITHELIAL Na^+ TRANSPORT AND REPAIR

Interestingly, the effects of long-term elevated CO_2 levels on the Na,K-ATPase are reversible. Exposing rats to elevated CO_2 concentrations for up to 1 week leads to a sustained and marked decrease in AFC (43). Similarly, exposure of AEC to elevated CO_2 levels for up to 24 h causes a sustained reduction of Na,K-ATPase abundance at the PM (43). However, when exposing rat lungs to normocapnia after a hypercapnic treatment for 1 h, levels of AFC rapidly return to normal (41). Furthermore, treatment of rat lungs with the β-adrenergic receptor agonist, isoproterenol not only prevents but also reverses the hypercapnia-induced decrease in AFC, confirming that the high CO_2-induced AFC impairment is reversible at least in the first hour of hypercapnia (43). Moreover, sustained hypercapnia induces the microRNA, miR-183, which down-regulates isocitrate dehydrogenase 2, an enzyme that catalyzes the conversion of isocitrate to α-ketoglutarate during the tricarboxylic acid cycle (54). This effect leads to mitochondrial dysfunction thus, inhibiting proliferation of AEC, which may impair repair mechanisms and resolution of lung injury.

CONCLUSION

Gas exchange disturbances are hallmarks of ALI and ARDS. Both low O_2 and elevated CO_2 levels are rapidly sensed by the alveolar epithelium, the site of oxygen uptake and CO_2 elimination, leading to adaptation but also deleterious effects on cellular function. Both hypoxia and hypercapnia are intimately coupled to inflammation and by highly specific and partially described signaling pathways, which inhibit epithelial sodium transport processes impair AFC. As alveolar hypoxia and hypercapnia cannot always be corrected at the areas of severe injury, interfering with these deleterious signals may lead to novel therapies against ARDS.

AUTHOR CONTRIBUTIONS

IV: drafting the work and preparing figures. JS and IV: revising it critically for important intellectual content. Both authors approved the final version of the manuscript.

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Cytokine–Ion Channel Interactions in Pulmonary Inflammation

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The lungs conceptually represent a sponge that is interposed in series in the bodies' systemic circulation to take up oxygen and eliminate carbon dioxide. As such, it matches the huge surface areas of the alveolar epithelium to the pulmonary blood capillaries. The lung's constant exposure to the exterior necessitates a competent immune system, as evidenced by the association of clinical immunodeficiencies with pulmonary infections. From the *in utero* to the postnatal and adult situation, there is an inherent vital need to manage alveolar fluid reabsorption, be it postnatally, or in case of hydrostatic or permeability edema. Whereas a wealth of literature exists on the physiological basis of fluid and solute reabsorption by ion channels and water pores, only sparse knowledge is available so far on pathological situations, such as in microbial infection, acute lung injury or acute respiratory distress syndrome, and in the pulmonary reimplantation response in transplanted lungs. The aim of this review is to discuss alveolar liquid clearance in a selection of lung injury models, thereby especially focusing on cytokines and mediators that modulate ion channels. Inflammation is characterized by complex and probably time-dependent co-signaling, interactions between the involved cell types, as well as by cell demise and barrier dysfunction, which may not uniquely determine a clinical picture. This review, therefore, aims to give integrative thoughts and wants to foster the unraveling of unmet needs in future research.

Keywords: epithelial sodium channel, Na^+/K^+-ATPase, tumor necrosis factor, TNF tip peptide, pneumonia, acute respiratory distress syndrome, lung transplantation, ischemia–reperfusion injury

INTRODUCTION

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are both clinical syndromes with a high morbidity and mortality rate. Although of a different degree of severity, both ARDS and ALI are characterized by critical gas exchange disturbances, an inflammatory reaction, and an associated alveolar fluid overload (edema). The etiology of ALI and ARDS can be differentiated between direct and indirect lung injury.

The conceptual work presented here discusses the mechanisms regulating alveolar fluid clearance (AFC) during inflammation. As recently demonstrated by several groups, the interaction between cytokines and ion channels may play a critical role in this setting. The presented review does not cover all cytokines and ion channels, but rather focuses on a selection of mainly pre-clinical pathophysiological models and addresses clinical needs and difficulties to effectively translate pre-clinical data into the clinical field. **Tables 1–3** give an overview on ion channels and mediator interaction. The ultimate aim of this translational research should be to improve patient care and to reduce morbidity and mortality. This can be achieved by reducing long-term residual sequelae and time on the ventilator, which can improve long-term lung function and health status or health-related quality of life.

The main task of the lungs is to account for the efficient external gas exchange between air and the blood. Only a thin barrier of several micrometers separates the pulmonary capillaries from the immense alveolar surface, mainly made up by alveolar type I cells. An intimately fine, deformable, tensible, flexible, and continuous net of interstitial tissue integrates the interstitial net around vessels and bronchi. The whole system has to be "breathable," i.e., has to be efficiently moved by the thoracic cage to transport fresh air in the alveolar space that matches to the vascular bed for gas exchange. A number of structural and physiological features prevent alveolar flooding. These protective mechanisms include the very low vascular resistance in the pulmonary circulation, the high capillary colloid-osmotic pressure and, on the other hand, the diminished interstitial colloid-osmotic pressure in case of increased filtration. The minimal mechanic stress of alveolar septa due to surface tension reduction by surfactant as well as the optimal active fluid reabsorption out of the alveolar space are further measures that optimize fluid clearance. Structurally, a rather tight pulmonary microvascular endothelium allows for a minimal continuous filtration of water, micro-and macromolecules, with an even tighter alveolar epithelium (1). All three fluid compartments, the capillaries, the interstitium, and the alveoli are in a complex dynamic equilibrium. The continuous pulmonary interstitial space is a drainable continuum that is ultimately emptied by the lymphatic vessels. There is a basal transendothelial filtration of about 10 ml/h that increases up to tenfold during physical activity. When such filtered fluid enters the alveolar interstitial space, it moves proximally to the peri-bronchovascular space (2). Under normal conditions, most of this filtered fluid will be removed by the lymphatics from the interstitium and returns to the systemic circulation (2).

The interstitial compartment is a reversible store of excess fluid. In the adult lung, interstitial fluid—or interstitial edema—can mount up to a volume of 500 ml. However, at that volume there is usually already some alveolar edema (3). It was formerly wrongfully postulated that the Starling filtration forces, which essentially represent the balance between oncotic and hydrostatic pressures in the capillaries and the interstitial space, are the only driving forces for liquid flow from the bloodstream into the extravascular space. In the last four decades, four important refinements have been made. The first one is that fluid reabsorption from the alveolar space is mainly performed by active vectorial Na^+ transport (4). Moreover, also Cl^- transport was suggested to be important, leading to consecutive counter-ion transport, as well as to an osmotic water shift. In the last few years, a second refinement has been made which mainly occurs in heart failure, namely that pumps which usually free the alveolus of ions can also provide inverse transport (5). This biological "emergency plan" in case of hydrostatic pulmonary edema widens the scope of mechanisms in cardiogenic lung edema, as one can argue that in heart failure these mechanisms could be rescue fluid shifts including into the alveolar space, and that a concerted fluid management in vascular, renal, and intestinal and pulmonary vascular beds might occur in severe cardiac failure or fluid overload, taking into account some degree of alveolar pulmonary edema. A third rather novel field is the research on emptying of the alveolar space from its protein load; but so far only few insights in this clinical topic exist (6). The fourth refinement is the close relationship of ion channel activation with barrier tightness. Interactions between the lectin-like domain of tumor necrosis factor (TNF), mimicked by its amino acid-identic TNF tip peptide (a.k.a. AP301 and Solnatide) and the epithelial sodium channel (ENaC) were shown to have a clear effect on epithelial (7) and endothelial barrier tightness (8, 9). As such, ion channel activity and barrier tightness may be key survival factors for tissue function, be it the lung or the kidney, the brain or other organs, and for tissue stability (9–11).

Alveolar fluid reabsorption is a very physiological process that is even required directly after birth where the lung has to be cleared from liquid as it has been so far immersed in the amnionic fluid. In premature infant, insufficient clearance of lung liquid at birth may lead to respiratory distress syndrome (RDS). The key clinical relevance of the physiological role of αENaC in the lungs has been confirmed in the mouse in which the ENaC-α gene was deleted by a homologous recombination. These animals were not able to remove alveolar fluid from their lungs and died shortly after birth (12). Surprisingly, in humans this situation seems more complex, as a child with an inactive homozygous ENaC-α mutation did not suffer perinatal respiratory failure (13).

Likewise in adults with heart failure or RDS, while they show no active fluid clearance greater morbidity and mortality rate is probable (14). In clinical studies using quantification of protein in alveolar liquid, prognosis was dependent on the estimated AFC. In a recent study, 56% had impaired AFC, and only 13% a maximal AFC rate (**Figure 1**). Survival was higher and days on mechanical ventilation were less in those patients with maximal alveolar clearance rate compared to patients with impaired clearance rate. With hydrostatic edema, by contrast, 75% of patients had submaximal to maximal AFC (15). Of note is that in hydrostatic edema alveolar fluid shift may even actively be reversed (5, 16) as discussed above.

PULMONARY EDEMA

Pulmonary alveolar edema is a life-threatening state that results from an imbalance between passive and active forces driving fluid into the airspaces and those mechanisms involved in its removal (1, 4). Based on the underlying cause, in the next two chapters we will discuss two main fundamentally different types of pulmonary edema occur in humans (2).

TABLE 1 | Role of different mediators on fluid transport through impacting on ion channels in the apical and basolateral membrane of epithelial cells.

Channel name	Mediator	Impact on pulmonary barrier function	Mechanism of action
Apical membrane			
Epithelial sodium channel (ENaC)	Transforming growth factor beta (TGF-β)	–/+	Decrease in expression during bacterial infection (132) Decreases expression of the αENaC mRNA and protein (132) Internalization of αβγENaC complex from the lung epithelial cell surface and, hence, block the sodium-transporting capacity of alveolar epithelial cells (AECs) (133) Increases the function of ENaC (134)
	Tumor necrosis factor (TNF) receptor binding site	–	Decreases the expression of ENaC mRNA in AECs *in vitro* (135)
	TNF lectin-like domain	+	Activates ENaC (37, 136) Increases ENaC open probability (102)
	Interleukin-1β (IL-1β)	–/+	Decreases the expression of ENaC during bacterial infection (113) Decreases expression of αENaC *via* a p38 MAPK-dependent signaling pathway (113) Suppresses expression of βENaC (137) Decreases ENaC function (138) Augments *in vitro* alveolar epithelial repair (139) Increases ENaC subunits expression in a specific fetal context (140)
	Interleukin-4 (IL-4)	–	Decreases in ENaC expression during bacterial infection (141) Decreases ENaC activity by decreasing the mRNA levels of γENaC and, to a lesser extent, that of the β subunit (142)
	Keratinocyte growth factor (FGF-7)	–	Decreases the expression of αENaC (143)
	Protein kinase C (PKC)	–	Inhibits ENaC function (144–147)
	Cycloheximide (CHX)	–	Downregulate αENaC mRNA abundance similarly *via* the ERK and p38 MAPK pathway (148); Chx effect involves post-transcriptional mechanisms (148)
	Lipopolysaccharide (LPS)	–	Downregulates αENaC mRNA abundance similarly *via* the ERK and p38 MAPK pathways (148); inhibits αENaC promoter activity (148)
	Pneumolysin (PLY)	–	Inhibits ENaC expression upon activation of ERK (102) and inhibits ENaC open probability, by reducing its association with myristoylated alanine-rich C kinase substrate (10, 149)
	Glutathione disulfide (GSSG)	–	Inhibits ENaC activity in primary AECs (150, 151)
	Reactive oxygen species (ROS)	–/+	Inhibit ENaC (150, 152) Decrease channel activity (117) Increases ENaC activity through: (i) Enhancing ENaC gating (153) (ii) Increasing channel abundance (153)
	Ethanol	+	Increases ENaC open-state probability (153) Increases ENaC abundance (153)
	Superoxide ($O2^a$)	+	Elevating endogenous (O_2^-) levels with a superoxide dismutase inhibitor, prevents NO inhibition of ENaC activity (111)
	Nitric oxide (NO)	–	Inhibits highly selective sodium channels (52, 53)
	Inter-α-inhibitor (Iαl)	–	Inhibits ENaC activity in CF patients (154)
	NEDD4-2	–	Decreases the expression of the epithelial ENaC (155)
	Hypoxia	–	Decreases apical expression of ENaC subunits (especially beta and gamma) (156)
	Purinergic receptors (P2YR)	–	Inhibits ENaC expression (157, 158)
	Muscarinic cholinergic	+	Increases ENaC activity. RhoA activity is essential for this process (159)
	Estriadol	+	Increases activity of the non-selective ENaC channels, and these effects are mediated through the G protein-coupled estrogen receptor (160)
	Glucocorticoids	+	Increased in expression of ENaC during bacterial infection (161–164)
	Thyroid hormone	+	Thyroid hormone in concert with glucocorticoids increased the expression of ENaC (165, 166)
	Corticosteroids	+	Increase expression of the γ-ENaC subunit which leads to increase ENaC activity (167)

(*Continued*)

TABLE 1 | Continued

Channel name	Mediator	Impact on pulmonary barrier function	Mechanism of action
	Prostasin [channel activating protease 1 (168)]	+	Activates ENaC (169)
	Urokinase-like plasminogen activator	+	Increases the ENaC activity (154, 170–173)
	Cyclic adenosine monophosphate (cAMP)	+	Increases channel activity either by increasing its open probability or by increasing the number of channels at the apical membrane (174)
	Cystic fibrosis transmembrane conductance regulator (CFTR)	+	Activated CFTR can inhibit ENaC (175)
	Dopamine	+	Increases ENaC activity by a cAMP-mediated alternative signaling pathway involving EPAC and Rap1, signaling molecules usually associated with growth-factor-activated receptors (176)
	β2-agonists	+	Activates ENaC (159) Enhancing the insertion of ENaC subunits into the membrane of AECs (156)
	Human AGEs (receptor for advanced glycation end product ligand)	+	Increases ENaC activity through oxidant-mediated signaling (177)
CFTR (Cl^- channel)	Interferon-gamma (IFN-γ)	–	Decreases the expression of CFTR mRNA (142, 178)
	TGF-β	–	Decrease CFTR expression and function (179)
	Interleukin-4 (IL-4)	+	Increases the expression and function of CFTR (142)
	Interleukin-13 (IL-13)	+	Increases the CFTR expression (180)
	Interleukin-1β (IL-1β)	+	Increases CFTR expression trough increasing mRNA levels (138, 181)
	β2-agonists	+	β2AR mediates enhancement of AFC *via* increasing Cl^- flux through CFTR (182, 183) It activates CFTR by raising cAMP intracellular levels and mediating protein kinase A (PKA) activation (184)
	Na^+/K^+ ATPase (Na^+/K^+-ATPase)	–	Inhibition of the Na^+/K^+-ATPase lead to a reduced transcription of CFTR (185) CFTR dysfunction occurs through Na^+/K^+-ATPase inhibition by ouabain (186)
Cyclic nucleotide-gated cation channels (CNG) (Na^+ channel)	Glucocorticoids	+	Increases mRNA for alphaCNG1 (187)
	mineralocorticoids	+	Increases mRNA for alphaCNG1 (187)
TMEM 16a (CaCC) (Ca^+ activated Cl^- channel)	CFTR	–	Can inhibit TMEM 16a through attenuation of ionophore-induced rise in Ca^{2+} (188)
	IL-4	+	Increases the expression of CaCC (189)
	IL-9	+	Increases the expression of CaCC (189)
	IL-13	+	Increases the expression of CaCC (189)
ClC-2 (Cl^- channel)	TNF	–	Inhibits Aquaporin 5 (AQ-5) Expression (190)
AQ-5 (H_2O channel)	Transient receptor potential vanilloid 4 (TRPV4)	–	Reduction of AQP5 abundance (191)
	IFN-γ	+	Increases ClC-2 transcripts *via* mRNA stabilization (192)
	cAMP	+	Increasing synthesis of AQP5 mRNA (193) Triggering translocation of AQP5 to the plasma membrane (193)
	Progesterone	+	Increases abundance of AQP5 (194)
	Estradiol	+	Increases in the AQP5 protein level (194)
Basolateral membrane			
Na^+/K^+ ATPase (Na^+, K^+ pump)	IFN-γ	–	Inhibits Na^+/K^+-ATPase activity (195)
	Interleukin-1β (IL-1β)	+	Increases Na^+/K^+-ATPase subunit expression (140)
	TNF lectin-like domain	+	Increased Na^+/K^+-ATPase activity (196) Activation of Na^+/K^+-ATPase by TIP probably occurs indirectly upon prior activation of ENaC

(*Continued*)

TABLE 1 | Continued

Channel name	Mediator	Impact on pulmonary barrier function	Mechanism of action
	TGF-β	−/+	Decrease in Na^+/K^+-ATPase β1 subunit expression, resulting in decreased Na^+/K^+-ATPase activity(197, 198) Increases the expression of Na^+/K^+-ATPase α 1- and β 1-subunits (134)
	TNF-related apoptosis-inducing ligand (TRAIL)	−	Influenza A virus (IAV)-induced reduction of Na^+/K^+-ATPase is mediated by a host signaling pathway that involves epithelial type I IFN and an IFN-dependent elevation of macrophage TRAIL (199)
	Leukotriene D4	+	Activates Na^+/K^+-ATPase (200)
	Acetylcholine	+	Activates Na^+/K^+-ATPase (201)
	NO	−	Inhibits Na^+/K^+-ATPase (53, 202)

[a]*There is growing evidence that ROS are important regulators of ENaC activity and, hence, of epithelial Na^+ absorption (153). But there is an important question here. Why does ROS increase ENaC activity under some circumstances (e.g., ethanol) but inhibit ENaC under others (e.g., influenza) (153)?*

Cardiogenic or Hydrostatic Edema

Cardiogenic pulmonary edema (also called hydrostatic or hemodynamic edema) (2) is caused by an increased capillary hydrostatic pressure, secondary to an elevated pulmonary venous pressure (18) (**Figure 2**, left panel). This type of edema can occur following left ventricular heart failure, renal failure, or fluid overload, or arteriovenous shunts or fistulas. Left heart failure is most commonly caused by myocardial ischemia with or without myocardial infarction, exacerbation of chronic systolic or diastolic heart failure, or dysfunction of the mitral or aortic valve. Acute cardiogenic pulmonary edema is a frequent medical emergency that accounts for up to 1 million hospital admissions per year in the United States and for about 6.5 million hospital days each year, and is typically present during acute cardiac failure in 75–80% of patients (19). Coronary heart disease may account for about half to two-thirds of heart failures. There has been an increase in cardiac failure patients as well as in hospitalization rate during the last decade (20). As a matter of fact, heart failure is the most rapidly growing cardiovascular condition globally. The reported Western world life time risk is typically about 33% for men and 29% for women for our population, and depends, besides sex, on comorbidities and cardiovascular risk factors, such as arterial hypertension, diabetes, obesity, sleep related disorders, smoking, sedentary lifestyle, and ethnic background (20). In patients aged 65 years and older, more than 10% suffer from congestive heart failure (21). Interstitial pulmonary edema and alveolar flooding impair lung mechanics and gas exchange, thus causing dyspnea and tachypnea, which ultimately results in an age-dependent in-hospital mortality rate of about 15% (22).

The development of pulmonary edema is characterized by increased transcapillary hydrostatic pressure gradients. Moreover, a reversed and active electrolyte flow and its resulting active fluid transport can be involved (5, 23). This is possible by the bidirectional permeation permitting anion channels cystic fibrosis transmembrane conductance regulator (CFTR) and NKCC1 (16), which seems to account for up to 70% of the total alveolar fluid influx at elevated hydrostatic pressure. It is supporting the concept that alveolar fluid secretion is a secondary consequence of impaired alveolar Na^+ uptake (16). Both CFTR and NKCC1 are inhibited by furosemide. This might explain why in the clinical heart failure setting furosemide immediately relieves patients, i.e., by inhibition ion and, thus, fluid transport into the alveolus during alveolar lung edema generation when furosemide is administered, and not only after a huger delay of about half an hour or more when the renal effect of relevant diuresis has occurred. However, also a venous vasodilation, direcly reducing preload, occurs immediately after systemic furosemide administration (24).

A rapid increase in hydrostatic pressure in the pulmonary capillaries, leading to increased transvascular fluid filtration, and even active fluid transport as mentioned above, is the sign of acute cardiogenic or volume-overload edema (**Figure 2**, left panel). Such an increase could be usually due to elevated pulmonary venous pressure from increased left ventricular end-diastolic pressure and left atrial pressure (2). Mild elevations of left atrial pressure (18–25 mmHg) cause edema in the peri-microvascular and peri-bronchovascular interstitial spaces (1). Excess interstitial fluid is transported by lung lymphatics into the vascular system. A negative interstitial pressure gradient, even under conditions of edema, is the major force for the removal of pulmonary interstitial edema fluid into the lymphatics (25). If left atrial pressure rises further (>25 mmHg), edema fluid passes through the lung epithelium, in part by active transport, flooding the alveolar space with protein-poor fluid (**Figure 2**, left panel) (1, 2, 5). By contrast, non-cardiogenic pulmonary edema is based on increased pulmonary vascular permeability, resulting in an increased flux of fluid and macromolecules into the pulmonary interstitium and airspaces (**Figure 2**, right panel) (2).

There is a considerable link between inflammation and heart failure. The Val-HeFT study demonstrated a direct correlation between elevated levels of C-reactive protein and heart failure severity, and C-reactive protein predicts the risk of death and early readmission in acutely decompensated heart failure (26). As reviewed by Azzam et al. in this topic issue, one hypothesis is that heart failure is accompanied by systemic and mesenteric venous congestion, which may in turn cause bowel edema and a consecutive increased permeability, leading to bacterial translocation, endotoxin release, and resultant systemic inflammation. A second hypothesis postulates that the failing, but not the healthy,

TABLE 2 | Impact of different factors on the alveolar–capillary barrier.

Mediator	Impact on pulmonary barrier function	Mechanism of action
Alveolar epithelium		
TGFβ1	–	Decreases lung epithelial barrier function (203–205) Increases the permeability of pulmonary endothelial monolayers (206) Increases the permeability of alveolar epithelial monolayers (206)
Tumor necrosis factor (TNF)	–	Causes alveolar epithelial dysfunction (207)
Lectin-like domain of TNF	+	Increases occludin expression, and improved gas–blood barrier function (7)
TNF-related apoptosis-inducing ligand (TRAIL)	–	Disruption of alveolar epithelial barrier (199, 208, 209)
Interleukin-1β (IL-1β)	+	Augments *in vitro* alveolar epithelial repair (139)
Protein kinase D3	–	Dysfunction of airway epithelial barrier through downregulation of a key tight junctional protein claudin-1 (210)
Claudin-3	–	Decreases alveolar epithelial barrier function (211)
Claudin-4	+	Improves the barrier function of pulmonary epithelial barrier by promoting pulmonary fluid–clearance function (211, 212)
Transient receptor potential vanilloid 4 (TRPV4)	–	Disruption of alveolar type I epithelial cells leading to lung vascular leak and alveolar edema (213)
Ethanol	–	Disruption of alveolar epithelial barrier function by activation of macrophage-derived TGFβ1 (214)
Acetoin (butter), diacetyl, pentanedione, maltol (malt), ortho-vanillin (vanilla), coumarin, and cinnamaldehyde	–	Impairment of epithelial barrier function in human bronchial epithelial cells (215)
Asbestos	–	Increases lung epithelial permeability through increasing epithelial fibrinolytic activity (216)
Pneumolysin (PLY)	–	Impairs epithelial barrier (217)
Fas-ligand system	–	Causes alveolar epithelial injury in humans with ALI or ARDS (218) Impairs alveolar epithelial function in mouse lungs by mechanisms involving caspase-dependent apoptosis (219) Inducing apoptosis of cells of the distal pulmonary epithelium during ALI (57)
CO	–	Enhances pulmonary epithelial permeability (220, 221)
Tight junctions (TJ)		
Purinergic receptor	+	Preserving integrity of endothelial cell (EC)-cell junctions (222)
Na^+/K^+ ATPase	+ +	Formation of TJs through RhoA GTPase and stress fibers (223) Gene transfer of β1-Na^+, K^+-ATPase upregulates TJs formation by enhancing expression of TJ protein zona occludins-1 and occludin and reducing pre-existing increase of lung permeability (224)
Nitric oxide (NO)	–	Decreases expression and mistargeting of TJ proteins in lung (225)
Influenza A virus (IAV)	–	Disruption epithelial cell TJs (226)
Caveolin-1	+	Regulates the expression of TJ proteins during hyperoxia-induced pulmonary epithelial barrier breakdown (227)
IL-4	–	Causes TJ disassembly and epithelial barrier permeability alteration *via* an EGFR-dependent MAPK/ERK1/2-pathway (228) Reduce protein density at the TJ without causing major changes in cldn1, cldn2, cldn3, and occludin protein levels (229)
IL-13	–	Reduction of protein density at the TJ without causing major changes in cldn1, cldn2, cldn3, and occludin protein levels (229)
TNF	–	Causes TJ permeability (230)
Interferon-gamma (IFN-γ)	–/+	Disorganization of the TJ and an increase in paracellular permeability (231) Promotes epithelial restitution by enhancing barrier function and wound healing (232) It can also reverse IL-4- and IL-13-induced barrier disruption (232)
Trypsin	–	Destroys the TJs which lead to airway leakage
Cigaret smoke	–	Causes disassembly of TJs, modulated through the EGFR–ERK1/2 signaling pathway (233)
Cadmium	–	Causes disruption of TJ integrity in human ALI airway cultures both through occludin hyperphosphorylation *via* kinase activation and by direct disruption of the junction-interacting complex (234)

(*Continued*)

TABLE 2 | Continued

Mediator	Impact on pulmonary barrier function	Mechanism of action
Capillary endothelium		
TGFβ 1	–	Induces endothelial barrier dysfunction *via* Smad2-dependent p38 activation (235)
TNF	–	Disruption of the lung vascular barrier (236, 237) Augmenting endothelial permeability (67, 238) Apoptosis of lung microvascular ECs (39, 239, 240)
Lectin-like domain of TNF	+	Strengthens barrier function or increasing endothelial barrier tightness (9) Protective effect in PLY-Induced endothelial barrier dysfunction (9) Can reduce PLY-induced RhoA/Rac-1 balance impairment and MLC phosphorylation (10) Protects from listeriolysin-induced hyperpermeability in human pulmonary microvascular ECs (241) Reducing vascular permeability (196) Increases in membrane conductance in primary lung microvascular ECs (242)
IFN-γ	–	Increases vascular permeability (243)
Interleukin-1β (IL-1β)	–	Given intratracheally, IL-1β increased endothelial permeability and lung leak (244–247) Increases vascular permeability (243)
Interleukin-2 (IL-2)	–	Increases vascular permeability (248)
Interleukin-6 (IL-6)	–	Increases endothelial permeability (249)
Interleukin-8 (IL-8)	–	Increases endothelial permeability (250)
Interleukin -12 (IL-12)	–	Upregulate the release of the vascular permeability factor which is a lymphokine derived from LN peripheral blood mononuclear cells (251)
Neutrophils	–	Inducing endothelial barrier disruption through secretion of leukotrienes or heparin-binding protein, direct signaling into the EC *via* adhesion-dependent mechanisms and production of ROS (252)
ENaC	+	ENaC-α can strengthen capillary barrier function (9)
TRPV4	–	Increases in vascular permeability thus promoting protein and fluid leak (253) Applying TRPV4 inhibitors exhibits vasculoprotective effects, inhibiting vascular leakage, and improving blood oxygenation (254)
Thrombin	–	Increase in endothelial permeability (255)
Platelet-activating factor	–	Increase in endothelial permeability (256)
Hydrogen peroxide	–	Increase vascular permeability through enhancing vascular endothelial growth factor expression (257)
Integrin αvβ5	–	Increases pulmonary vascular permeability (258)
T-cadherin	–	Causes enhancement of endothelial permeability (259)
Myosin light chain kinase	–	Vascular hyperpermeability (260)
Lipopolysaccharide (LPS)	–	Induces lung endothelial barrier dysfunction (261)
PLY	–	Impairs endothelial barrier (10, 262)
P2Y receptors	+	Regulators of lung endothelial barrier integrity (263)
CO	–	Enhances pulmonary epithelial permeability (221)
Soluble receptor for advanced glycation end products	–	Increase in alveolar–capillary barrier permeability (264)
EC adhesion		
Podocalyxin	+	Decreases vascular permeability of ECs by altering EC adhesion (265)
NLRP3	+	Protects alveolar barrier integrity by an inflammasome-independent increase of epithelial cell adherence (266)

heart has the ability to produce pro-inflammatory TNF during dilated myopathy. Third, decreased cardiac output could cause systemic tissue hypoxia with subsequent systemic inflammation, which might be the primary stimulus for increased TNF production (21).

Soluble TNF receptor-1 and interleukin-8 (IL-8) are independently associated with cardiovascular mortality, as is endothelin-1. In transgenic mice overexpressing TNF the left ventricular ejection fraction was depressed depending on TNF gene dosage (21). TNF has been associated with worsened prognosis. However, two studies aiming to neutralize the cytokine in heart failure, using the soluble human TNF receptor 2 construct etanercept, were stopped because of lack of clinical benefit and patients receiving the highest dose even had increased adverse

TABLE 3 | Comparison of the properties of highly selective and non-selective channels.

	Highly Selective	Non-selective
Na/K selectivity (267, 268)	>40	1.1
Unit conductance, pS (267–269)	6	21
Amiloride K_i, nM (268, 270)	38	2,300
Increased cellular cyclic adenosine monophosphate or β-adrenergic stimulation (271, 272)	Channel surface density increases	P_o increases
Increased cGMP or NO (273)	P_o decreases	P_o decreases
Protein kinase C activation (274, 275)	P_o decreases, surface density decreases	Channel surface density increases
Increased intracellular Ca^{2+} (271)	No effect	P_o increases
Purinergic stimulation (276–279)	P_o decreases	P_o increases
Dopaminergic stimulation (176, 280)	P_o increases	No effect
Superoxide production (111)	P_o increases	Channel surface density increases
Hypoxia (268)	Channel surface density decreases	Channel surface density increases

P_o, channel open probability; K_i, inhibitory constant, i.e., the dose that reduces open probability by 50%.

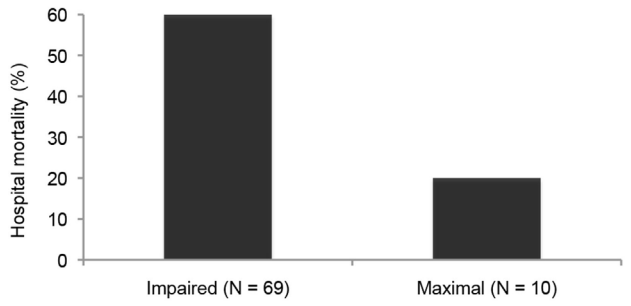

FIGURE 1 | Hospital mortality is increased in patients with acute lung injury or the acute respiratory distress syndrome with impaired fluid clearance (17).

outcomes (27). Similar results were observed with the neutralizing antibody infliximab (28). Whether the negative results are explained by inappropriate blocking of a "physiological" inflammation linked with tissue-reparative processes such as cardiac remodeling, or whether other mechanisms like too advanced heart failure, infections, toxicity of treatment, or genetic polymorphisms are involved, remains open, and should be further studied (21). Recently, it was suggested that beneficial or detrimental effects of TNF neutralizing agents depend on whether they spared or rather blunted discrete amounts of TNF that preconditioned cardiomyocytes to make them more resistant to high concentrations of the cytokine (29). The results, however, put forward that cytokines are effectors and not solely biomarkers in heart failure. Furthermore, reparative processes in the myocardium are accompanied by reactive or replacement fibrosis, mediated by TGF-β1, endothelin-1, and angiotensin-II (21). Angiotensin-II decreases AFC *via* cyclic adenosine monophosphate (cAMP) effect on the Na^+/K^+-ATPase pathway. It is involved through p38 and possibly p42/44 MAP kinases with myocardial hypertrophy, inflammation, and neurotransmitter and catecholamine synthesis and release in the brain. Angiotensin-II regulates the NF-κB-dependent gene expression in response to IL-1β stimulation by controlling the duration of ERK and NF-κB activation (21). Many immune cell functions are moreover coupled to intracellular pH. As such, a higher pH represents an important signal for cytokine and chemokine release, and a low pH can induce an efficient antigen presentation. The pH regulating Na^+/H^+ exchanger isoforms may play a role in these events (30).

The kidney is a major target organ and a modulator in the pathogenesis of heart failure at least partially by means of the renin–angiotensin system. In initial heart failure, it aims at blood pressure maintenance by direct systemic vasoconstriction, *via* augmentation of the sympathetic nervous system activity and by promoting renal Na^+ retention. The latter mechanism is deleterious in the progress of cardiac failure and is characterized by enhanced Na^+ reabsorption in the proximal tubule and collecting duct induced by effects of angiotensin-II and aldosterone on NHE3 and ENaC, respectively (21). Two-thirds of filtered Na^+ is reabsorbed in the proximal tubule *via* transporters for amino acids, glucose, phosphate and *via* NHE3. At the distal tubule, Na^+ is reabsorbed by Na^+, K^+ co-transporter, which is sensitive to thiazide. In the collecting ducts, a minimal amount of sodium is reabsorbed by ENaC and this is increased by aldosterone. The counterbalance by the natriuretic and vasodilatory atrial natriuretic peptide is dominated at that point by angiotensin-II and aldosterone effects, attenuates endothelial-dependent renal vasodilation and leads to endothelial dysfunction characteristic of cardiac heart failure (21). Heart failure also causes a vasopressin-dependent water reabsorption which maintains blood pressure in the failing heart and further increases fluid retention. The renin–angiotensin system, especially angiotensin-II, activates the immune system and *vice versa*. TNF and IL-6 stimulate the generation of angiotensinogen, exaggerate sodium retention and enhance renal fibrosis. Angiotensin-II enhances TNF and IL-6 in cardiomyocytes and in renal cortical and tubular cells, impairs mitochondrial function, and is pro-oxidative (21). CRP also directly activates endothelin and by this may potentiate a pulmonary vasoconstriction. The review by Azzam et al. in this issue further discusses the causative role of cytokines in the development of cardiogenic edema.

Non-Cardiogenic or Permeability Pulmonary Edema

Non-cardiogenic pulmonary edema, also known as permeability pulmonary edema, accompanies ALI, pneumonia, pulmonary reimplantation response after lung transplantation, or ARDS (2, 31) (**Figure 2**, right panel). During the course of these diseases, the interstitium and the alevolus are sites of intense inflammation by an innate immune cell-mediated damage of the alveolar endothelial and alveolar epithelial barrier, with consecutive exudation of protein-rich pulmonary edema fluid (31–33), as recently reviewed by Thompson et al. (31).

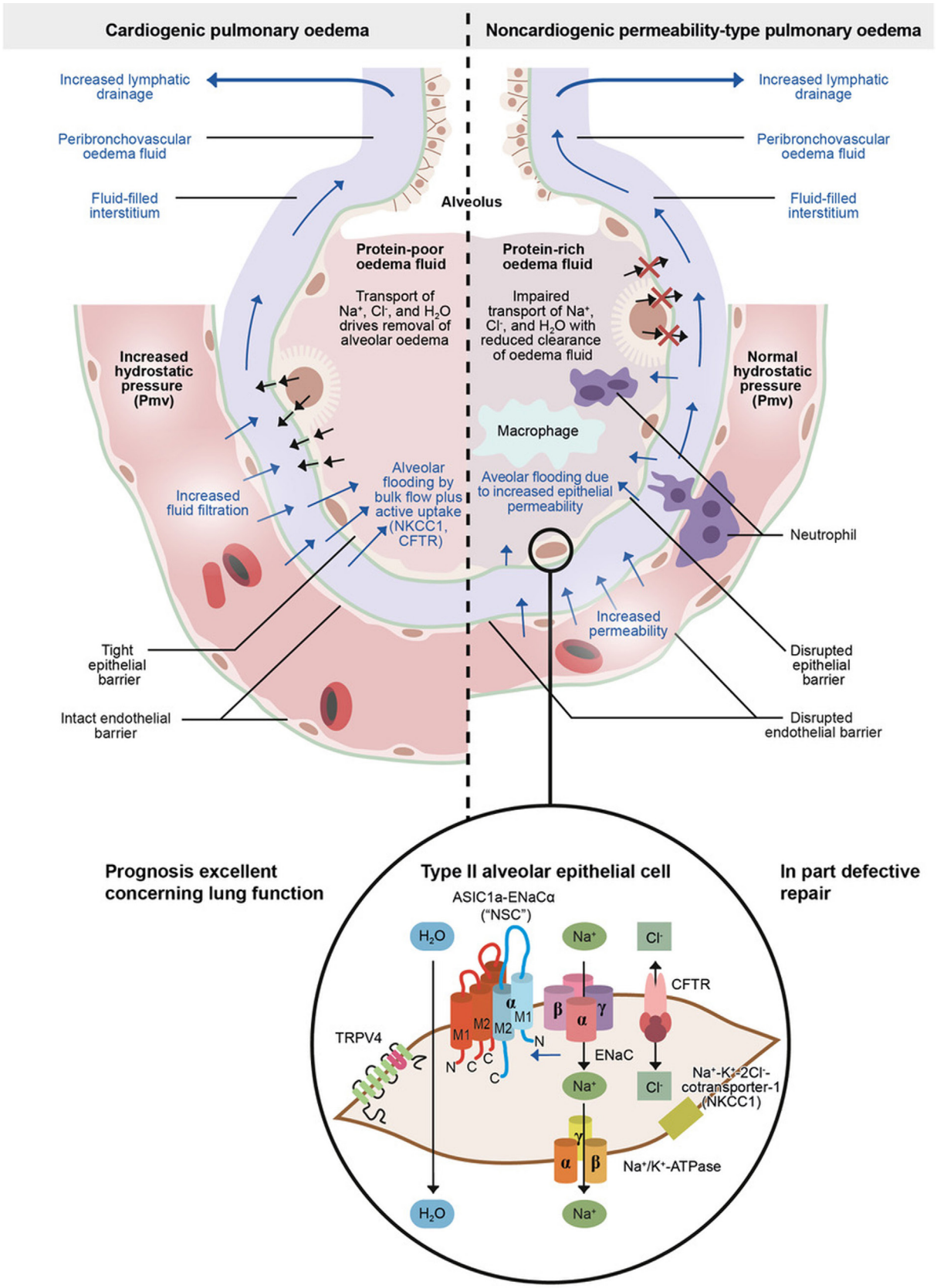

FIGURE 2 | Physiology of microvascular fluid exchange in the lung.

This type of pulmonary edema occurs due to modifications in barrier function of the pulmonary capillary or alveolar epithelial compartments as a consequence of either a direct or an indirect pathological process (31). There is some evidence that direct injury, such as pneumonia, aspiration, or pulmonary contusion, mainly affects epithelial barriers, whereas indirect blood-borne insults such as severe sepsis, non-thoracic trauma, pancreatitis, or burns may predominantly target the capillary endothelium (34). Permeability edema accompanies a spectrum of illnesses, ranging from the less severe form of ALI to ARDS (18). Variations in histology and in fluid management strategies suggest different ARDS subphenotypes (31). Apart from ARDS, ALI and severe pneumonia, also lung transplantation can be accompanied by acute pulmonary edema by the pulmonary reimplantation response (35). Ischemic vascular injury of the allograft results in increased permeability of the lung after reperfusion and in turn leads to interstitial and alveolar edema (33).

The extent of alveolar edema depends on the competing effects of increased permeability and the active edema fluid clearance from the alveolar space in regions where the epithelium is undamaged (31, 36). Inflammation plays a key role in the pathogenesis of permeability edema (37, 38) and can lead to the orchestration of a great variety of inflammatory and non-inflammatory cells, the former of which can locally release pro-inflammatory mediators such as TNF, LTD4 (32). There may also be endothelial and alveolar epithelial cell (AECs) death, which can further contribute to organ dysfunction and leak (39, 40). Moreover, a cascade of inflammation and a downregulation of repair mechanisms may occur (**Figures 3**, **4**).

Cells of the innate immune system, such as activated alveolar macrophages and recruited polymorphonuclear granulocytes (PMN) and also cells from the adaptive immune system, such as T_H17 cells can interact in ALI and ARDS and release huge amounts of mediators (31). Thrombo-coagulative processes ensue, e.g., TNF-mediated by tissue factor, with a proaggregatory role for platelets. Preventive aspirin was recently shown to protect from ARDS (41). Regional tissue overdistension especially during ventilation and repetitive opening and closing of inflamed alveolar spaces amplify the regional inflammation, further denaturing surfactant, underlining the vital importance of protective ventilation strategies and positions.

Although pulmonary edema is one of the most frequent medical emergencies, clinically it is sometimes difficult to differentiate between its two main subtypes: cardiogenic and non-cardiogenic edema (2). Moreover, to date, no proven drug therapy is available for permeability edema associated with ALI and ARDS (2, 31, 38). Morbidity and mortality inversely correlate with AFC capacity in this setting (42, 43). The severity of shock in sepsis-induced ARDS is associated with lower AFC (44).

As mentioned above, 56% of patients with permeability pulmonary had an impaired AFC, and only 13% a maximal AFC rate (**Figure 1**). Survival of patients with maximal alveolar clearance rate was higher, as compared to patients with abnormal clearance rate, and the days on mechanical ventilation was less in this group. Clinically impressive is also a series of post-lung transplant patients showing a relation between total ischemic time and the degree of post-transplantation protein-rich and

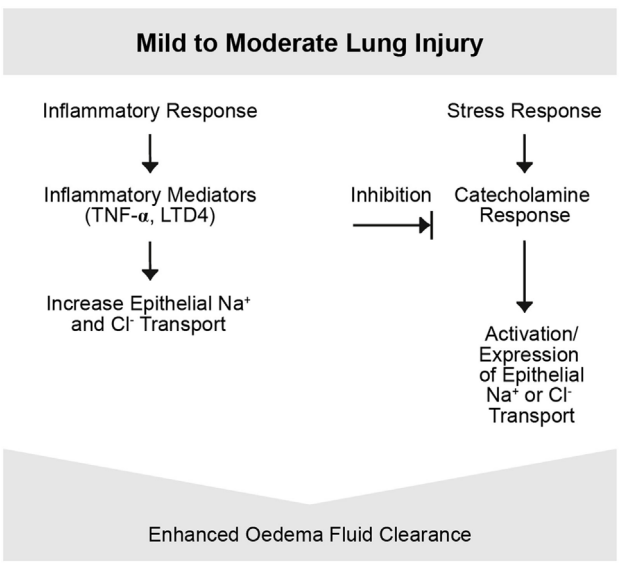

FIGURE 3 | Mild-to-moderate lung injury. Mild-to-moderate lung injury may lead to enhanced edema clearance. This response is due to an activation of epithelial Na^+ transport probably based on the increased endogenous catecholamine production associated with the insult. However, in certain types of injury, other pathways may be involved. Other inflammatory mediators such as tumor necrosis factor (TNF) potentially participate (4).

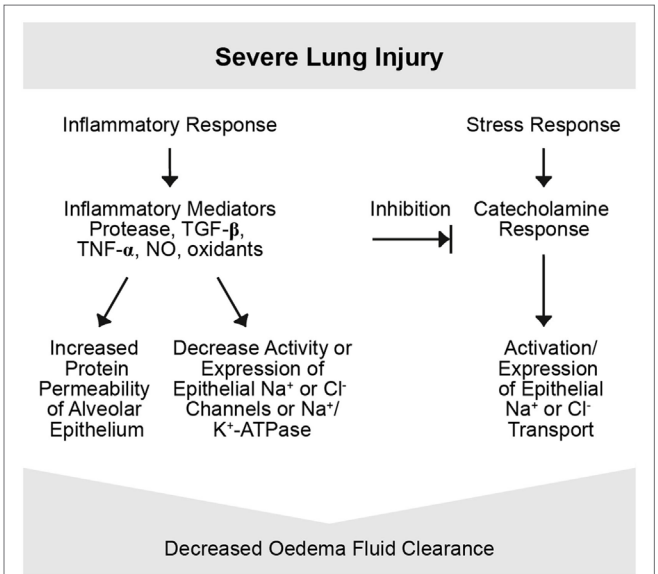

FIGURE 4 | Severe lung injury. Severe lung injury may usually lead to decreased edema clearance. Severe injury usually includes alveolar epithelial injury and, thus, increases epithelial permeability and electrolytes and is associated with reduced epithelial Na^+ transport. Inflammatory mediators are involved in this response, such as proteases, tumor necrosis factor (TNF), TGF-β, nitric oxide (NO), and oxidants. Possibly the intensity of the inflammatory response may transform a mild to a severe lung injury form by inducing changes in function and integrity of the alveolar epithelium and endothelium (4).

highly neutrophil-rich (71–99% of cells) permeability edema. Those patients with the best AFC had the best clinical outcomes, including the least and the fastest resolving pulmonary reimplantation response (45). Thus, the ability to reabsorb fluid from the alveolar space was a marker of less severe reperfusion injury. These findings indicate that intact alveolar epithelial fluid transport is critically important for a timely recovery from post-transplantation reperfusion pulmonary edema.

PULMONARY FLUID BALANCE THROUGH BARRIERS

Airways normally have a critically regulated fluid layer essential for normal gas exchange and removal of foreign particulates from the airway. Maintaining this fluid layer in the alveoli also depends critically on sodium reabsorption. The pulmonary epithelium serves as a barrier to prevent access of the inspired luminal contents to the subepithelium (11) and modulates the initial responses of the airways and lung to both infectious and non-infectious stimuli (11). One mechanism by which the epithelium achieves this is by coordinating transport of diffusible molecules across the epithelial barrier, both through and between cells (11). Specific elements of pulmonary alveoli play different roles as a barrier maintaining the pulmonary fluid balance (38). These barriers will be discussed in more detail below.

Epithelial Barrier

Lung epithelium is a mucosal surface composed of ciliated cells, mucus-producing cells, and undifferentiated basal and progenitor cells. This dynamic barrier forms the interface between the lumen and the parenchyma from the upper airways to the alveoli. The lung epithelium constantly responds to luminal stimuli and coordinates its response to maintain homeostasis in the lung (11). A breakdown in this coordinated response can cause different lung diseases (11). The alveolar epithelium (0.1–0.2 μm) covers 99% of the airspace surface area in the lung (46) and contains a number of important cell types. Type I cells (AT1) cover at least 95% of the alveolar surface and are the apposition between the alveolar epithelium and the vascular endothelium. This provides a tight barrier that facilitates efficient gas exchange and which is involved in fluid and protein movement from the interstitial and vascular sites (38, 47) and its reabsorption *vice versa* (4, 5). The role of aquaporin 5 (AQ-5) in AFC is not clear, in view of the normal AFC capacity in physiological situations in AQ-5 knock out mice (48). The osmotic clearance of water secondary to the ion transport gradient across the alveolar epithelium probably occurs by paracellular pathways and not by the assumed transcellular using aquaporin 5 (25); however, their role in injury is not fully excluded (4). Type II cells (AT2) cover about 5% of the alveolar surface and are known especially for their key function in surfactant secretion and in vectorial transport of Na^+ (49), a major driving force for fluid removal from the alveolar space. Amiloride-sensitive sodium channels on the apical, "air-faced," surface, mainly the ENaC, are key channels in alveolar fluid transport (50, 51), with the driving force stemming from the Na^+/K^--ATPase on the basolateral, "blood-faced," surface (46). Dysfunction of these Na^+ transporters during inflammation can contribute to pulmonary edema (52–54). Tight junctions (TJ) that connect adjacent epithelial cells near their apical surfaces and maintain apical and basolateral cell polarity are fundamental to create a permeability barrier required to preserve distinct compartments in the lung (55).

Alveolar and distal airway epithelia are surprisingly resistant to injury, particularly if compared to the adjacent lung endothelium. When lung endothelium gets injured, the alveolar epithelial barrier may retain its normal impermeability and its normal fluid transport capacity, as seen in animal models with LPS given intravenously or intratracheally (4). This might explain why in mild-to-moderate lung injury AFC may not only be preserved, but even upregulated by stress hormones—an effect that may be inhibited by amiloride or propranolol.

However, in severe ALI, ARDS, and pneumonia, epithelial cell death may occur, as has been shown in a seminal morphological study published 4 decades ago by Bachofen and Weibel (56). A central role for soluble Fas ligand (FasL) has been proposed in AT1 and AT2 cell death, and an association between its levels in bronchoalveolar lavage level on day 1 of ARDS and patient death has been proposed (57, 58). However, there may be extensive crosstalk between injurious, inflammatory, and death cascades and repair in the lungs, as well as in other organs in patients with ARDS. Direct alveolar cell death may probably also occur due to bacterial exotoxins or stresses like overdistension. Such epithelial cell death may make the lungs prone to increased permeability and thus disturb AFC, as well as to the danger of disordered repair, such as in fibroproliferative ARDS.

Recent work on different predictors of ARDS suggests that the degree of AT1 cell injury is a central determinant of outcome in ALI and ARDS. Receptor for advanced glycation end products (RAGE) is an immunoglobulin superfamily member, involved in propagating inflammation. RAGE is abundant in the lungs and can be primarily found in AT1 cells. Higher baseline plasma levels of RAGE were found to be associated with worse outcome, including less ventilator-free days and increased mortality, and it excellently discriminated in sepsis patients for the diagnosis of ARDS. Higher levels in bronchoalveolar lavage also predicted post-lung-transplant primary graft failure and correlated with its grade of severity (59). Apart from RAGE, also surfactant protein D level, an AT2 cell product, was, together with the neutrophil chemokine IL-8 (CXCL8), the best performing biomarker for poorer outcome in terms of mortality (60).

Endothelial Barrier

The capillary endothelial barrier also functions as a key component to maintain the integrity of the vascular boundaries in the lung. The gas exchange surface area of the alveolar–capillary membrane is extremely huge and optimized to facilitate perfusion–ventilation matching (61). Pulmonary endothelium separates also the intravascular marginated pool of polymorphonuclear neutrophils from the airspaces. The endothelium, the most abundant cell relative to the total cell population in the lung, has additional key regulatory roles apart from gas exchange,

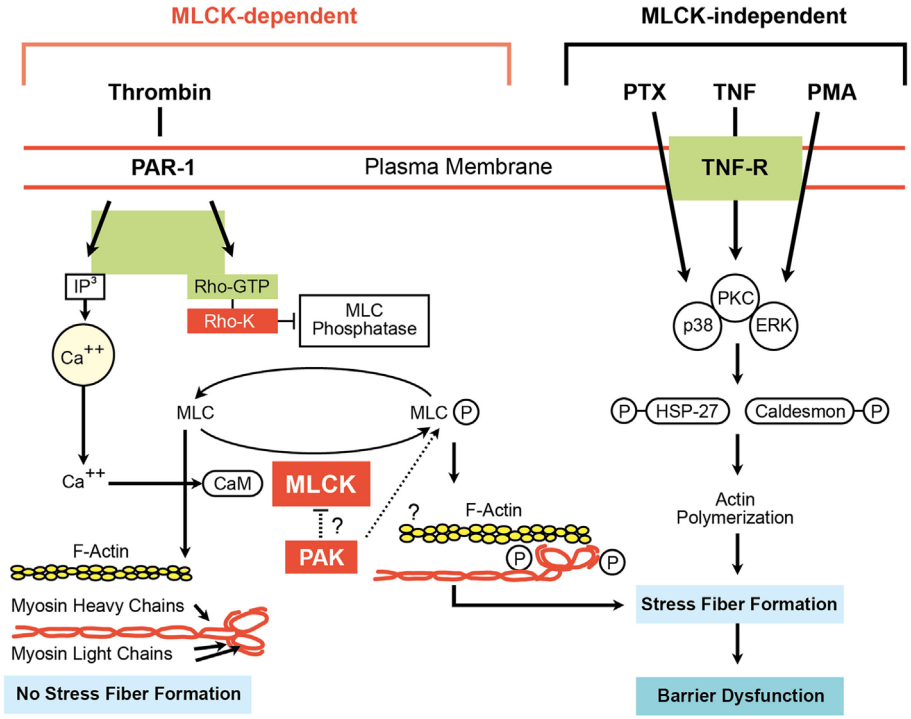

FIGURE 5 | Myosin light chain kinase (MLCK)-dependent and MLCK-independent pathways involved in endothelial cell (EC) barrier dysfunction (66). Adapted from Ware and Matthay (2).

namely vascular tone *via* nitric oxide (NO) and endothelin-1, and coagulation, as recently discussed in depth in a review on the endothelium and ARDS (34).

In the pulmonary microvasculature, the endothelial cells (ECs) form a semi-permeable barrier between the blood and the lung interstitium (38). Disruption of this barrier may occur during inflammatory disease such as pneumonia, ALI, ARDS, or ischemia–reperfusion injury. In sepsis, early microcirculatory perfusion indices are more markedly impaired in non-survivors, as compared to survivors and correlate with increasing severity of vascular dysfunction (62). Lung ECs are considered orchestrators of the inflammatory response. These cells can directly sense pathogens *via* toll-like receptors and may contain local bacterial spreading by coagulation, leading to capillary thrombosis and extravascular fibrin deposition (34). This contributes to an increased dead-space fraction that correlates with clinical outcome (63). In sepsis, overwhelming EC activation can lead to apoptosis within minutes to hours (64), which in turn increases barrier permeability and subsequent mortality (65). In ARDS, EC death can occur in by mechanical insults, like shear stress, and by pro-inflammatory mediators, including TNF, angiostatin, and TGF-β (39).

Intercellular junctions act as dynamic structures and do not statically resist entry to all substances. They that can open or close in response to physiological or pathological stimuli. **Figure 5** presents some potential pathways regulating EC barrier function (66). Endothelial barrier dysfunction can result in the movement of both fluid and macromolecules into the interstitium and pulmonary air spaces. This can contribute to important morbidity and mortality (66). TNF can reduce capillary endothelial barrier function (67, 68).

REGULATION OF AFC

In the normal lung, fluid and protein leakage is thought to occur primarily through small gaps between capillary ECs (2, 3). Since both capillary endothelial and AECs have TJ, fluid, and macromolecules that are filtered from the circulation into the alveolar interstitial space normally do not enter the alveoli (2).

The hydrophobic plasma membranes composed of phospholipids, act as a huge energy barrier for transporting ions (69–71). Yet, physiological processes assure for the continuous in- and outflow of ions, as such overcoming the plasma membrane barrier, which is impermeable to ions. Due to their biological complexity, interactions between cytokines and ion channels may be under-recognized (72). A group of plasma membrane proteins, including active transporters, generate and maintain ion concentration gradients for particular ions. These active transporters carry out this task by forming complexes with the ions they are translocating. The process of ion binding and unbinding for transport typically requires several milliseconds. As a result, ion translocation by active transporters is much slower than ion movement through ion channels, which can conduct thousands of ions across a membrane each millisecond. Active transporters effectively store energy in the form of ion concentration gradients, whereas the opening of ion channels

rapidly dissipates this stored energy during relatively brief electrical signaling events.

Several types of active transporters have now been identified. Although the specific roles of these transporters differ, all must translocate ions against their electrochemical gradients (energetically "uphill"). Moving ions uphill requires the use of energy, and neuronal transporters fall into two classes based on their energy sources. Some transporters acquire energy directly from the hydrolysis of ATP and are called ATPase pumps. The most prominent example of an ATPase pump is the Na^+/K^+-ATPase pump, which is responsible for maintaining transmembrane (TM) concentration gradients for both Na^+ and K^+ (73). Another one is the Ca^{2+} pump, which provides one of the main mechanisms for removing Ca^{2+} from cells. The second class of active transporters does not use ATP directly as an energy source, but rather the electrochemical gradients of other ions. This type of transporter carries one or more ions up its electrochemical gradient, while simultaneously taking another ion, most often Na^+, down its gradient. These transporters are usually called ion exchangers. An example of such a transporter is the Na^+/Ca^{2+} exchanger, which shares with the Ca^{2+} pump the important task of keeping intracellular Ca^{2+} concentrations low. Other exchangers regulate both intracellular Cl^- concentration and pH by swapping intracellular Cl^- for another extracellular anion, bicarbonate, or the Na^+/H^+ exchanger that regulates intracellular pH, by regulating the concentration of H^+. Although the electrochemical gradient of Na^+ (or other counter ions) is the immediate source of energy for ion exchangers, these gradients ultimately depend on the hydrolysis of ATP by ATPase pumps, such as the Na^+/K^+ ATPase pump (74).

Alveolar fluid clearance is mainly regulated by Na^+ uptake through the apically expressed ENaC and the basolaterally localized Na^+/K^+-ATPase in type II AECs (**Figure 2**, lower panel) (54). Dysfunction of these Na^+ transporters during pulmonary inflammation can contribute to pulmonary edema (54). In this context, the movement of larger plasma proteins is restricted (2). The hydrostatic force for fluid filtration across the lung microcirculation is approximately equal to the hydrostatic pressure in the pulmonary capillaries, which is partly compensated by a protein osmotic pressure gradient (2). The net quantity of accumulated pulmonary edema is logically determined by the balance between the rate at which fluid is filtered into the lung (1) and the rate at which fluid is removed from the air spaces and lung interstitium (46). In mild-to-moderate lung injury, the capacity of the alveolar epithelium to transport salt and water is not only preserved but may also even be upregulated by stress hormones (**Figure 3**) (4). In severe lung injury, pulmonary fluid clearance can also be stimulated in lung injury by catecholamine-independent mechanisms (**Figure 4**) (4).

Moderate hypoxemia was shown to reduce AFC by 50%. This is caused by decreasing apical sodium uptake, at least partially through impaired trafficking of ENaC to the surface membrane (75–77). Hypoxia, moreover, inhibits the function of Na^+/K^+-ATPase in AECs, in part by triggering endocytosis through reactive oxygen species (ROS) and phosphorylation of the α1 subunit (78) (**Figure 6**). Restoration of normoxia rapidly reversed the depressant effects of hypoxemia in rats. Therefore, the simple administration of supplemental oxygen to patients with pulmonary edema may enhance the resolution of alveolar edema. As discussed more in detail in a contribution by Vadasz and Sznajder in this topic issue, hypercapnia can also impair AFC by the mechanisms of ubiquitination-mediated retrieval of ENaC from the plasma membrane, i.e., a post-translational modification of βENaC by regulating trafficking and stability, thereby modifying, and in this case reducing cell surface expression of the channel through βENaC ubiquitinylation in the alveolar epithelium (78–80). This mechanism seems of importance in ARDS as well in COPD. Hypercapnia and the associated acidosis have been shown to have anti-inflammatory effects, which might be advantages at sites of excessive inflammation, whereas

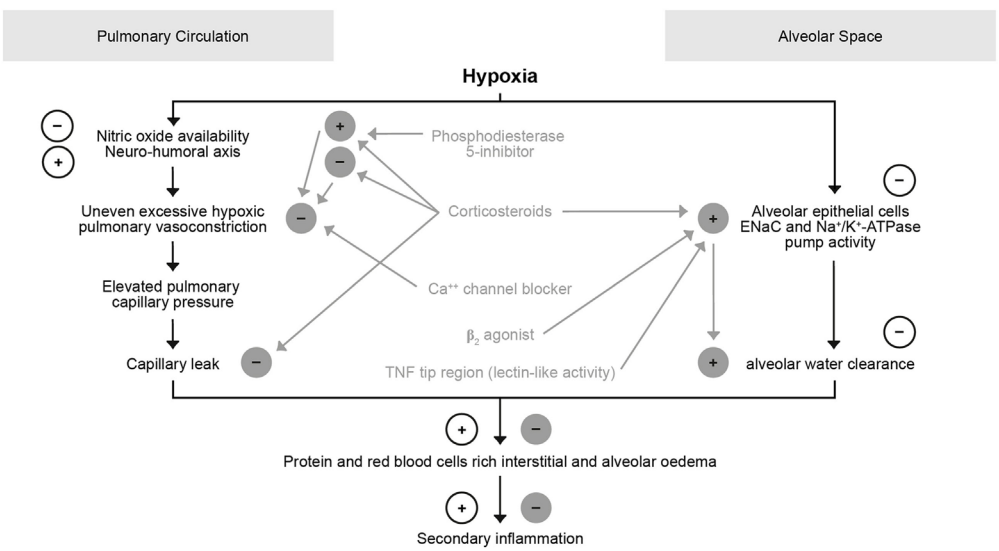

FIGURE 6 | Role of hypoxia in the pulmonary circulation and alveolar space.

on the other hand, ARDS and COPD studies showed that both patient groups had worse outcome when they were hypercapnic (78). In a randomized controlled trial Köhnlein, Windisch et al. showed that in severely sick, chronic hypercapnic COPD patients non-invasive ventilation, when targeted to reach noromocapnia (PaCO2 < 6.5 kPa/48.1 mmHg) or to improve hypercapnia by at least 20%, is associated with much better outcome (81). Survival was impressively improved, and also quality of life and lung function in terms of FEV1 improved. Possibly further effects exist such as sometimes improved cardiac output (82, 83), although interactions between ventilation and cardiac output are complex.

Ion Channels and Pumps/Transporters and AFC

Ion channels are integral membrane proteins that form a pore to allow the passage of specific ions by passive diffusion (84). Most ion channels undergo conformational changes from closed to open states. Once open, ion channels allow the passage of thousands of ions (84). This distinguishes them from transporters and pumps, which can also transport ions, but only a few at a time (84). The opening and closing of channels can be controlled by various means, including voltage, the binding of ligands such as intracellular Ca^{2+} or extracellular neurotransmitters, and post-translational modifications such as phosphorylation (84).

Ion channels and pumps also play multiple important roles in cell homeostasis (84). Their function promotes passive, agonist-induced, or voltage-dependent flux of specific ions in and out of the cell (84, 85). The mchanisms of removing the infiltrated fluid from the alveoli is called AFC (84).

The ENaC in Type I and II Alveolar Epithelial Cells

Epithelial sodium channel, a member of the ENaC/degenerin (ENaC/DEG) family of ion channels, constitutes the rate-limiting entry step in Na^+ reabsorption across epithelial in colon, kidney, and lungs (86). ENaC is inhibited by the drugs amiloride, benzamil, and triamterene, some of which are clinically used as potassium-sparing diuretics (87, 88). ENaC is a heteromultimeric protein (89) and is composed of at least four homologous subunits, α, β, γ, and δ (89–91) which are able to compose an ion channel (50, 92). A functional, pore-forming channel usually comprises one or two α subunits, together with a β - and a γ -subunit (89, 91, 93, 94). δ as a fourth unique subunit can form ion channels joining the β and γ subunits but exhibits biophysical and pharmacological features that are different compared to α ENaC channels (95). Investigations of the biological role of αENaC in the mouse lungs underlined the crucial role of this subunit in AFC (12). The β subunit is highly glycosylated and an important regulator of ENaC (4). In the lungs, ENaC is expressed not only in alveolar type II and type I cells (96), but also in capillary ECs (97).

Epithelial sodium channel was shown to exert a crucial role in pulmonary fluid reabsorption (46). Accordingly, ENaC is responsible for the maintenance of Na^+ balance, extracellular fluid volume and blood pressure (98). ENaC activity is determined by the number of channels in the surface membrane *N*, which can change according to membrane insertion, degradation, or retrieval, as well as by the open probability time *Po* of individual channels (86, 99, 100). The basolaterally expressed, ouabain-inhibitable Na^+/K^+-ATPase then further drives the vectorial transport into the interstitium and, finally, into the lymphatic and blood vessels (73).

In order to maintain the correct composition and volume of alveolar lining fluid, Na^+ transport through apically located ENaC in the alveolar epithelium is critical for gas exchange (92).

Epithelial sodium channel expression was shown to be decreased in transplanted lungs, both at the messenger RNA and protein level (8, 101).

Physiological ENaC Regulation

Epithelial sodium channel activity is important for fluid homeostasis and blood pressure control, but its regulation is complex and remains in many aspects incompletely understood (102) (**Table 1**). ENaC channels are also called highly selective cation (HSC) channels, and are presumed to be made up by the three ENaC subunits, α, β, and γ (103).

Epithelial sodium channel function can be affected by direct modulation of channel activity (92), subunit degradation, and membrane trafficking/recycling (104). cAMP indirectly increases ENaC activity, since it activates Cl^- uptake through CFTR (105). Intracellular as well as extracellular proteases, including prostasin and furin can affect the activity of the channel by modulating the Na^+ self-inhibition (106, 107). Another important system that modulates ENaC activity is trafficking of the channels to the membrane, which involves a complex system of ubiquitination and binding to Nedd-4-2 (108). Na^+ transport can also be regulated by gene expression (4). The two major hormonal modulators of pulmonary ENaC expression are catecholamines (50) and corticosteroids (109).

Many agents that increase Na^+/K^+-ATPase activity also increase ENaC activity (36). Negative ENaC regulators are activated purinergic P2Y receptors (110), NO (111, 112), Il-1β (113), hypoxia (46), and TGF-β (46).

ENaC Dysfunction

Dysfunction of the ENaC, which regulates salt and water homeostasis in epithelial, causes several human pathological conditions, including pulmonary edema (114). As ENaC regulates the airway surface liquid layer, its exaggerated activity might lead to airway dehydration, mucus stasis and bacterial overgrowth, as can be seen in cystic fibrosis and chronic bronchitis (115–117). ENaC hypo-activity, by contrast, can dramatically impair AFC, which is particularly important in conditions of pulmonary edema and correlates with mortality and morbidity in patients with ALI and ARDS (33).

The significant role of ENaC in inherited diseases associated with mutations in ENaC which increase or decrease channel activity regarding salt and water homeostasis has been well-documented (118). Mutations in the PPxY motif of β- and γ-subunits cause a severe form of hypertension, associated with ENaC in Liddle's syndrome (OMIM: 177200) (119–123). A decrease in ENaC function can also cause a rare, life-threatening salt-wasting syndrome in pseudohypoaldosteronism type 1B

(PHA1B) (OMIM: 264350) (124–127). This disease does not improve with age and patients are at risk from life-threatening, salt-losing crises, combined with severe hyperkalemia and dehydration throughout their entire lives (128, 129). Additionally, dysregulation of channel function and/or expression can lead to organ dysfunction and severe disease (84, 85, 130).

The Hybrid Acid-Sensing Ion Channel 1a (ASIC1a)/α-ENaC (NSC) Channels in Alveolar Type I and Type II Cells

Apart from ENaC, another apically expressed channel was recently shown to promote AFC. This hybrid channel is relatively non-selective for Na^+ over K^+, has a larger conductance, and shorter mean open and closed times (103, 131). In elegant assays, Trac et al. showed that the channel included ASIC1a as the mandatory counterpart to α-ENaC. These hybrid channels are, thus, composed of, at a minimum, one α-ENaC subunit and one or more ASIC1a subunits. The biological significance is great, as the regulation of these NSC channels is dramatically different from ENaC. Thus, treatments to reduce alveolar flooding based on the known properties of ENaC (HSC) could be suboptimal because ASIC1a/α-ENaC-channels are regulated differently (see **Table 3**). Indeed, NSC channels are less sensitive to inhibition by amiloride than ENaC HSC channels.

As the proton-gated ASIC1a plays a role in the formation of channels, its properties determine the pharmacological ASIC1a/α-ENaC-channels (NSC) modulation. The MitTx agonist, derived from Texas coral snake toxin, strongly activates ASIC1a/α-ENaC-channels (NSC) (**Table 3**).

Why Do Alveolar Epithelial Cells in the Lungs Have Several Types of Channels That Mediate Na^+ Uptake?

As shown, an important functional role of non-selective cation (NSC) channels, which consist of ASIC1a and of ENaC-α subunits (281), is Na^+ uptake by AT2 cells in the lung (103). By contrast, other sodium-transporting epithelial tissues such as the distal nephron of the kidney and the colon were not reported to have these functional NSC channels, and mainly transport Na^+ through ENaC. In the lungs, the alveolar fluid layer must be very tightly controlled. Therefore, it may be important to have alternative ion transport pathways that respond differently to physiological stimuli, such as to acidification, which accompanies ALI and which activates NSC channels (282). An alternative hypothesis is that NSC channels provide a stable driving force for cation and anion movement across the alveolar epithelium. Indeed, NSC channels contribute to the apical membrane potential, causing the membrane potential to be close to zero. This will ensure that there is a driving force for the unidirectional movement of anions, through CFTR and for movement of Na^+ through classical ENaC and NSC into cells. This is necessary because of the requirement to move salt, i.e., anions plus cations. Other epithelia tend to have counter-ion pathways for cations that obviate the need to maintain a strong potential driving force.

In an evolutionary context, the lung has been the most recent organ to adapt to a terrestrial environment. Typical for evolutionary processes is the modification of existing mechanisms to produce a different evolutionary outcome, in this case, the formation of a new channel type out of parts from two pre-existing channels of the same channel family. Of further evolutionary interest is that the activity of both HSC channels HSC (ENaC) and NSC channels is increased by a peptide mimicking the lectin-like region of TNF, which binds to ENaC-α, as shown below and in Czikora et al. (9), in this issue (9).

The Na^+/K^+-ATPase

Apart from apical ENaC and, potentially NSC, the basolaterally expressed Na^+/K^+-ATPase, a.k.a. the sodium-potassium pump is also a crucial driver of AFC (73, 78). Na^+/K^+-ATPase activity regulation also involves complex patterns, including modulation of the trafficking of the protein to the membrane (73). The Na^+/K^+-ATPase is a ubiquitous enzyme consisting of α and β subunits and a less well-characterized regulatory FXYD subunit. The Na^+/K^+-ATPase is responsible for the generation and preservation of the Na^+ and K^+ gradients across the cell membrane by transporting 3 Na^+ out and 2 K^+ into the cell (283).

Changes in intracellular Na^+ concentration and hormones, such as mineralocorticoids, glucocorticoids and thyroid hormones as well as adrenoceptor stimulants modulate Na^+/K^+-ATPase activity (284). Like ENaC, increase of Na^+/K^+-ATPase expression is considered central to enhance transepithelial Na^+ transport (4). In addition, thyroid, mineralocorticoid and glucocorticoid hormones modulate Na^+/K^+-ATPase expression (4). Likewise, β adrenoceptor activation upregulates Na^+/K^+-ATPase expression in AECs (50).

The Na^+/K^+-ATPase contains one principal catalytic subunit, designated α and one sugar-rich auxiliary subunit, designated β. There is also a regulatory subunit FXYD subunit, which was recently shown to play an important role in regulation of lung inflammation (285). The α-subunit carries the catalytic function of the enzyme, and this is reflected in its possession of several binding and functional domains (283). The α subunit (4) transports Na^+ out of the cell, providing the driving force for Na^+ reabsorption (286). It is clear that an essential role for β subunit lies in the delivery and the appropriate insertion of the α subunit in the membrane (287). In recent years, a variety of studies have suggested that the β subunit may be more intimately involved in the mechanism of active transport (287–290).

FXYD5 or Dysadherin or RIC is a pro-inflammatory type I membrane protein, which belongs to seven members of the FXYD family named by their shared TM amino acid motif. FXYD5 is an established tissue-specific modulatory subunit of Na^+/K^+-ATPase, expressed in a variety of epithelial cells. Recent work shows a role for FXYD5 as a key mediator of the inflammatory response during ALI (285). It impairs adherens junctions by downregulating the markers zona occludins-1 (ZO-1) and occludin and redistributing beta catenin (291). It is required for the secretion of NF-κB, e.g., upon lipopolysaccharide (LPS), and inflammatory mediators, including TNF and interferon-α (IFN-α) and C-C chemokine ligand-2 (CCL2) from AECs that activate alveolar macrophages, amplify lung injury by orchestrating an overly exuberant inflammatory response, and recruit monocytes into the alveolar compartment, or in bronchoalveolar lavage

fluid (285). The presence of FXYD5 is an important component for NF-κB activation pathway as shown in AECs induced by LPS, TNF, or interferon-α, as its silencing prevented IκB-α phosphorylation and reduced cytokine secretion in response to these stimuli. Probably FXYD5 increases CCL2 transcription by inducing Akt-dependent activation of NF-κB signaling. Binding of IFN-α activated phosphoinositide 3-kinase (PI3K) *via* STAT5, which in turn activates NF-κB. Activation of PI3K seems downstream of TLR4 and TNFR1. Possibly, FXYD5 modulates NF-κB signaling by regulating the location of TNF receptor 1, by modulation associations with other proteins and their location and mobility in the membrane (285). It is of interest that FXYD5 regulates inflammation, activates NF-κB dependent cytokine secretion and infiltration of immune cells to the alveolar spaces as well as alveolar barrier tightness, and is closely linked to one key ion transport channel.

The Cystic Fibrosis Transmembrane Conductance Regulator

Cystic fibrosis transmembrane conductance regulator is a cAMP-regulated and post-translationally modified chloride channel of 1,480 amino acids, which is mainly expressed in epithelial cells. The non-glycosylated form of CFTR has a molecular weight of 127 kDa, with 160 kDa for the glycosylated form. CFTR can either take up or release Cl^- ions from the AT1 and AT2 cells. Apical to basolateral chloride transport may be important because the maximal rate of sodium and water transport from the airspaces appears to be limited by the concomitant chloride transport (115–117). An important part of transepithelial chloride transport occurs through the paracellular route in the alveolar epithelium. The selectivity and magnitude of paracellular ion conductance may influence net transport capacity. Upon increasing Cl^- influx, CFTR will activate ENaC-mediated Na^+ uptake, as such activating AFC, but the channel will inhibit AFC upon increasing Cl^- efflux. Increased cAMP generation will open CFTR in the apical membrane of AT1 and AT2 cells for Cl^- uptake, as such increasing Na^+ uptake and AFC. Therefore, factors that can activate cAMP-mediated Cl^- uptake by CFTR, such as β2 agonists, have been investigated as potential therapeutic candidates for pulmonary edema (105). Cystic fibrosis, a disease characterized by impaired airway dehydration, is caused by a loss of function of CFTR, accompanied by an excessive activity of ENaC. A peptide mimetic of SPLUNC, i.e., SPX-101, was shown to promote internalization of the three ENaC subunits and to restore mucus transport in a mouse and a sheep model of CF (292).

The Transient Receptor Potential Vanilloid 4 (TRPV4) Channel

Transient receptor potential vanilloid 4 is a TM cation channel and a vanilloid-type member of the transient receptor potential (TRP) protein superfamily (293). TRPV4 is ubiquitously expressed in many cell types in the respiratory system (294). It is part of an integrated system, consisting of ion channels and membrane pumps, which tightly regulates intracellular calcium levels in a spatiotemporal manner (295). TRPV4 counts 871 amino acids and contains six TM domains, an ion pore located between TM5 and 6, an NH2 terminal intracellular sequence with several ankyrin-type repeats, and a COOH-terminal intracellular tail (296, 297). Both the NH2 and COOH termini interact with signal kinases, other molecules (e.g., NO), and scaffolding proteins (298). The intracellular tails contain several activity-modifying phosphorylation sites (294). In the setting of pulmonary inflammation, TRPV4 has been found to be highly expressed and upregulated in airway smooth muscle, vascular ECs, AECs, as well as in immune cells, such as macrophages and neutrophils (298–303).

The Role of TRPV4 in Pulmonary Edema

Transient receptor potential vanilloid 4 mediates cellular responses to both physical (such as osmotic, mechanical, and heat) as well as chemical stimuli (304). It is also involved in lung diseases associated with parenchymal stretch and inflammation or infection (254, 294). Target diseases include cough, asthma, cancer, and pulmonary edema associated with ARDS (253, 294, 305–310).

These studies support a role for TRPV4 in a broad spectrum of lung and airway functions and disease processes. TRPV4 also has been implicated as a key regulator of lung endothelial barrier integrity, specifically, the integrity of the lung alveolar–capillary endothelium, which is most relevant to alveolar edema generation in ALI (311). TRPV4 activation increases vascular permeability, thus promoting protein and fluid leak (254).

Several studies have shown that TRPV4 can regulate generation of inflammatory cytokines that play key roles in orchestrating lung tissue homeostasis and inflammatory lung disease (301, 307, 309, 310, 312–314). Therefore, TRPV4 could be considered a potential target for lung disease pathogenesis, including to alveolar–capillary barrier function (300). TRPV4 has been proposed as a candidate target for the management of ALI that develops as a consequence of aspiration of gastric contents, or acute chlorine gas exposure (254). Protection from the ALI response to intratracheal HCl and a key role *in vivo* of polymorphonuclear neutrophil TRPV4 (294) was noted in mice that lack TRPV4 (TRPV4 KO), or in mice that were treated with three different small molecule inhibitors of TRPV4 (253, 301, 307, 309, 312, 313, 315).

However, in view of its ubiquitous expression, and the multitude of functions attributed to the channel, including its role in pulmonary vasomotor control, endothelial barrier tightness, inflammatory response and systemic blood pressure regulation, TRPV4 blockade may represent a double-edged sword. Therapeutic benefits of TRPV4 inhibition have, therefore, to be carefully weighed against potential adverse effects (254).

Transient receptor potential vanilloid 4 activation and its downstream signaling pathways differ in response to varying stimuli, cell types, and contexts (294). For instance in asthma, TRPV4 mediates hypotonicity-induced airway hyperresponsiveness, but not release of Th2 cytokines (312, 316). In CF, TRPV4 appears to play paradoxical roles in CBF/mucociliary clearance and epithelial cell pro-inflammatory chemokine (IL-8/KC) secretion (317, 318). Depending on the underlying etiology, TRPV4 may play different roles in ARDS (307, 310, 314, 319). Also, in pulmonary fibrosis, TRPV4 has been shown to mediate the

mechano-sensing that drives myofibroblast differentiation and experimental lung fibrosis in mice (308).

TRPV4 and Macrophage Function in Lung Injury

Alveolar macrophages are known to be effector cells in bacterial and particle clearance but also in any injury and repair process (320). Since intracellular Ca^{2+} is known to be required for the phagocytic process, and because TRPV4 plays a role in force-dependent cytoskeletal changes in other systems/cell types, the role of TRPV4 in macrophage phagocytosis was extensively studied by Scheraga and colleagues (213, 253, 307, 315, 321–323). The process of phagocytosis in macrophages requires integration of signals from macrophage surface receptors, pathogens, and the extracellular matrix (324–326). However, the effects of matrix stiffness on the macrophage phenotypic response or its signal transduction pathways have yet to be fully elucidated (294). TRPV4 mediates LPS-stimulated macrophage phagocytosis of both opsonized particles [immunoglobulin G (IgG)-coated latex beads] and non-opsonized particles (Escherichia coli) *in vitro* (294). Inhibition of TRPV4 by siRNA or pharmacologic inhibitors completely abrogated both the LPS effect and the matrix stiffness effect on phagocytosis (294). These data indicate that both the LPS and stiffness effect on macrophage phagocytosis are TRPV4 dependent (310). Concordant with their *in vitro* data, also LPS-induced alveolar macrophage phagocytosis was proposed to be TRPV4 dependent (294).

Collectively, obtained data demonstrate that TRPV4 responds to extracellular matrix stiffness, thereby altering the LPS signal to mediate macrophage phagocytosis and cytokine production (310). Furthermore, TRPV4 regulates a feed-forward mechanism of phagocytosis in activated lung tissue macrophages when they interact with stiffened infection/injury-associated lung matrix. This concept is further supported by the observation that surfactant protein B-deficient mice have altered alveolar macrophage shape and function in association with increased alveolar surface tension (327).

Other Ion Channels

Recent research has given much more detail to a number of further ion channels and their interactions, such as Cl^- regulators in the paracellular TJ area including claudin-4 and -18 implicated in epithelial ion and fluid transport and ARDS regulation in specific infectious, inflammatory, or other stimulatory situations. The reader is referred to further reviews as that of Brune et al (11). and Weidenfeld and Kübler (5). The transient receptor potential channel 6 (TRPC6), a Ca^{2+}-permeable non-selective cation channel, widely expressed in the lungs, was proposed to be a key regulator of acute hypoxic pulmonary vasoconstriction and was demonstrated to be implicated in pulmonary hypertension. TRPC6 is also involved in pulmonary vascular permeability and lung edema formation during LPS- or ischemia/reperfusion-induced ALI as discussed in this topic issue (328).

CYTOKINE-ION CHANNEL INTERACTION

Cytokines, which are organized in a cytokine network, play a major role in maintaining lymphocyte and leukocyte homeostasis under both steady-state and inflammatory conditions (329). Regulatory cytokines have to function in combination with other environmental signals to properly modulate the function and the extent of lymphocyte and leukocyte activation (329). Increased generation of pro-inflammatory cytokines represents a first-line defense mechanism against bacterial infections of the lung (102). Dysregulation of cytokine generation leads to alterations in cell–cell interactions (330). Cytokines, such as TNF, IL-1, IL-6 activate host defense by promoting the production of a wide spectrum of other cytokines and chemokines, including GM-CSF, G-CSF and IL-8 in inflammatory processes (331, 332). They moreover mediate the increase of surface adhesion molecule expression through activation of leukocytes and ECs (38). As such, cytokines can contribute to the pathogenesis and development of pulmonary edema (37, 99, 333–338). During the acute phases of ARDS, higher levels of TNF were detected in the BALF from patients with early-stage ARDS (39).

The Dichotomous Yin and Yang Effects of TNF in Pulmonary Edema

Tumor necrosis factor is a homotrimeric 51 kDa protein, binding to two types of membrane receptors: TNF receptor 1, which signals either apoptosis, necroptosis or inflammation; and TNF receptor 2, which is mainly implicated in inflammation and which is devoid of a death domain (239, 339, 340). TNF is one of the central cytokines in inflammation and moreover modulates ion channel activity (341–344). An intriguing feature of the ligands of the TNF and TNFR family is that when certain members are shed, they inhibit the function of the ligand-receptor complex and act as inhibitors (345). A central regulatory process may, therefore, be the proteolytic release of soluble bioactive oligomers from membrane-bound forms, e.g., for TNF by the protease TACE. The existence of TM forms of most of the TNF-superfamily ligands indicates that they are meant to act locally. Only under non-physiological conditions, when these ligands are released, they may prove to be harmful (345) or beneficial, as is the case of immune defense to bacterial infection (346). As a consequence, long-term treatment with TNF neutralizing substances can cause increased sensitivity to tuberculosis (346).

Tumor necrosis factor contributes to the pathogenesis and development of pulmonary edema (38), but, paradoxically, also plays an important role in edema reabsorption (347–350). It was assumed for a long time that cytokines exert their activities solely upon activating their respective receptors, but in the case of TNF, this is not true, which broadens this concept (38). TNF was shown to exert a lytic, i.e., killing effect on certain bloodstream stages of African trypanosomes, by means of a lectin-like interaction with trimannoses and *N,N'*-diacetylchitobiose oligosaccharide residues in the variant surface glycoprotein on the surface of the parasites (344). Later investigations could demonstrate that this lectin-like activity can be attributed to a special 17 amino acid long domain, named the lectin-like domain of TNF in the molecule's tip region (351, 352) (**Figure 7**). This special region is spatially distinct from its receptor binding sites (353) and is not present in lymphotoxin, which has a highly similar tertiary structure as TNF. Comparative sequence analysis of TNF and

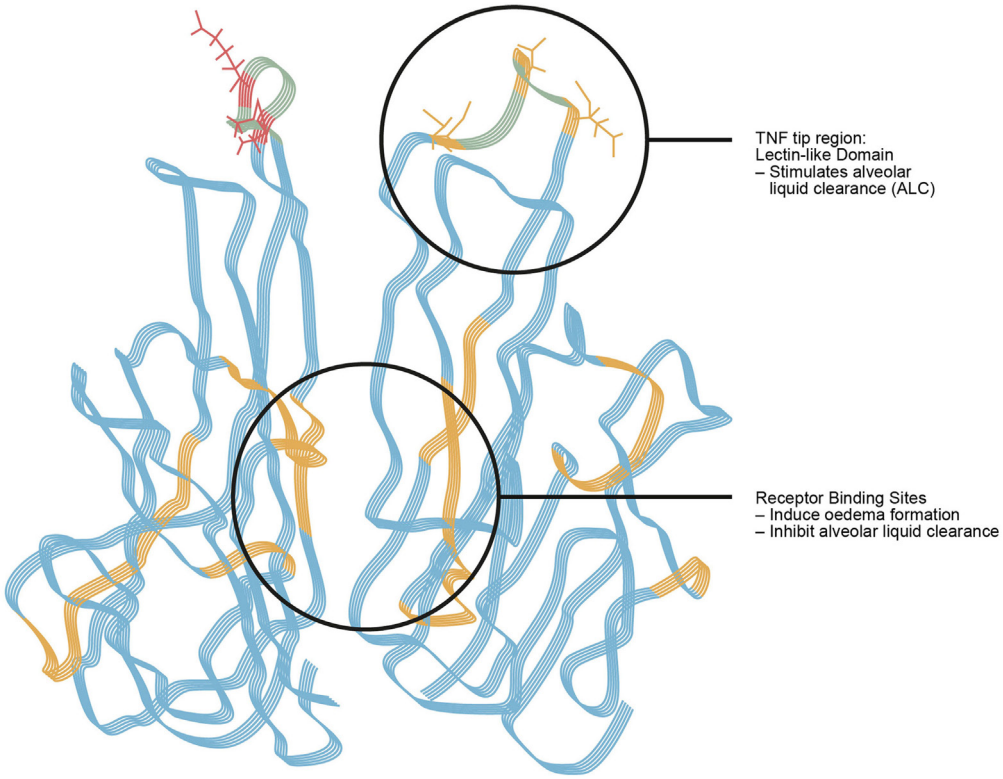

FIGURE 7 | Tumor necrosis factor. Tumor necrosis factor (TNF) as a "moonlighting" or dual role, or dichotomal yin-yang cytokine. The TNF receptor 1 binding sites within the TNF homotrimer mediate edema formation and blunt edema reabsorption. The lectin-like domain of the same cytokine activates epithelial sodium channel function and as such promotes alveolar fluid clearance and acts on endothelial cell barrier tightness (360).

LT allowed for the identification of the lectin-like domain of TNF (353).

For experimental purposes to mimic the TNF lectin-like domain, the amino acid sequence-identic synthetic 17 amino acid peptide which has shown to biologically mimic the lectin-like tip domain of TNF (353–355), as described above, has been used in a variety of experimental researches. It, moreover, gave rise to a therapeutic candidate that was recently evaluated in clinical trials (a.k.a AP301 and Solnatide) (356–358).

There are conflicting data about the critical involvement of TNF in the regulation of AFC (359). *In situ* and *in vivo* investigations conducted by Braun et al. in flooded rat lungs demonstrated a dual role for TNF in pulmonary edema (37, 38). This is possibly due to the opposite effects of, on the one hand, the classical TNF receptor 1 binding sites and, on the other hand, the lectin-like domain of TNF on pulmonary fluid reabsorption (37). In fact, the TNF tip region with its lectin-like activity is spatially distinct from the cytokine's receptor binding sites and causes an increase of alveolar fluid reabsorption, which is completely independent of the TNF receptors type 1 and 2, and further increases the cell–cell barrier tightness as shown in the alveolar EC barrier (**Figure 7**) (38, 99).

As discussed more in detail in this issue (361), in murine models of ventilator-induced ALI, TNF receptor 2 can have protective effects, whereas TNF receptor 1 is deleterious, thus adding another level of complexity to the role of TNF in edema (362). As such, the complex between soluble TNF receptor 1 and TNF can stimulate fluid reabsorption. TNF causes receptor-mediated edema formation in part by decreasing the expression of ENaC mRNA in AECs *in vitro* (135) leading to decreased amiloride-sensitive sodium uptake (135). Moreover, TNF receptor 1 signaling initiates the process of neutrophil migration (363) which can also contribute to the formation of pulmonary edema. It is also involved in orchestrating mechanisms, such as complement activation, cytokine regulation, chemokine production, and activation of adhesion molecules as well as their respective adhesion molecule receptors (364).

A TNF-dependent and amiloride-sensitive increase in AFC occurs in a rat model of *Pseudomonas aeruginosa* pneumonia (365). Other studies have shown in rats that intestinal ischemia–reperfusion leads to stimulation of AFC. This stimulation is at least in part mediated by a TNF-dependent mechanism which is independent of catecholamine release, because propranolol did not influence the AFC, and there was no observed cAMP stimulation (366). This indicates a protective effect of TNF-dependent stimulation of AFC in the early phase of injury (366).

Fukuda et al. could show that in ventilated rats TNF increased AFC by about 67% (136). This increase was inhibited by amiloride, but not by propranolol, indicating the mechanism is catecholamine-independent. A triple TNF mutant, in which

three crucial residues for the lectin-like activity were mutated to alanines, did not show any increase in AFC. The effect of TNF occurred within 30 s from the onset of perfusion in A549 cells and within 1 h in the distal airspaces of the rat. This shows that the primary mechanism does not depend on a transcriptional effect of TNF. This indicates that TNF increased AFC most probably by an amiloride-sensitive mode of action, independent of any TNF receptor binding and mediated through the lectin-like region.

These antagonistic functions of the same molecule on pulmonary edema refer to the complex biology of the TNF molecule (361). Indeed the TNF receptor 1 binding sites of TNF inhibit, whereas its lectin-like domain activates edema reabsorption (**Figure 7**) (37), and, as described above, tightens intercellular epithelial and endothelial barrier function (8, 9).

The Impact of TNF on Pulmonary Edema Generation by TNF Receptor-Mediated Effects

Tumor necrosis factor is mainly known for its receptor-mediated pro-inflammatory functions in the systemic inflammatory response and the induction of apoptosis on a cellular level (339, 367). Both of these activities of TNF are implicated in the pathogenesis of pulmonary edema, which is often associated with ALI (37).

Tumor necrosis factor promotes pulmonary dysfunction through edema formation and inhibition of edema reabsorption by several procedures (37), for instance:

- TNFR-dependent upregulation of chemokine production (338, 363) and adhesion molecule expression (333, 334, 368), which leads to neutrophil attraction and sequestration.
- Decrease in barrier function in human pulmonary artery ECs and rearrangement of microtubules (67).
- Induction of reactive oxygen intermediates (336).
- Down-regulation of ENaC expression in alveolar type 2 cells (135)

TNF Inhibits Transcription of All Three ENaC Subunits

Seminal studies conducted by Dagenais et al. clearly demonstrated the involvement of TNF in modulation of Na^+ absorption in cultured AECs is investigated. The results show that TNF decreased the expression of the α-, β-, and γ-subunits of ENaC mRNA after 24-h treatment and reduced to 50% the amount of ENaC-α protein in these cells (135). There was no impact, however, on α1 and β1 Na^+/K^+-ATPase mRNA expression (135). Amiloride-sensitive currents and ouabain-sensitive Rb^+ uptake were reduced. A strong correlation was found at different TNF concentrations between the decrease of amiloride-sensitive current and ENaC-α mRNA expression (135). All these data show that TNF has a profound effect on the capacity of AECs to transport Na^+ (135). In another study performed by Yamagata et al., mRNA expression of all three ENaC subunits in whole lung tissue was inhibited by TNF (359). TNF also inhibited ENaC function, as indicated by the reduction of amiloride-sensitive current (359). These data suggest that TNF may affect the pathophysiology of ALI and pulmonary edema through the inhibition of AFC and sodium transport (359).

TNF Increases Permeability of the Epithelial–Endothelial Barrier

The activation of TNF receptor 1 by TNF modulates the integrity of the alveolar barrier, in addition to its direct effects on ion channels and pumps of the alveolar epithelium. TNF increases the endothelial expression of chemo-attractants and adhesion molecules including IL-8 (formerly called neutrophil chemotactic factor), the IL-8- receptor 2, the intercellular adhesion molecule-1 (ICAM-1), platelet endothelial cell adhesion molecule-1 (PECAM-1), and vascular adhesion molecule-1, thus promoting excessive recruitment of mononuclear phagocytes and neutrophils during lung inflammation (71, 369–371).

Tumor necrosis factor is released in acute inflammatory lung syndromes linked to the extensive vascular dysfunction associated with increased permeability and EC apoptosis (372). The critical importance of the pulmonary vascular barrier function is shown by the balance between competing EC contractile forces, which generate centripetal tension, and adhesive cell–cell and cell matrix tethering forces, which regulate cell shape. Both competing forces in this model are intimately linked through the endothelial cytoskeleton, a complex network of actin microfilaments, microtubules, and intermediate filaments, which combine to regulate shape change and transduce signals within and between ECs (66).

Tumor necrosis factor can activate ECs, cause acute pulmonary vascular endothelial (VE) injury or even EC death and increase pulmonary vascular permeability *in vivo* as well as *in vitro* (39, 67, 373). Also, TNF increases the permeability of EC monolayers to macromolecules and lower molecular weight solutes by involving pertussis toxin-sensitive regulatory G protein (374). Furthermore, it is reported that TNF can increase the permeability of lung EC monolayers and that fibronectin can blunt this effect (375). In addition, TNF-induced increase in endothelial permeability involves the loss of fibronectin and remodeling of the extracellular matrix (376). Moreover, it has also been shown that TNF can increase capillary permeability causing transcapillary filtration *in vivo* (377).

TNF Increases ROS Generation

In addition to the above-mentioned mechanisms, TNF can induce pulmonary edema indirectly through increasing ROS (336). ROS have been shown to be able to disrupt the pulmonary endothelial barrier (336) and to decrease Na^+ channel activity (378).

Identification of the Alveolar Liquid Clearance-Promoting Effects of TNF

Lung Transplantation and Primary Graft Dysfunction (PGD)/Ischemia–Reperfusion Injury

The receptor-independent lectin-like domain of murine TNF has a potential physiological role in the resolution of alveolar edema in an *in situ* mouse lung model and an *ex vivo* rat lung model (99). The lectin-like domain of TNF can activate amiloride-sensitive sodium uptake in type II AECs (99, 100). Therefore this TNF domain is a potential therapeutic candidate (360).

As there is no specific treatment for ischemia–reperfusion-mediated lung injury, which is accompanied by a disrupted

capillary barrier integrity and an impeded AFC, the capacity of the TNF tip peptide to improve lung function after unilateral orthotopic lung iso-transplantation was tested *in vivo* in adult rats (8).

The unilateral rat transplant study showed that a highly severe lung injury with blood gas parameters qualifying for severe ARDS could be virtually prevented by the activation of the TNF lectin-like region. Furthermore, a significant reduction in polymorphonuclear neutrophilic leukocytes (PMN) infiltration in the bronchoalveolar lavage fluid was observed. The TNF tip peptide reduced ROS generation in the transplanted rat lungs *in vivo* and diminished ROS generation in pulmonary artery ECs *in vitro* under hypoxia and reoxygenation (8). ROS, the generation of which is increased during ischemia–reperfusion ALI (379–381), have been shown to be able both to disrupt pulmonary endothelial barrier integrity (378) and to inhibit ENaC activity (382).

Moreover, the effect of the lectin-like domain of TNF likely has physiologic relevance during inflammation and infection (8). As the soluble TNF receptors are cleaved by the same enzyme that generates soluble TNF, i.e., TACE (383), complexes between soluble TNF receptors and TNF can form (8). Soluble TNF receptors do not inhibit the activity of the lectin-like domain of TNF and complexes between these receptors and TNF are even able to stimulate AFC in *in situ* flooded rat lungs (37, 99, 353). At the same time, unfavorable actions of TNF on edema reabsorption and formation that are mediated by TNF receptor 1 activation are being blocked by the soluble receptors (37). Therefore, the favorable actions of the lectin-like domain of TNF might occur in conditions where both TNF and its soluble receptors are being generated (8).

A recent pilot study of 20 patients on treatment of PGD by twice daily nebulized 125 mg inhalation of the TNF tip peptide (AP301, solnatide) randomized 1:1 showed an improved gas exchange (mean and SD, daily measured up to 72 h, PaO2/FiO2 365.6 ± 90.4 versus 335.2 ± 42.3 mm Hg; $p = 0.049$) and clearly less time intubated (2 ± 0.82 versus 3.7 ± 1.95 days, $p = 0.02$) in the verum group, which also seems clinically relevant (357).

In summary, the lectin-like activity of TNF, and thus, the TNF tip peptide significantly improves lung function after lung transplantation in the rat. Pilot studies confirm a relevant effect in clinical treatment (8, 357). The experimental model showed a reduced alveolar neutrophil content and less ROS generation. It exerts a favorable effect on organ function in terms of gas exchange (8). It was furthermore shown that the apically expressed ENaC was found to be decreased at the messenger ribonucleic acid and the protein level in transplanted lungs, suggesting that ENaC, rather than the basolaterally expressed Na^+/K^+-ATPase, is important in the abnormal AFC (101). These studies reinforce the idea that the TNF tip peptide acts as an agent with potential therapeutic traits against the ischemia–reperfusion injury associated with lung transplantation.

The Lectin-Like Region of TNF Ameliorates High-Altitude Pulmonary Edema (HAPE) in Rats

About 100 million people live at altitudes greater than 2,500 m, about 15 million above 3,000 m, and some above 5,000 m (384). Most of these individuals have developed the ability to live and reproduce at elevation as high as 5,000 m, but in some cases, develop chronic medical problems due to their high-altitude residence. At 5,500 m barometric pressure is about only half of the one at sea level. Furthermore, many lowlanders venture to high altitude for work and recreation. The prevalence of HAPE depends on an individual's susceptibility, the rate of ascent, the final altitude, but also on heavy and prolonged exercise, and is higher in males (385). Although the mechanism underlying HAPE remains incompletely understood, it appears that the elevated pulmonary artery pressure plays a pivotal role in the process. Multiple studies demonstrated that susceptible individuals have abnormally high pulmonary artery pressure in response to hypoxic breathing, during normoxic and hypoxic exercise, and on high altitude before the onset of edema. Increased sympathetic tone, and alteration in vasoactive mediators such as endothelin-1, NO produced by pulmonary ECs, may also lead to stronger hypoxic pulmonary vasoconstriction (384). In autopsies, a red cell rich proteinaceous alveolar exudate with hyaline membrane is characteristic. In all autopsies, areas of pneumonitis with neutrophil accumulation but no evidence of bacterial accumulation have been observed. The estimated death rate of altitude illness is about 7.7/100,000 trekkers, with increasing mortality during the last decade (386). Treatment of HAPE consists, if ever possible, in descent from altitude, rest, oxygen supplementation, and administration of drugs like corticosteroids and furosemide.

Prophylactic inhalation of salmeterol, an inhalative β2-adrenergic receptor (β2AR) agonist, decreased the incidence of HAPE by more than 50% (387). The most pertinent explanation was that salmeterol would enhance the clearance of alveolar fluid since β2-adrenergic agonists upregulate AFC by stimulating transepithelial sodium transport. This hypothesis is supported by the fact that the level of sodium transport in the respiratory epithelium is lower in patients prone to HAPE. However, the study results cannot exclude the possibility that the β2 agonist could have modulated vascular permeability or the hemodynamic response associated with hypoxemia and HAPE (4).

In an experimental rat model simulating HAPE by hypobaric and hypoxic conditions equivalent to an altitude of 4,500 m with exhaustive treadmill exercise of 15 m per minute for 24 h, then for an equivalent of altitude of 6,000 m for further 48 h, the TNF tip peptide reduced pulmonary edema and increased expression of the epithelial TJ protein occludin, as compared to high-altitude controls. Compared to untreated high-altitude control animals, TNF tip peptide significantly lowered levels of the inflammatory cytokines TNF, IL-1β, IL-6 and the chemokine IL-8 in bronchoalveolar lavage. TNF tip peptide-treated animals experienced less pulmonary edema, as compared to dexamethasone-treated animals, and was more effective than its comparators in reduction of bronchoalveolar lavage protein content and inflammatory parameters (7).

Identification of the Mechanism of ENaC Activation by the Lectin-Like Region of TNF

It has been shown that the lectin-like domain of TNF can activate ENaC (353) and increases sodium uptake capacity in type II AEC (38). Intriguingly, the TNF tip peptide was shown to directly

bind to the α subunit of ENaC (54, 102) in a two-hit manner, first interacting with the glycosylated extracellular loop of the subunit and subsequently in the TM 2 domain, where the actual activation of the channel occurs (54, 102, 114). The former interaction was proposed to increase the expression of ENaC at the surface membrane in the presence of bacterial toxins, whereas the latter increases the channel's open probability time (102). Indeed, the binding of ENaC to the lectin-like domain of TNF or to the TNF tip peptide stabilizes the channel's complex formation with myristoylated alanine-rich C kinase substrate and with phosphatidylinositol 4,5-bisphosphate, both of which are important for the open conformation of the channel (388), in the presence of the pneumococcal pore-forming toxin pneumolysin (PLY), an important mediator of permeability edema in pneumococcal pneumonia (54). Knock-in mice expressing a TNF mutant lacking a functional lectin-like domain was shown to be more prone to develop capillary leak and permeability edema than their wild-type counterparts after instillation of a low dose of PLY, which did not induce significant barrier dysfunction in control mice (54). In short, these results demonstrate a novel TNF-mediated mechanism of direct ENaC activation and indicate a physiological role for the lectin-like domain of TNF in the resolution of alveolar edema during inflammation (54).

The Lectin-Like Region of TNF Increases Activity of Na^+/K^+-ATPase

Vadasz et al. investigated the impact of the TNF tip peptide on fluid balance in experimental lung injury. Alveolar–capillary permeability and fluid clearance were assessed in adult male rabbits. Aerosolized TNF tip peptide improved ALC by both reducing vascular permeability and by enhancing the absorption of excess alveolar fluid in experimental lung injury. TNF tip peptide increased Na^+/K^+-ATPase activity by promoting its exocytosis to the AEC surface and increased amiloride-sensitive sodium uptake, which increased the active Na^+ transport 2.2-fold and consecutively the AFC (196). Together with its previously discussed effects on ENaC, these data suggest a role for the TNF tip peptide as a potential therapeutic agent in pulmonary edema (196), since the two main mediators of Na^+ transport are both activated by the TNF tip peptide. It should be noted that the primary target is likely ENaC and that the activation of Na^+/K^+-ATPase could be through the indirect increase in intracellular Na^+ upon prior stimulation of ENaC (8). Moreover, the TNF tip peptide was recently also shown to increase the activity of NSC channels (9).

The Lectin-Like Region of TNF Restores ENaC Function in PHA1B Mutants

The lectin-like domain of human TNF activates the ENaC in various cell- and animal-based studies. The synthetically produced cyclic peptides Solnatide (a.k.a. tip peptide or AP301) and its congener AP318 possess molecular structures that mimic the TNF tip region. AP318-mediated ENaC activation was shown to rescue loss of function in a phenotype of ENaC carrying mutations and restored the amiloride-sensitive Na^+ current to physiological levels or even higher (118). This implies that the TNF tip domain can activate ENaC by a mechanism which remains intact even in the presence of various mutations occurring in different subunits, because binding to the putative binding site in the TM 2 domain of the glycosylated α subunit apparently remains basically unaffected in all tested point mutations or was compensated in frame shift mutations *via* a moderate activation of αβ- and βγ-ENaC, respectively (389). Apart from the mechanism responsible for loss of the ENaC performance in the studied ENaC mutations, the synthetic TIP and AP318 peptides could restore ENaC function up to or even higher than current levels of wild-type ENaC (118). As therapy of PHA1B is only symptomatic so far, these TNF tip peptides, which directly target ENaC, are promising candidates for the treatment of the channelopathy-caused disease PHA1B (118).

Clinical Trials on the Effect of the Lectin-Like Region of TNF

In a recent phase 2a clinical trial with ALI, patients received inhalable TNF tip peptide in the ventilator twice daily over a 7-day period. There was no significant improvement in lung liquid clearance over all patients, as assessed by the PiCCO method. However, there was a significant increase in extravascular lung water removal in those patients with a sequential organ failure assessment score higher than or equal to 11, representing more than 50% of the subjects in this trial (358). One hypothesis for this observation is that patients in this group, apart from suffering from impaired AFC capacity, might also suffer from more severe capillary barrier dysfunction. The TNF tip peptide was recently shown to not only improve AFC (54, 102), but also capillary barrier function (97) in the presence of bacterial toxins.

As mentioned before, in a randomized pilot study performed with 20 patients on the treatment of established PGD after lung transplantation by twice daily inhalation of the TNF tip peptide (AP301, solnatide) versus placebo, the TNF tip peptide improved gas exchange and clearly reduced the intubation—and thus mechanical ventilation—time in a probably clinically relevant manner (357).

TNF-Related Apoptosis-Inducing Ligand (TRAIL)

TNF-related apoptosis-inducing ligand, a member of the superfamily of TNF ligands, is a homotrimeric type II TM protein with a conserved C-terminal extracellular domain that mediates receptor binding and which can be cleaved by metalloproteinases to generate a soluble mediator (390). TRAIL is produced by several cell types, including immune cells such as macrophages and T cells and can be induced by both type I and type III Interferons (IFNs), a family of cytokines with fundamental importance in the innate immune response to viral infections (209, 391). Macrophages generate both soluble and membrane-bound TRAIL, which operate through distinct receptors on infected and non-infected, neighboring cells (209). TRAIL is a potent activator of cell death in transformed cells and activates cellular stress pathways in epithelial cells, as such finally leading to caspase-dependent or -independent cell death (209). In view of the prominent role of IFNs in antiviral response, IFN-dependent induction of TRAIL is a prominent regulator of

disease outcome especially in respiratory viral infection, enters into the scene (209). As such, the IFN/TRAIL signaling axis is of potential interest in disease progression and attenuation of tissue injury during respiratory viral infection (209). Here we focused on the role of TRAIL in edema reabsorption and in alveolar epithelial function.

TRAIL Disrupts the Alveolar Epithelial Barrier

TRAIL plays adverse roles in viral infection (392–394). On the one hand, TRAIL drives infected cells into apoptosis in order to limit virus distribution (209). On the other hand TRAIL can induce functional and structural damage not only in infected cells, but also in bystander cells, such as uninfected cells of the alveolar epithelium (199, 208). As such TRAIL can at the same time prevent viral spreading, but also cause lung injury in acute respiratory viral infection (209). Accordingly, in influenza A virus (IAV) infection, TRAIL acts as a detrimental factor contributing to tissue injury and impaired inflammation resolution when released in excessive amounts by recruited immune cells (209). The activation of proapoptotic and pro-necroptotic pathways in respiratory infection can result in a structural disruption of the airway and the alveolar epithelial barrier, which is a major hallmark of respiratory disease and its progression to the ARDS (395, 396).

TRAIL Decreases Na^+/K^+-ATPase Expression and Impairs AFC

Peteranderl et al (199). have investigated whether IAV infection alters Na^+/K^+-ATPase expression and function in AECs and the ability of the lung to clear edema. IAV infection reduced α1 Na^+/K^+-ATPase expression in the plasma membrane of human and murine AECs and in distal lung epithelium of infected mice. Accordingly, the decreased Na^+/K^+-ATPase expression impaired AFC in IAV-infected mice. A paracrine cell communication network between infected and non-infected AECs and alveolar macrophages was identified, which led to decreased alveolar epithelial Na^+/K^+-ATPase function, thus to AFC inhibition (199). The IAV-induced reduction of Na^+/K^+-ATPase was mediated by a host signaling pathway that involved epithelial type I IFN and an IFN-dependent elevation of macrophage TRAIL (199). In non-infected cells within the IAV-infected lung, TRAIL severely compromised the function of the ion channel Na^+/K^+-ATPase, which was mediated by induction of the stress kinase AMPK (199) thereby potentially revealing a cross-link to TRAIL-induced autophagic cell stress pathways in bystander cells both *in vitro* and *in vivo* (199). The TRAIL-induced and AMPK-mediated downregulation of the Na^+/K^+-ATPase, a major driver of vectorial ion and fluid transport from the alveolar airspace toward the interstitium, resulted in a reduced capacity of IAV-infected mice to clear excessive fluid from the alveoli (395). Thus, TRAIL signaling contributes to intensive edema formation, a hallmark of disease in virus-induced ARDS (395). Notably, this effect of TRAIL on Na^+/K^+-ATPase expression was induced independently of cell death pathways elicited by caspases, as treatment of cells and mice with a specific caspase-3 inhibitor diminished apoptosis in AECs but still allowed for the reduction of the Na^+/K^+-ATPase (199).

Transforming Growth Factor-β (TGF-β)

Transforming growth factor-β is a pleiotropic cytokine with a broad regulatory role in the immune system. Three highly homologous isoforms - TGFβ1, TGFβ2, and TGFβ3—share a receptor complex and signal transduction pathway, but their tissue expression levels are different (397). All are produced as inactive complexes, which must be activated to bind to their receptors (398). Platelets, T lymphocytes, macrophages, ECs, keratinocytes, smooth muscle cells, fibroblasts, i.e., a wide range of cells, can produce TGF (399). Following wounding or inflammation, all these cells are potential sources of TGF-β (400). Receptors for TGF-β have been found almost on every cell type tested so far, which enables this cytokine to exert its effects on almost any body tissue (401). Classically, TGF-β receptor signaling occurs by activating the Smad-dependent intracellular signaling pathway (398). The TGFβ receptor complex consists of two receptor subunits, TGF-β receptor (TGF-βR) I and II (398). These receptors mediate multiple responses (401).

TGFβ Context-Dependent Mode of Action

Transforming growth factor-β action is highly context-dependent and can be influenced by cell type, culture condition, interaction with other signaling pathways, developmental or disease stage *in vivo* and innate genetic variation among individuals (402). As such, TGF-β can be both a pro- and anti-inflammatory cytokine, which affects the growth and proliferation of many cell types (399). During inflammation, TGF-β1 is also able to effectively inhibit inflammatory response (403). The action of TGF-β following inflammatory responses is characterized by increased production of extracellular matrix components, as well as mesenchymal cell proliferation, migration, and accumulation (404). Pleiotropic nature of TGF-β modulates expression of adhesion molecules, provides a chemotactic gradient for leukocytes and other cells participating in an inflammatory response in one hand and, in contrast, inhibits them once they have become activated (405). Also in autoimmunity, TGFβ represents a double-edged sword (406). It can cause both T-cell growth promotion, as well as immune suppression (406).

Role of TGF-β Role in Pulmonary Edema

Transforming growth factor-β has a dual role in pulmonary edema. It can up- or downregulate alveolar ion and fluid transport, through its impact on ion channels/pumps (ENaC, CFTR and Na^+/K^+-ATPase) or on the pulmonary barrier. As such, TGF-β can decrease the expression of ENaC through decreasing expression of its α subunit mRNA and protein during bacterial infection (132). During ALI/ARDS, increased TGF-β1 activity in the distal airspaces promotes alveolar edema by reducing distal airway epithelial sodium and fluid clearance (132). Moreover, TGF-β can induce the internalization of βENaC from the lung epithelial cell surface and, hence, block the sodium-transporting capacity of AECs (133). In fact, TGF-β causes the subsequent activation of phospholipase D1, phosphatidylinositol- 4-phosphate 5-kinase 1α, and NADPH oxidase 4 (Nox4) (133). Nox4 activation moreover results in the production of ROS, which in turn reduces cell surface stability of the αβγENaC complex and thus leads to edema fluid accumulation (371). Apart from its effects

on ENaC expression, TGF-β can also decrease CFTR expression and function (179) and it, moreover, impairs expression of the Na^+/K^+-ATPase β1 subunit, resulting in decreased Na^+/K^+-ATPase activity in lung epithelial cells (197, 198).

Transforming growth factor-β decreases lung epithelial barrier function (203–205) *in vitro* by a mechanism that involves depletion of intracellular glutathione (206, 407). The cytokine moreover induces endothelial barrier dysfunction *via* Smad2-dependent p38 activation (235).

The integrin αvβ6 (408) can activate latent TGF-β in the lungs and skin (409). Using this clue, Pittet et al. have shown that mice lacking integrin αvβ6 are completely protected from pulmonary edema in bleomycin-induced ALI. Furthermore, pharmacologic inhibition of TGF-β also protected wild-type mice from pulmonary edema induced by bleomycin or *Escherichia coli* endotoxin (206). In short, integrin-mediated local activation of TGF-β is critical for the development of pulmonary edema in ALI, and blocking TGF-β or its activation attenuates pulmonary edema. This neutralization can be done e.g., by the administration of a soluble type II TGF-β receptor, which sequesters free TGF-β during lung injury (206).

All of the deleterious actions of TGF-β discussed above will ultimately lead to decreased ion transport and may, therefore, promote and worsen pulmonary edema. However, TGF-β can also positively impact pulmonary edema. Intriguingly, TGF-β was proposed to increase the function of ENaC, *via* enhancing the expression of Na^+/K^+-ATPase α1- and β1-subunits (134).

Interleukin-8

Interleukin-8 is a pro-inflammatory chemokine produced by a variety of tissue and blood cells (410), including bronchial epithelial cells (411), that correlates with neutrophil accumulation in distal airspaces of patients with ARDS. IL-8 is also a predictor of mortality in ALI (412–414). As such, significantly higher concentrations of IL-8 are found in the pulmonary edema fluid and plasma of patients with a septic versus a non-septic etiology of ARDS (415). Moreover, IL-8 promotes edema formation by blocking AFC (105).

The Role of IL-8 in Inhibiting β2AR Agonist

Roux et al (105). have shown that IL-8 or its rat analog cytokine-induced neutrophil chemokine-1 significantly decreased β2AR agonist-stimulated vectorial Cl^- and net fluid transport across rat and human alveolar epithelial type II cells, through reducing CFTR activity and biosynthesis (105). This reduction process was mediated by heterologous β2AR desensitization and downregulation (50%) *via* the G-protein-coupled receptor kinase 2 (GRK2)/PI3K signaling pathway (105) (**Figure 8**). Consistent with the experimental results, high pulmonary edema fluid levels of IL-8 (>4,000 pg/ml) were associated with impaired AFC in patients with ALI. Taken together, these results suggest a role for IL-8 in inhibiting β2AR agonist-stimulated alveolar epithelial fluid transport *via* a GRK2/PI3K-dependent mechanism (105). On top of this, IL-8 can promote edema formation by increasing endothelial permeability (250).

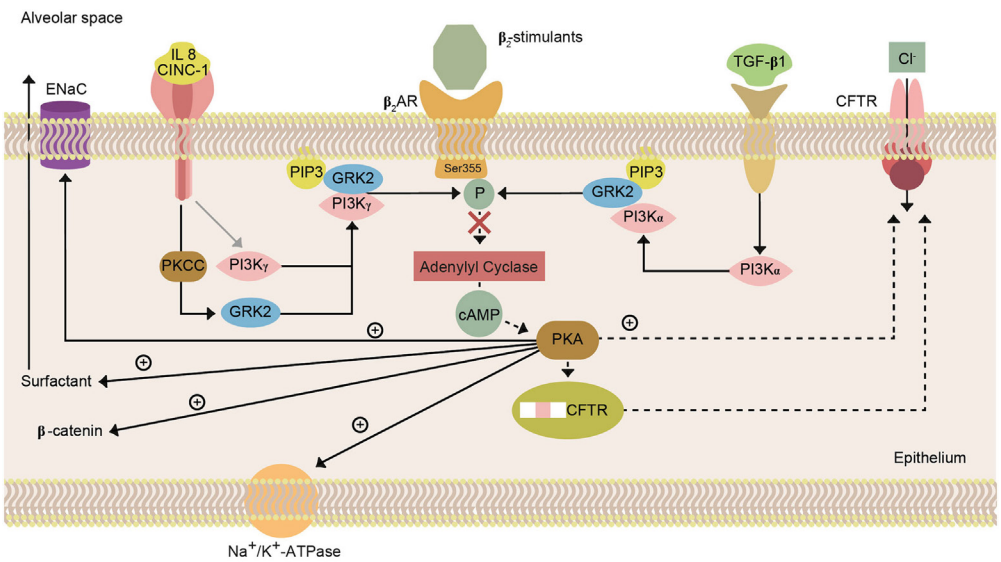

FIGURE 8 | Rationale for the problematic role of β2 adrenergic agonists in clinical trials. Schematic representation of the mechanisms by which interleukin-8 (IL-8)/cytokine-induced neutrophil chemokine (CINC)-1 and transforming growth factor (TGF)-β1 have a synergistic inhibitory effect on the β2-adrenergic receptor (β2AR) signaling pathway in type II alveolar (ATII) cells. IL-8/CINC-1 and TGF-β1 cause the activation of different phosphoinositide 3-kinase (PI3K) isoforms. However, IL-8/CINC-1 but not TGF-β1 phosphorylates G-protein-coupled receptor kinase 2 (GRK2) *via* a protein kinase C-zeta (PKC-ζ)-dependent mechanism explaining why the blockade of IL-8/CINC-1 prevents the TGF- β1-mediated inhibition of the β2AR signaling pathway in ATII cells. This results in the translocation of the protein complex GRK2 and PI3K to the cell membrane. This protein complex causes phosphorylation at the Ser355 heterologous desensitization and downregulation of the β2AR in ATII cells. IL-8/CINC-1 and TGF-β1 then prevent the activation of 3′-5′-cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) pathway that upregulates the vectorial fluid transport across the alveolar epithelium *via* phosphorylation and increased expression of cystic fibrosis transmembrane conductance regulator (CFTR) at the plasma membrane of ATII cells. The solid lines indicate the pathways stimulated by IL-8/CINC-1 and the dashed lines indicate the pathways inhibited by these mediators (416).

Interleukin-1β

Interleukin-1β is associated with decreased alveolar fluid reabsorption and thus with worse outcome in ALI and sepsis. IL-1β primarily decreases alveolar fluid reabsorption *via* a p38 MAPK, reducing the expression of the α-subunit of ENaC (113) as well as the β-subunit (137). In ARDS patients, the mean initial plasma levels of TNF IL-1β, IL-6, and IL-8 were significantly higher in non-survivors and in patients with sepsis. High plasma levels of IL-1β were associated with poor patient outcome (417). Likewise, high levels of IL-1β in the lungs of patients with ARDS were associated with an increased risk of mortality (417). The FAS/CD95 system acts together with TNF and IL-1β (57, 219, 418–420), leading to NF-κB production and neutrophil accumulating IL-8 secretion. Of note, an epithelial repair effect for type II pneumocytes *via* IL-1β was described in the injured alveolus (139), possibly in a specific context of cytokines, mediators and growth factors (139). Only in a specific fetal context IL-1β may increase alveolar fluid reabsorption by a hypothalamus-pituitary-adrenal gland axis (421) and an increase of both ENaC and Na^+/K^+-ATPase expression (140).

Fas/FasL System (CD95/CD95 Ligand System)

Fas is a 45-kDa type I cell surface receptor that belongs to the TNF receptor family. It can cause cytokine and chemokine release, especially the neutrophil attractant IL-8, *via* MAP kinase activation in lung epithelial cells, as such promoting inflammation (219). Binding of FasL to membrane Fas activates apoptosis through activation of caspases, which seems the key to AEC apoptosis, thus epithelial barrier breakdown and its consequences in ALI (57, 418).

Keratinocyte Growth Factor (KGF, FGF-7)

KGF is an epithelial cell-specific growth factor that has been shown to exert beneficial actions in many animal models of ALI and ARDS as well as in the *ex vivo* human lung (143, 422–430). Rats in which KGF was intratracheally administered increased AFC by about up to 50%, and this was further increased by the β2 agonist terbutaline (427). *In vitro* studies using mesenchymal stem cell-derived medium suggested that this growth factor plays a dominant role in tissue repair, even in the presence of the inflammatory cytokines IL-1β, TNF-α, and interferon-gamma, as well as in hypoxia. The observation that no downregulation of ENaC-α expression occurred despite of the presence of three key inflammatory cytokines suggested a dominant biological role of KGF in the acutely injured alveolar milieu (431). There is a currently a large interest in stem cell therapies as therapeutic approaches in clinical disorders like myocardial infarction, limb ischemia, diabetes, hepatic and renal failure, and ALI/ARDS. Stem and progenitor cell therapies as well as work with factors influencing those cells to reduce injury and increase repair have been performed. KGF has been proposed to be one of the main candidates to promote the repair capacity of stem cells in ALI. A recently performed double-blind, placebo-controlled phase 2 clinical trial—the KARE trial—tested the effects of KGF in 29 verum patients versus 31 placebo patients (432). There was no difference in the primary outcome variable, the oxygenation index, at day 7, and the treatment group had a trend to higher mortality, and more adverse events in terms of pyrexia. Nevertheless, these data do not exclude that the combined use of KGF and stem cells might provide protection in ALI.

Soluble Receptor for Advanced Glycation End Products (sRAGE)

Receptor for advanced glycation end products, first characterized in 1992 by Neeper et al. is a 35 kDa TM receptor which belongs to the immunoglobulin superfamily (433). RAGE is one of the AT1 cell-associated proteins in the lungs (434, 435). RAGE and its ligands have been recognized to be involved in the pathobiology of a wide range of diseases which are accompanied by symptoms, like enhanced oxidative stress, immune/inflammatory responses, and altered cell functions (436). RAGE is highly expressed in the lungs at readily measurable levels and its level increases quickly at sites of inflammation, mainly in inflammatory and epithelial cells (437). RAGE has three forms, consisting of N-truncated, dominant-negative, and soluble RAGE, which can be produced either by natural alternative splicing or by the action of membrane-associated proteases (438). The correlation between sRAGE levels and AFC rate was investigated in both a clinical study of patients with ARDS, as well as in an experimental model of acid-induced lung injury in mice (264). The results obtained showed a correlation between elevated levels of sRAGE with lung injury and an impairment of AFC (264). Accordingly, an increase in alveolar–capillary barrier permeability, arterial oxygenation impairment, lung injury scores, and the extent of human lung damage on CT scan are all associated with sRAGE levels (264). Conversely, it has been shown that RAGE regulates lung fluid balance *via* protein kinase C-gp91(phox) signaling to ENaC (177). In fact, hAGE, a RAGE ligand, increases ENaC activity through oxidant-mediated signaling, which can ultimately impact lung fluid clearance (177).

β2ARs AS IMPORTANT MODULATORS OF AFC

Structure and Subtypes

β2-adrenergic receptors are G protein-coupled receptors with seven-TM domains (439). Their three subtypes are β1, predominantly found in the heart, β2 in the respiratory system, and β3 in adipose tissue (440). β2 adrenergic agonists activate the β2-adrenoceptors (β2AR) on airway smooth muscle and are used to treat bronchoconstriction in asthma and chronic obstructive pulmonary disease (COPD) (441). In their canonical signaling pathway, agonist binding couples the β2AR to the Gs subtype of G protein. Gs activation leads to adenylyl cyclase, production of cAMP and activation of the cAMP-dependent protein kinase A (PKA), which mediates most of the functional consequences of Gs-coupled receptor activation (442). In airway smooth muscle, β2AR-stimulated PKA activity mediates relaxation through

phosphorylation of multiple proteins involved in regulating intracellular calcium levels, calcium sensitivity, and cross-bridge cycling (442).

The Role of β2AR Agonists in AFC

The presence of pulmonary β2ARs includes the alveolar space and provides the possibility to modulate the active Na^+ transport. β2adrenoceptors and the β-adrenergic agonists accelerate AFC (439) due to Na^+ transport *via* an amiloride-sensitive pathway (443) as shown *in vitro* (444), *ex vivo* (445), and *in vivo* in rat (446), dog (447), sheep (448), guinea pig (449), mouse (443, 450), and human lung tissue (451). β2AR knockout mice results suggest that the β2AR is responsible for most of the β-adrenergic–mediated upregulation of AFC (452). Therefore, β2ARs appear to be responsible for the bulk of the β-receptor–sensitive alveolar active Na^+ transport likely due to direct and indirect up-regulation of the alveolar active Na^+ transport (445, 449, 452–454). β-agonists *via* activation of β2ARs regulate necessary key proteins for the process of alveolar epithelial active Na^+ transport such as ENaC, Na^+/K^+-ATPase and CFTR in animal models as well as in human lung tissue (445, 449, 453, 455). β2ARs mediate short-term regulation of Na^+ pumps which occurs within minutes of receptor engagement *via* highly regulated recruitment of assembled Na^+/K^+-ATPase from intracellular compartments through phosphorylation of intermediary proteins and RhoA-kinase (456, 457). Long-term regulation is carried out *via* transcription (458) and translation of α1-subunit of Na^+/K^+-ATPase and ENaC subunits through PKA induced phosphorylation of cAMP-responsive elements and post-transcriptional regulation *via* mitogen-activated protein kinase/extracellular signal–regulated kinase and rapamycin sensitive pathways (455, 459) by direct modulation of Na^+ channels at the apical surface of the cells (460) or an activation of PKA to modulate a cation channel (92, 453).

Impact of β2AR Agonists on ENaC

Protein kinase A-mediated β2-agonist action phosphorylates cytoskeleton proteins and promotes trafficking of Na^+ channels through the cell membrane and direct phosphorylation of epithelial Na^+ channel β and γ subunits stimulate the β2AR and increases the number of epithelial Na^+ channels and their open time in alveolar type II cells (453) and enhances the expression of the α-subunit of the epithelial Na^+ channel ENaC (458). β-agonists and cAMP analogs increase the open probability and open time of amiloride-sensitive Na^+ channels (161). β2AR agonists thus increase Na^+ flux across the apical cell membrane by increasing both membrane-bound channel abundance and Na^+ flux through ENaC (439).

Impact of β2AR Agonists on Na^+/K^+-ATPase

β-adrenergic agonist modulate Na^+/K^+-ATPase partially through adenosine 3',5'-cyclic monophosphate (461). β2-adrenergic agonists increase the gene expression of Na^+/K^+-ATPase which leads to:

- Increased expression of α1-Na^+/K^+-ATPase mRNA and protein (458).
- Increase of the quantity of Na^+/K^+-ATPase (458)
- Increased activity of Na^+/K^+-ATPase (456, 458, 462–464).

Impact of β2AR Agonists on CFTR

Cystic fibrosis transmembrane conductance regulator is required for cAMP-mediated upregulation of fluid clearance, but is not necessary for basal fluid absorption (183), thus for alveolar fluid homeostasis in the uninjured lung (182, 183). β2-adrenergic stimulation activates CFTR by cAMP and PKA activation (184). In airway epithelial cells, the interaction of β2-AR with CFTR is mediated by scaffold proteins, such as NHERF1, allowing its interaction with PKA and stabilizing it on the plasma membrane (465). β2-adrenergic stimulation increases CFTR regulator expression in human airway epithelial cells through a cAMP/PKA-independent pathway (466).

β2-Adrenergic Agonists Are at Least in Part Not of Clinical Benefit in ALI/ARDS Studies and May Increase Mortality

In mild-to-moderate lung injury, alveolar edema fluid clearance is often preserved by catecholamine-dependent or -independent mechanisms (467). Stimulation of AFC is then related to activation or increased expression of sodium channels like ENaC or the Na^+/K^+-ATPase pump and may involve CFTR (467). In severe lung injury, AFC perturbation result through increased endothelial-interstitial-epithelial alveolar permeability and changes in activity or expression of sodium or chloride transport molecules (467). Improved barrier function and increased alveolar fluid reabsorption, theoretically by β-adrenergic agonists or the lectin-like TNF activity or alternatives, vasoactive drugs, regenerative or repair measures are therefore therapeutic alternatives (467). Whereas in the BALTI-2 study with salbutamol given as an intravenous infusion for up to 7 days, compared with a placebo, more than 160 patients [age 55 (SD 17) years] per group were studied, the study was stopped as salbutamol treatment was associated with increased 28-day mortality of 34% compared to 23% (risk ratio 1.47, 95% confidence interval 1.03 to 2.08) (468).

Salbutamol early in the course of ARDS was poorly tolerated. The authors concluded that such a β2-agonist therapy is unlikely to be beneficial and could worsen outcomes. Follow-up data further suggested worse outcome at 6 and 12 months in ARDS patients treated with salbutamol. They discussed that further trials of β-agonists in patients with ARDS were therefore unlikely to be conducted.

Some questions remained open, such as whether or not there may be benefit at a different dose or in specific populations (468). The survival curves for salbutamol and placebo appeared to continue to diverge after the end of the study drug infusion after 7 days, suggesting that the mechanisms may involve indirect effects as, e.g., more systemic disease under and after intravenous salbutamol. Concerning morbidity and mortality, Salbutamol can cause arrhythmia and tachycardia, and electrolyte and metabolic disturbances such as hypokalemia, hypomagnesemia, and lactic acidosis, which was observed in the study, and led to more salbutamol discontinuation. The used salbutamol dose of 15 μg/kg ideal body weight/hour i.v. was considered the maximum that critically ill patients could receive without an increase in ventricular or atrial tachycardia or ectopy. It was at the higher end of the recommended dosing regimen, and it is possible that lower

doses might have been better tolerated and caused fewer adverse outcomes (468).

Rather similar results were observed in the USA in the ALTA trial (Albuterol for the Treatment of ALI). ALTA was a placebo-controlled multicentre study of nebulized salbutamol in patients with ALI. Patients were randomized to receive either salbutamol 5 mg every 4 h or saline placebo, for up to 10 days. The primary outcome was ventilator-free days. Recruitment started 2007 with a target sample size of 1,000 patients. It was terminated after 282 patients had been enrolled because of futility. There was no clear difference observed in both ventilator-free days between the salbutamol and placebo arms (14.4 versus 16.6 days; 95% CI −4.7 to 0.3 days) or in hospital mortality (salbutamol 23.0% versus placebo 17.7%; 95% CI −4.0% to 14.7%). Although the β2 stimulator intervention was delivered by a different route in ALTA, and the early termination of recruitment caused that confidence intervals are wide, the results seemed much consistent with the BALTI-2 trial.

One alternative way was to use combination of inhaled corticosteroid and inhaled β2 agonist. In a recently published pilot study, a typical asthma treatment combination of twice daily inhaled formoterol and budesonide for 5 days showed its feasibility and promising results. The rationale was to reduce by both budesonide and formoterol alveolar inflammation, and to further improve by formoterol AFC. The aim was to reduce ARDS. More patients in the placebo group developed ARDS (7 versus 0) and required mechanical ventilation (53% versus 21%) (469).

Further Potentially Critical Mechanisms of Action β-Adrenergic Agonists

Besides two futile ARDS trials, further factors might restrict the β2 receptor agonist usage as a therapy to increase the resolution of pulmonary edema (467). Prolonged stimulation of β-adrenergic receptors with endogenous catecholamines could desensitize the β-receptors and prevent their stimulation with exogenous catecholamines (467). For instance, in some patients the alveolar epithelium might be too injured to respond to β-adrenergic agonist therapy (467), likewise circulating factors could limit the action of β-adrenergic agonists (467). Also, in the presence of left atrial hypertension, atrial natriuretic peptide can inhibit the stimulatory effect (467). Similarly in prolonged hemorrhagic shock and resuscitation, cAMP agonists may not stimulate AFC because oxidant-mediated injury may reduce the response of the alveolar epithelium to β-2 agonists (467).

An important clinical aspect is the potential to increase cardiac index by β2 receptor agonists (470), by both cardiac stimulation and pulmonary arterial vasodilation. Cardiac stimulation can lead to a higher cardiac index. This is potentially dangerous, as due to the injured lung put in the circulation in series, there is an increase in filtration, which further increases alveolar fluid and gas exchange disturbance. An interrelated second, and in ALI most probably untoward "Robin Hood effect" of potential opening of vascular beds that are closed by vasoconstriction is, e.g., observed in COPD patients inhaling β2 receptor agonists and developing more hypoxemia (471). This is probably due to increased perfusion in badly ventilated ALI/ARDS alveolar areas. As shown by Briot et al., β2 receptor agonist therapy seems therefore to have the potential to heighten the protein leakage from plasma to alveoli in the acutely injured lung (470).

PROTEIN CLEARANCE OUT OF THE ALVEOLAR SPACE

Clearance of serum and inflammatory proteins from the alveolar space is an important and possibly vital process in recovery from pulmonary edema. Albumin and IgG are present in pulmonary edema fluid in concentrations that are 40–65% of plasma levels in hydrostatic pulmonary edema and 75–95% in non-cardiogenic pulmonary edema. Concentrations of albumin, for example, may be 5 g/100 ml or more. Protein concentrations rise during recovery from alveolar edema because the salt and water fraction of edema fluid is cleared much faster than albumin and IgG. Clearance of alveolar protein occurs by paracellular pathways in the setting of pulmonary edema. Transcytosis may be important in regulating the alveolar milieu under nonpathological circumstances. Alveolar protein degradation may become important in long-term protein clearance, clearance of insoluble proteins, or under pathological conditions such as immune reactions or ALI.

Early since the first descriptions of ARDS, we know that protein content is high, "haemorrhagic," and about the same as plasma proteins. Plasma and coagulative products such as fibrin strands are degraded or modified, e.g., also to hyaline membranes in a high number of patients (31). They are observed in ARDS, are especially covering denuded basement membranes where pneumocytes are missing, and may be related to adverse outcome (56).

Recent research hints to a better understanding of the resolution of those alveolar proteinaceous contents and debris out of the distal airways. Counterintuitively, neither macrophages, nor the mucociliary transport processes seem to play major roles in protein clearance also over several days time (472). Protein clearance from the distal air spaces is in part facilitated by active endocytotic processes including for albumin by the 600 kDa TM glycoprotein called megalin or LDL-receptor related protein-2, a member of the low-density lipoprotein-receptor superfamily (6). Again, its important functional inhibition seems TGF-beta1 related. Megalin seems negatively regulated by glycogen synthase kinase 3b (GSK3b). An important regulator for this protein kinase signaling molecule seems the RNA binding protein Embryonic Lethal, Abnormal Vision, Drosophila Like 1/Human antigen R (ELAVL-1/HuR) as an upstream regulator of GSK3b (6). ELAVL-1/HuR is an RNA binding protein that increases mRNA stability. Its importance has been shown in ventilator-induced and acid-induced mouse lung injury. In EC lines it induces ICAM-1 and IL-8 after TNF stimulation.

Endocytosis of macromolecules can be mediated by a non-selective fluid phase uptake, which is a very slow process in alveolar epithelium. A receptor-mediated endocytosis is much faster and occurs when specific high-affinity receptors are implicated. Two pathways are described, called caveolae-mediated and clathrin-mediated endocytosis.

Detailed research on alveolar protein and debris clearance have only recently begun. Judging their roles is more complex, as hyperosmotic stimuli might be of anti-inflammatory action,

and possibly there is even more biological signaling as formerly assumed that may influence underlying lung disease.

POTENTIAL NOVEL APPROACHES TO UNDERSTANDING THE EFFECTS OF ION CHANNEL STIMULANTS IN LUNG DISEASE

Hyperosmolarity, High Na^+ Content, or High Oncotic Pressure

One biological effect that has, to our knowledge, not yet been assessed is the question whether due to fluid reabsorption out of the alveolus the hyperosmolarity or hyper-oncotic situation is of biological effect. Several limitations have to be mentioned: Certainly the pulmonary surfaces including the mucus and the surfactant system and its layers are complex and disease-prone systems, as suggested in cystic fibrosis. Dose- and time response have to be taken into account. Actually, there are contradictory results on those effects: Some observations described anti-inflammatory effects of hyperosmolarity in the airways, as in the nose and sinuses with a few randomized controlled trials that compared isoosmotic versus hyperosmotic irrigating solutions (473, 474). Honey is hyperosmotic and antibacterial, and in wound healing it seems frequently beneficial (475). This is also the case for hyperosmotic salt pastilles in throat and neck infections. However, nebulized hypertonic saline is still disputed in infants with acute viral bronchiolitis (476). There are also *in vitro* cell model results showing a switch from adaptive to inflammatory gene expression by hyperosmotic stress by protein kinase R activation, NF-kappaB p65 activation with responsive genes including inducible NO synthase, interleukin-6, and interleukin-1β (477), others with some protection *via* p53 gene regulation (478). In a rat seawater drowning model, alveolar hypertonicity, but not iso-or hypotonicity-induced inflammation and vascular leak, thus edema probably by hypoxia-inducible factor-1 and including ataxia telangiectasia mutated kinase and PI3 kinase (479).

Local Na^+ accumulation and enhanced availability have been linked to activation of tonicity-responsive enhancer binding protein (TonEBP) *via* the mononuclear phagocyte system in the skin (480), a system also widely represented in the lung. Enhanced local Na^+ has been shown to boost pro-inflammatory TH17 cell production and, finally, IL-17 release (481). The pro-inflammatory phenotype is maintained in high-salt conditions with upregulation of TNF-α and IL-2. As it is currently unclear what is the mechanism of enhanced Na^+ presentation to activate the TonEBP, an enhanced Na^+ accumulation in the extracellular matrix (482), the activation of Na^+ channels or even a permissive role of an altered Na^+/K^+-ATPase activity *via* endogenous ouabain have to be considered (483). As all of these mechanisms are also represented in the lung, both Na^+ presentation and availability should, therefore, be considered in pulmonary fluid regulation.

Briefly, there may be important, but so far not yet well understood anti-, or even pro-inflammatory, stimuli, or signals by hyperosmotic stimulation, underlining the importance to investigate this subject further.

SPECIFIC CLINICAL SETTINGS WITH POTENTIAL SIGNIFICANCE OF ALVEOLAR FLUID REABSORPTION IN INFLAMED LUNGS

RDS in the Newborn

Respiratory distress syndrome is one of the most important causes of morbidity and mortality in newborns and has a prevalence of about 1%. It is clinically manifesting as respiratory distress accompanied by abnormal pulmonary function and hypoxemia directly in the first minutes or hours after birth. RDS prevalence increases with decreasing gestational age. As such the incidence of RDS is highest in extremely preterm infants, affecting more than 90% of infants at a gestational age of 28 weeks or less. In a birth cohort of more than 230,000 deliveries, the syndrome was observed at 34 weeks gestation in 10.5%, at 35 weeks in 6%, at 36 weeks in 2.8%, at 37 weeks in 1%, at 38, and more in 0.3%. Therapy is supportive, includes surfactant replacement, fluid restriction, and glucocorticoids. Whereas a viewpoint has been that qualitative and quantitative surfactant deficiency, inflammation including alveolar neutrophil influx, and fluid overload (in part by low urine output) account for this syndrome, some reports hint to a suboptimal Na^+ transport. During gestation, the lung epithelium secretes Cl^- and fluid and develops the ability to actively reabsorb Na^+ only during late gestation. At birth, the mature lung switches from active Cl^- and consecutive fluid secretion to active Na^+ and consecutive fluid absorption in response to circulating catecholamines. Changes in oxygen tension augment the Na^+-uptake capacity of the epithelium and increase ENaC gene expression. The inability of the immature fetal lung to switch from fluid secretion to fluid absorption results, at least in large part, from an immaturity in terms of low expression of ENaC, where all three ENaC subunits are low in preterm relative to full-term infants. ENaC-α is increased in the respiratory epithelium by therapeutic glucocorticosteroids (484, 485).

However, in the last years the incidence of near-term and term infants with RDS has increased, and their clinical characteristics differ from those of premature infants with RDS. Li et al. found that death was virtually inevitable for some babies, despite intensive care and surfactant replacement therapy, particularly in near-term and term infants. Lung tissue slices taken during autopsies of near-term and term infants who died of neonatal RDS showed that some alveoli were obviously dilated, with a large amount of lung fluid. This was in addition to an alveolar collapse from a lack of surfactant, and suggested that lung fluid absorption disorders might be an important additional cause of RDS by influencing gas exchange or surfactant function (486). In their study on 120 neonates with RDS and 129 controls, 7 newborns died despite of intensive care and surfactant replacement therapy. All of them received surfactant more than once and four of them were near-term or term infants. Preterm babies (less than 35 weeks of gestational age) had a better response to surfactant treatment than near-term and term babies. These results were consistent with the finding that the surfactant therapy was not effective for all newborns with RDS. The authors assessed the

relationship between RDS and 7 candidate polymorphisms of the *SCNN1A* gene that encodes α-ENaC. One single nucleotide polymorphism (rs4149570) of the *SCNN1A* gene was associated with RDS. Moreover, in a group of term infants (gestational age was 37 weeks or greater), another single nucleotide polymorphism locus (rs7956915) was associated with RDS. These results are consistent with the hypothesis that the causes of RDS are multifactorial, and that in term infants it might differ from those in preterm infants (487). Alveolar fluid reabsorption and, thus, α-ENaC might play a key role in the pathogenesis by influencing the amount of lung liquid absorption, especially in term infants with RDS.

Acute Infection-Related Respiratory Failure

Pulmonary infections are the most prevalent infections worldwide, most of bacterial or viral origin. Community-acquired pneumonia is a frequent infectious respiratory disease with an annual incidence of about 5–12/1,000, and leads to hospitalization in 20–50% of patients. Mortality in hospitalized patients ranges from 5 to 15%. The most common reason for hospital admission in childhood is pneumonia and accounts for up to 50% of admissions. The high morbidity, mortality, and epidemiologic dangers with viral or bacterial pneumonias are of high concern. Pneumonia mortality is typically caused by flooding of the pulmonary alveoli preventing normal gas exchange and consequent hypoxemia. We refer to excellent recent reviews (117, 371). Of note is that pneumonia and sepsis are by far the leading causes of ALI and ARDS. Sepsis is a major healthcare burden, mirrored by up to 45% of intensive care unit costs (64) and bearing a high mortality of about 30%. Cytokines and ion channels are key elements in this common health problem.

Lung Transplantation

Lung transplantation is a substitutive treatment of various end-stage pulmonary disease. Cystic fibrosis, COPD, and idiopathic pulmonary fibrosis (IPF) are the most important transplanted patient groups (488). The high mortality rate relative to other solid-organ transplants is in part due to chronic rejection. The limited availability of donor lungs results in a highly limited treatment strategy for patients in whom a survival benefit—estimated 5-year survival is about 60%—is expected (488).

Primary graft dysfunction (PGD) is termed the development of allograft infiltrates within 72 h of transplantation together with impaired oxygenation, when other identifiable insults such as volume overload, pneumonia, acute rejection, atelectasis or vascular compromises are excluded. PGD is usually referred to ischemia–reperfusion injury, but additionally to any further mechanical, surgical or chemical trauma such as inflammatory, neural or hormonal events of the donor, high oxygen fraction during reperfusion, or lymphatic disruption. PGD is mild and transient in most cases, but 10–20% of patient situations are sufficiently severe to cause life-threatening hypoxemia similar to ARDS, based on the same mediators and cytokines and a diffuse alveolar damage resembling ARDS. Similar to ARDS, it is considered a systemic disease, not only affecting the lung, but the whole patient. Thereby, the increased occurrence of cerebral dysfunction, i.e., patient delirium, worsens prognosis. Severe PGD quadruples perioperative mortality, the leading cause of early death of lung transplant recipients. In one study it is associated with a 30 day mortality of 63 versus 9%, and associated duration of mechanical ventilation is 15 versus 1 day (489) (**Figures 9** and **10**). The risk of higher morbidity and death risk persists even after an often protracted recovery, suggesting that PGD triggers an increased risk for bronchiolitis obliterans syndrome as a manifestation of chronic allograft rejection (490).

Ischemia–reperfusion injury is the main mechanism for PGD (503, 504). With logarithmic function ischemia time is associated with reperfusion injury: Whereas 4 h ischemia is associated with about 13% more risk than 2 h, 6 h ischemia increases the risk by more than 50%, 8 h by a factor of 3, and 10 h by a factor of about 8 (494). The hypothermic preservation increases oxidative stress, leads to accumulation of intracellular sodium and loss of intracellular potassium and an intracellular calcium overload, cell death with apoptosis (240) and necrosis. The release of pro-and anti-inflammatory cytokines such as TNF, INF-γ, IL-8, IL-10, IL-12, and IL-18 and complement cause smooth muscle contraction and increase vascular permeability, amplify by C5a the inflammatory response and are chemoattractant. Soluble complement receptor-1 is an accepted, but underused treatment based on a placebo-controlled clinical trial with 59 patients (505).

A huge part of the ischemia–reperfusion injury of lung allografts is mediated by the change in vascular shear stress due to the blood flow cessation. The endothelial sensing mechanism called mechanosome chiefly consists of PECAM-1, VEGR receptor-2 (VEGFR2) and VE cadherin in the EC caveolae (499). It closes the K_{ATP} channel of the EC membrane, depolarizes it and leads to NADPH oxidase 2 activation as the main source to generate ROS. EC depolarization results in opening of T-type voltage-gated Ca^{2+} channels, increase intracellular calcium, and NO synthase activation and consecutive NO-mediated vasodilation, and an overproduction of ROS that causes oxidative injury which triggers inflammation or even cell death (499). PI3K-Akt leads to NADPH activation, producing ROS. With ischemia, there is also an NO production by endothelial NO synthase, probably as a physiological response to the loss of blood flow. The ROS generated in ECs interact with signaling-related proteins and thus with enzymatic activity. NF-ƙB, activator protein 1 (AP-1), and c-Jun and c-Fos and the redox-sensitive HIF-1α, Nrf2, ATR/CREB are increased (499). Even although PMN are then recruited into lungs, the production of ROS by the endothelium is the initial signal.

Reperfusion further activates NADPH oxidase-2 leading to lipid peroxidation, which can be several fold more extensive than ischemia alone. Opening of an inward K^+ channel was accompanied with hyperpolarization and ROS as well as NO production. Mainly a PMN influx and macrophage activation contribute to that injury. There is a strong correlation between excessive oxidative stress markers and the acute donor lung injury extent, and immunological rejection including later chronic rejection in terms of bronchitis obliterans syndrome as both the major causes for lung graft failure (499) (**Figure 9**).

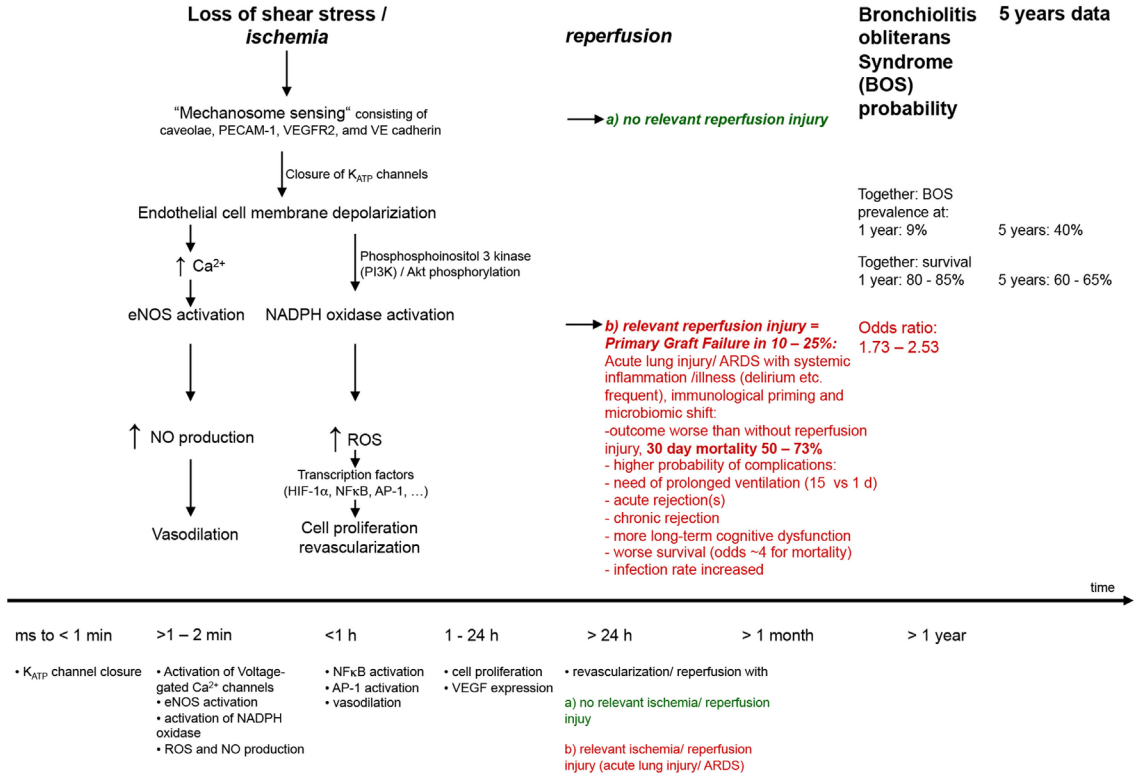

FIGURE 9 | Pathophysiology and biological significance of pulmonary reimplantation response after lung transplantation (491–501). Adapted from Chatterjee et al (499), Whitson et al (496), Hartert et al (501), Basseri et al (498), Bharat et al (495), Bharat et al (497), Christie et al (492, 493), Huang et al (502), and King et al (491).

The success of lung transplantation is much tempered by the limited organ supply. Many potential recipients are dying on the waiting list or being removed from the list because of clinical decline (506). Groups have therefore tried to expand the donor polls using extended criteria donors, with efforts to suggest rates of PGD, bronchiolitis obliterans syndrome, early morbidity and mortality to have equivalent to those with standard criteria donors (506). Most lung grafts come from brain-dead donors, but only about 15–20% of donors provide lungs that are satisfactory for lung transplantation (506). Strategies to expand the donor pool include the use of donation after cardiocirculatory death by doing a normothermic *ex vivo* lung perfusion (507), resulting in a study an about 28% increase in lungs suitable for transplantation. Problems are the increased risk of perioperative hypotension, warm ischemia time, a higher rate of aspiration, and more uncertainty to predict the lung's usability for transplantation. *Ex vivo* assessment and reconditioning might overcome some issues in the longer term (506) (**Figure 9**).

As shown before, using TNF tip peptide as preventative strategy in the left-sided unilateral orthotopic rat lung transplant model of prolonged cold ischemia we could show important biological effects, as highly severe lung injury with blood gas parameters qualifying for severe ARDS could be virtually prevented by the activation of the TNF lectin-like region (8) (**Figure 11**). The clinical pilot study of Aigner et al. suggests relevant improvement during established PGD by the TNF tip peptide (357). Both studies underline the biological potential of the TNF lectin-like region, i.e., the cytokine's ion channel activation, thus its potent modulation of ALI, and thus its potential effect to prevent untoward long-term effects.

Interstitial Lung Disease, Especially Acute Exacerbation of Idiopathic Pulmonary Fibrosis (aeIPF)

Idiopathic pulmonary fibrosis is a chronic and progressive lung disease of unknown etiology that occurs primarily in adults in their 50s and 60s and higher. Annual incidence is about 7–16 cases per 100,000 in the USA and 0.2 – 7 per 100,000 in Europe. Prognosis is severe with a median survival of about 2–3 years after diagnosis (508).

Acute exacerbation of idiopathic pulmonary fibrosis is a highly important disease progression of high morbidity and an extremely high mortality of 50–90% (509). It is typically reported to have an annual incidence of 5–15 or more %, with a higher incidence in advanced disease, and is defined as an acute worsening of dyspnea and lung function without an identifiable cause. Intriguingly, aeIPF has quite similar clinical features and similar prognosis compared with non-idiopathic causes of acute respiratory worsening in IPF such as infection or aspiration. It is, therefore, debated whether etiologies are to be separated (509).

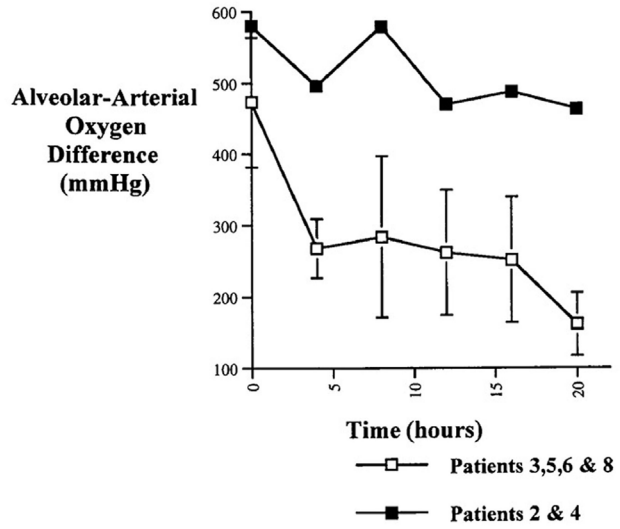

FIGURE 10 | Time course of mean AaPO2 after the onset of reperfusion pulmonary edema. Comparison of mean AaPO2 in four patients with intact alveolar epithelial fluid clearance (open squares) to the patients with no net alveolar epithelial fluid clearance (solid squares). The data for Patients 3, 5, 6, and 8 are expressed as mean 6 SD. The data for Patients 2 and 4 are expressed as the average of the AaPO2 at each time point (45). Reprinted with permission of the American Thoracic Society. Copyright © 2017 American Thoracic Society.

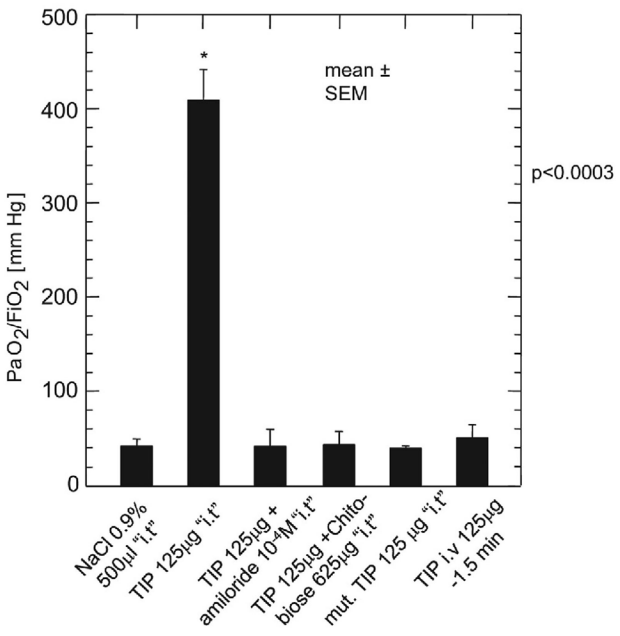

FIGURE 11 | Oxygenation at 24 h after transplantation. At sacrifice, 24 h after reperfusion of the left-sided lung transplant, the PaO2/FIO2 ratio was measured after excluding the native right-sided lung by clipping the right-sided stem bronchus and right-sided pulmonary artery. The animals were tracheotomized and ventilated with an FIO2 of 1.0. The tumor necrosis factor tip peptide significantly increased gas exchange compared with all other study groups. *$p < 0.003$ versus NaCl. Data are mean ± SEM. i.t., intratracheally (8).

There is some similarity between aeIPF and ARDS. However, the biological backgrounds are even much less understood. Gene expression profiles mainly show primarily infections or overwhelming inflammatory etiology, but more epithelial injury and proliferation as main profile, including gene expression of CCNA2, alpha-defensin, and apoptosis. Histopathologically, diffuse alveolar damage seems frequently observed in aeIPF. This finding is similar to ARDS and also has systemic multiorgan disease consequences, as evidenced by autopsy findings (510).

A number of current pharmacotherapies are under investigation for the therapeutic challenge of aeIPF as reviewed by Juarez et al., but no substance, combination of substances, or treatment modality (such as non-invasive ventilation which seems beneficial) has demonstrated such a clear benefit to become a new standard of therapy. This leaves clinicians with polypragmatic, mainly supportive care. Novel approaches are actually developed concerning immune suppression including calcineurin inhibitors, rituximab, removal of immune cells and mediators by either therapeutic plasma exchange or haemoperfusions with polymycin-B immobilized fibers aimed to remove not primarily endotoxin, but also contributing cytokines, and maybe hemostasis modulating agents such as intravenous recombinant thrombomodulin (508). The option of modulating the inflammation and to protect barrier function with, e.g., the biological action of TNF tip region is actually conceptualized in this group of severely sick patients.

Pre-eclampsia

Pre-eclampsia refers to the new onset of the combination of hypertension and proteinuria or of hypertension and end-organ dysfunction without or with proteinuria in previously normotensive pregnant women after at least 20 weeks of gestation. About 4–5% of pregnancies worldwide are complicated with pre-eclampsia, and first pregnancies are more frequently associated with this disease. Together with hemorrhage, thromboembolism, and cardiovascular disease, pre-eclampsia is one of the four leading causes of maternal death, accounting for 15% of them in the Western world. Prevalence is about 1 maternal death per 100,000 live births. When pre-eclampsia occurs, the fatality rate is about 6 per 10,000. Severe acute diastolic dysfunction in severe pre-eclampsia can lead to pulmonary edema in this patient group. Maternal and fetal/placental factors seem responsible, such as abnormal trophoblast invasion of the spiral arteria of the decidua and myometrium early in pregnancy, a suboptimal uteroplacental blood flow possibly leading to high oxidative placental stress, altering placental angiogenesis, poor feto-placental vasculature and abnormal vascular reactivity. Endothelial dysfunction can be caused by systemic anti-angiogenic signals by anti-angiogenic factors. Elevated levels of soluble fms-like tyrosine kinase 1 (sFlt-1; an inhibitor of vascular endothelial growth factor), reduced levels of placental growth factor (PlGF), and an increased sFlt-1: PlGF ratio have been reported both in women with established pre-eclampsia and in women before the development of pre-eclampsia (511). This is moreover accompanied by increased pro-inflammatory cytokine production, which in turn promotes renal and pulmonary barrier dysfunction and impaired ion channel activity. As a consequence, pulmonary edema is a severe feature

of the disease. In this case, the edema can be multifactorial, due to left heart failure, and thus excessive pulmonary vascular hydrostatic pressure, to decreased plasma oncotic pressure, to capillary leak, or to iatrogenic volume overload (511, 512).

High-Altitude Pulmonary Edema

About 100 million people live at altitudes greater than 2,500 m, about 15 million above 3,000 m, and some above 5,000 m (384). Most have developed the ability to live and reproduce at elevation as high as 5,000 m, but in some cases, develop chronic medical problems due to their high-altitude residence. At 5,500 m the pressure is about only half the normal. Furthermore, many lowlanders venture to high altitude for work and recreation. These more acute exposures also pose the hazards of acute altitude illness, e.g., in Colorado skiers in 15–40% of them with an incidence of HAPE then of 0.1–1%. The prevalence of HAPE depends on an individual's susceptibility, the rate of ascent, the final altitude, but also heavy and prolonged exercise, and is higher in male. At altitudes of 4,500 m the prevalence is between 0.2 and 6%, and at 5,500 m between 2 and 15% (385). Many adaptive processes can vastly reduce the risk of such sickness. Susceptibility to altitude illness varies considerably between individuals, but for a single individual, the symptoms are often reproducible given the same rate of ascent. High-altitude pulmonary odemea is the most important complication of high-altitude illness and its most common cause of death. It typically manifests with 2–4 days of ascent to altitudes above 2,400 m, most commonly beginning on the second night. In the early stage of disease, decreased exercise performance occurs and individuals require increased amount of time to recover from exertions. Individuals also complained of fatigue, weakness, and persistent dry cough, possibly combined with symptoms of acute sickness. As the disease progresses, individuals become short of breath with minimal exertion. Dyspnea at rest, audible chest congestion, generalized pallor, nail bed cyanosis and production of pink frothy sputum are late findings in severe disease. Even in the absence of concurrent high-altitude cerebral edema, severe hypoxemia may produce mental changes, ataxia, and altered levels of consciousness. In general blood gas analysis reveals severe hypoxemia. Pulmonary arterial pressure is high, but pulmonary wedge pressure is normal, and heart size is not increased. Although the mechanism underlying HAPE remains incompletely understood, it appears that the elevated pulmonary artery pressure plays a pivotal role in the process. Multiple studies demonstrated that susceptible individuals have abnormally high pulmonary artery pressure in response to hypoxic breathing, during normoxic and hypoxic exercise, and on high altitude before the onset of edema. Increased sympathetic tone, and alteration in vasoactive mediators-like endothelin-1, NO produced by pulmonary ECs may also lead to stronger hypoxic pulmonary vasoconstriction (384). In autopsies, a red cell rich proteinaceous alveolar exudate with hyaline membrane is characteristic. In all autopsies, areas of pneumonitis with neutrophil accumulation but no evidence of bacterial accumulation has been observed. Most reports mentioned capillary and arterial thrombi, fibrin deposits, hemorrhage, and infarcts. Uneven hypoxic vasoconstriction is discussed. Uneven perfusion is suggested clinically by the typical patchy radiographic appearance and by MRI studies in patients together with hypoxic blood gas parameters which demonstrates greater heterogeneous regional perfusion in HAPE-susceptible subjects (384). The estimated death rate of altitude illness is about 7.7/100,000 trekkers, with increasing mortality during the last decade (386).

Treatment of HAPE consists, if ever possible, in descent from altitude, rest, oxygen supplementation, and administration of drugs such as corticosteroids and furosemide.

Prophylactic inhalation the β2AR agonist salmeterol decreased the HAPE incidence by more than 50% (387). The most pertinent explanation was that salmeterol would enhance the clearance of alveolar fluid since β-adrenergic agonists upregulate the clearance of alveolar fluid by stimulating transepithelial sodium transport. This hypothesis is supported by the fact that the level of sodium transport in the respiratory epithelium is lower in patients susceptible to HAPE. However, the study results cannot exclude the possibility that the beta2 agonist could have modulated vascular permeability or the hemodynamic response associated with hypoxemia and HAPE (4).

In an experimental rat model simulating HAPE by hypobaric and hypoxic conditions equivalent to an altitude of 4,500 m with exhaustive treadmill exercise of 15 m per minute for 24 h, then for an equivalent of altitude of 6,000 m for further 48 h, it has been shown that the TNF tip peptide reduced pulmonary edema and increased the TJ occluding expression compared to high-altitude controls, dexamethasone, and aminophylline treated control animals (7). Compared to untreated high-altitude control animals, TNF tip peptide significantly lowered levels of the inflammatory cytokines TNF, IL-1β, IL-6 and IL-8 in bronchoalveolar lavage. TNF tip peptide-treated animals experienced less pulmonary edema also compared to dexamethasone-treated animals, and was more effective than its comparators in reduction of bronchoalveolar lavage protein content and inflammatory parameters (7). The higher expression of occludin may have translated in an increased stability of the alveolar–capillary barrier, probably related to the reduction in the extent of protein leakage in TNF tip peptide-treated animals. The results suggest that the biologic potential of the TNF tip region is more active in this model than dexamethasone as standard therapy on one hand, and as the glucocorticosteroids (7). The model suggests that HAPE can be treated with TNF tip peptide at least in a part of patients affected, and clinical studies are underway.

However, inhaled budesonide seems not consistently able to prevent acute mountain sickness and HAPE (513).

SUMMARY AND CONCLUSION

Alveolar fluid reabsorption is of high clinical importance in both cardiac and non-cardiac edema. Clinically, a conservative fluid strategy in ARDS patients resulted in more ventilator-free days (514). There is evidence that lower vascular pressures reduce pro-inflammatory pathways (515), and in chronic hydrostatic pulmonary edema tissue remodeling ensues (516).

Recent studies of cytokine-ion channel interactions have clearly shown that the concept of ion channel modulation to improve AFC has to be broadened, also taking into account

previously ignored functions of these mediators. The concept of active interactions between barrier function and ion transporters to maintain lung fluid balance plays a pivotal biological role. TNF's lectin-like domain, mimicked by the TNF tip peptide, was demonstrated to strengthen capillary barrier function in the presence of bacterial toxins in vitro and in vivo. Indeed, influx, efflux, and tightness of the EC layer are all biologically interrelated. Such a relationship is also present in the alveolar epithelium with interactions with ion transporters and TJs (11). These observations suggest that the biologic potential of ion channel modulation with drugs or peptides is more relevant than initially presumed.

A conceptual problem in ALI and other inflammatory conditions is how fluid reabsorption can function in such an "un-tight system" as in partially destroyed endothelial-interstitial or interstitial-alveolar barriers, and what is the expression level of ion channels in those conditions (25). The same may hold true in the context of hypoxia and the decreased expression of ENaC. Regeneration and repair of injured, apoptotic or necrotic endothelial or AECs can be fostered endogenously by local or bone-marrow derived precursors or by exogenously administered factors, as formerly studied in animal models using progenitor cell populations and stimulants. Clinical refinements are underway and update outcome parameters, such as AFC (517).

In clinical situations with cardiogenic as well as with non-cardiogenic pulmonary edema, i.e., ALI and ARDS, we have to be extremely cautious with prescribing drugs that might interfere with alveolar fluid transports or inflammation. Furosemide might further be the mainstay of diuretic drug and the alveolar flooding stopper especially in cardiogenic edema due to its effect on NKCC1 and CFTR. Amiloride should not be taken. Many clinical questions will be open around beta blocking agents as well as beta stimulating agents in the context of pulmonary edema and will probably depend on their indication. cAMP may play some role, but from which point those two drug classes are counterproductive, remains actually open.

There has been much work focused on one ion channel without considering the interconnection between major biological ion channels or its modulators, which may limit the validity of conclusions or findings of much published work. In future research it would be important to try to better integrate these channels, as well as their interactions with cytokines present in the lung milieu during the various pathologies. Many parallels exist between different organ systems and ion channels, underlining that interdisciplinary network is promising.

As shown in lung transplant primary graft failure, and thus probably also true in ARDS, ALI causes important and systemic long-term injury, especially brain injury. The critical step of high ethical impact for the scientific community is to expand integrative translational research in terms of clinical investigation with the known targets to improve clinical outcome. This is especially important in lung transplantation, as donor shortage still leaves many patients worldwide dying without this therapeutic option, and possibly in ALI and ARDS.

AUTHOR CONTRIBUTIONS

All authors significantly contributed to the conceptual work, the writing and editing of the work.

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FXYD5 Is an Essential Mediator of the Inflammatory Response during Lung Injury

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The alveolar epithelium secretes cytokines and chemokines that recruit immune cells to the lungs, which is essential for fighting infections but in excess can promote lung injury. Overexpression of FXYD5, a tissue-specific regulator of the Na,K-ATPase, in mice, impairs the alveolo-epithelial barrier, and FXYD5 overexpression in renal cells increases C-C chemokine ligand-2 (CCL2) secretion in response to lipopolysaccharide (LPS). The aim of this study was to determine whether FXYD5 contributes to the lung inflammation and injury. Exposure of alveolar epithelial cells (AEC) to LPS increased FXYD5 levels at the plasma membrane, and FXYD5 silencing prevented both the activation of NF-κB and the secretion of cytokines in response to LPS. Intratracheal instillation of LPS into mice increased FXYD5 levels in the lung. FXYD5 overexpression increased the recruitment of interstitial macrophages and classical monocytes to the lung in response to LPS. FXYD5 silencing decreased CCL2 levels, number of cells, and protein concentration in bronchoalveolar lavage fluid (BALF) after LPS treatment, indicating that FXYD5 is required for the NF-κB-stimulated epithelial production of CCL2, the influx of immune cells, and the increase in alveolo-epithelial permeability in response to LPS. Silencing of FXYD5 also prevented the activation of NF-κB and cytokine secretion in response to interferon α and TNF-α, suggesting that pro-inflammatory effects of FXYD5 are not limited to the LPS-induced pathway. Furthermore, in the absence of other stimuli, FXYD5 overexpression in AEC activated NF-κB and increased cytokine production, while FXYD5 overexpression in mice increased cytokine levels in BALF, indicating that FXYD5 is sufficient to induce the NF-κB-stimulated cytokine secretion by the alveolar epithelium. The FXYD5 overexpression also increased cell counts in BALF, which was prevented by silencing the CCL2 receptor (CCR2), or by treating mice with a CCR2-blocking antibody, confirming that FXYD5-induced CCL2 production leads to the recruitment of monocytes to the lung. Taken together, the data demonstrate that FXYD5 is a key contributor to inflammatory lung injury.

Keywords: alveolar epithelium, inflammation, FXYD5, acute lung injury, C-C chemokine ligand-2

INTRODUCTION

The alveolar epithelium not only is responsible for gas exchange but also acts as a physical and immunological barrier for all inhaled substances and microbial products. Alveolar epithelial cells (AEC) also contribute to innate immunity by secreting cytokines and chemokines, which recruit phagocytic myeloid cells and other inflammatory cells to the site of infection (1–3). During lung inflammation, the interaction of monocyte chemoattractant protein C-C chemokine ligand-2 (CCL2) secreted by AEC with its receptor, CC chemokine receptor 2 (CCR2), results in cellular recruitment to the lung. Present in a subset of peripheral monocytes, CCR2 serves as marker for classical monocyte inflammation (4–7). Recruitment of circulating monocytes to tissues is essential for effective control and clearance of infections, but if not controlled, it can become harmful, contributing to disease progression.

The alveolar epithelium is comprised of large flat type I alveolar (ATI) epithelial cells and cuboidal type II alveolar (ATII) epithelial cells. Both ATI and ATII cell types have important roles in airway surveillance through the initial recognition of microbial pathogens and bacterial toxins by various pattern recognition receptors (PRR) such as toll-like receptors (TLR) and nod-like receptors to activate the host defense (8). Lipopolysaccharide (LPS), a glycolipid of the outer membrane of Gram-negative bacteria is a major cause of morbidity and mortality in humans (9–11). Acute exposure to LPS increases cytokine release and disrupts the alveolo-capillary barrier, resulting in pulmonary edema and the recruitment of inflammatory cells into the lung (12–15). The response to LPS is initiated by interaction with TLR4 in association with the accessory proteins MD-2 and CD-14 (10, 16, 17). TLR4 is constitutively expressed in primary alveolar type II cells as well as in the adenocarcinoma cell line A549 (18). Exposure of lung epithelial cells to LPS leads to the activation of the NF-κB family of transcription factors, which in turn directs the expression of pro-inflammatory mediators (16, 19).

FXYD proteins, named after an invariant FXYD sequence, were first described as tissue-specific modulators of Na,K-ATPase activity (20–26). This family contains seven integral membrane proteins that interact with the Na,K-ATPase and regulate its function in a tissue-specific manner (27, 28). FXYD5, also known as dysadherin, is not only involved in the regulation of Na,K-ATPase activity (29, 30) but also acts as a tumorigenic protein when overexpressed (31, 32). Its expression is elevated in metastatic tumors, suggesting FXYD5 as an oncogenic marker (32–38). FXYD5 is also expressed in normal tissues, including the alveolar epithelium (24, 26, 29, 30, 39, 40). In cancer cells, CCL2 has been identified as a mediator of FXYD5 effects on cell migration (41). In these cells, FXYD5 has been shown to regulate CCL2 expression through the activation of the NF-κB signaling pathway.

Several publications have suggested a role of FXYD5 in the regulation of inflammation. In AEC, we have described that *in vivo* overexpression of FXYD5 impairs the interaction between Na,K-ATPase subunits in neighboring cells, disrupting the alveolar barrier (26), which might contribute to the recruitment of inflammatory cells into the alveolar compartment. Also, overexpression of FXYD5 in normal kidney epithelial cells increases the inflammatory response to LPS in a tumor necrosis factor α (TNF-α) receptor-dependent manner and the levels of FXYD5 are increased in lungs after treatment of mice with LPS (30). Supporting a role for FXYD5 in inflammatory diseases, the expression levels of FXYD5 are elevated in the lungs of patients with acute lung injury (42). However, whether endogenous FXYD5 plays a role in the epithelial inflammatory response remains mostly unknown. Here, using *in vivo* and *in vitro* models, we investigated the mechanism by which the increase of FXYD5 in AEC contributes to lung inflammation and injury.

MATERIALS AND METHODS

Reagents

Chemical and cell culture reagents were purchased from Sigma-Aldrich or Corning Life Sciences unless stated otherwise. LPS from Escherichia coli 0111:B4 was from Sigma-Aldrich.

Cell Culture

Mouse lung epithelial MLE-12 and human epithelial A549 cells (ATCC) were grown and maintained as previously described (43, 44).

LPS-Induced Lung Inflammation and Injury Model

Mice were provided with food and water *ad libitum*, maintained on a 14-h-light–10-h-dark cycle, and handled according to National Institutes of Health guidelines and an experimental protocol approved by the Northwestern University Institutional Animal Care and Use Committee. C57BL/6 mice (10–12 weeks of age) were given intratracheal instillation of LPS (3 mg/kg body weight) for up to 24 h as we previously described (45). Bronchoalveolar lavage fluid (BALF) was obtained through a 20-gage angiocath ligated into the trachea through a tracheostomy (26). A total of 1-ml of PBS was instilled into the lungs and then aspirated three times. BALF was collected for cell counts, protein quantification, and cytokine determination as we previously described (30, 46). RNA was isolated from lung peripheral tissue using an RNeasy kit (QIAGEN) and reverse transcribed using qScript cDNA synthesis (Quanta Biosciences). Quantitative PCRs were set up using iQ SYBR Green Super mix (Bio Rad). Data were normalized to the abundance of L19 mRNA. The primers for FXYD5, CCL2, GAPDH, and L19 were: FXYD5 5′ CAT CCT ACA TTG AAC ATC CA 3′ and 5′ TGA GAC AAC TGC CTA CAC 3′; L19 5′ AGC CTG TGA CTG TCC ATT C 3′ and 5′ ATC CTC ATC CTT CTC ATC CAG 3′; CCL2 5′ CCT GTC ATG CTT CTG GGC CTG C 3′ and 5′ GGG GCG TTA ACT GCA TCT GGC TG 3′; and GAPDH 5′ AAC TTT GGC ATT GTG GAA GGG CTC 3′ and 5′ TGG AAG AGT GGG AGT TGC TGT TGA 3′. Proteins were determined in cell lysates or total membranes as we previously described (26, 43).

Lentivirus Instillation

To knock down mouse FXYD5 protein *in vivo* in lung, we generated the VSVG pseudotyped lentiviruses (10^9–10^{10} TU/ml) expressing

mouse FXYD5 shRNA and non-silencing shRNA as control (47, 48) (provided by DNA/RNA Delivery Core, SDRC, Northwestern University, Chicago, IL, USA). For lentivirus packaging, 293T packaging cells (Gene Hunter Corporation) were transiently transfected using Transit-2020 reagent (Mirus) with the following vectors: second generation packaging vectors psPAX2 and pMD2.G (Addgene) and third generation lentiviral expression vector pLKO (Sigma). The pLKO vectors used encoded two specific shRNAs against mouse FXYD5 (Cat# TRCN0000079348, sense: CCTCCAAACTACACCAACTCA; and Cat# TRCN0000079352, sense: GTGCTGTTCATCACGGGAATT), and a non-silencing control shRNA (Cat# SHC002) (all from Sigma). FXYD5 shRNA and control non-silencing shRNA viruses were intratracheally instilled in mice in a volume of 50 µl. FXYD5 silencing was confirmed by RT-qPCR and Western blot analysis as described above.

Adenoviral Infection

CCR2$^{-/-}$ mice were purchased from Jackson Laboratories (49). WT C57BL/6 or *CCR2*$^{-/-}$ mice at 8–12 weeks of age were infected with Ad-mCherry-HA-FXYD5 (Ad-FXYD5; 1×10^9 plaque-forming units (pfu)/animal) in 50% surfactant vehicle as previously described (30, 50) and housed in a containment facility. After 72 h, BALF was collected and used as described above. Control adenovirus (Ad-Null) was purchased from Viraquest, Inc. Cells were infected with Ad-Null or Ad-FXYD5 20 pfu/cell as previously described (26).

Analysis of Cytokines and Chemokines

The concentration of CCL2/MCP-1 (Affymetrix), TNF-α (Affymetrix), and IL-6 (Life Technologies) in the BALF or cell culture supernatants were quantified by ELISA following the manufacturer's instructions.

In Vitro Treatment of AEC and siRNA Transfection

MLE-12 or A549 cells were transfected with 120 pmol of mouse or human FXYD5 siRNA duplex (final concentration 100 µM) (Santa Cruz Biotechnology), respectively, using Lipofectamine RNAiMAX (Invitrogen). A non-silencing negative control siRNA was purchased from Santa Cruz Biotechnology. Experiments were performed 24 h after transfection. Cells were starved for 2 h by incubation in culture media containing 2.5% fetal bovine serum and treated with LPS (100 ng/ml) for the indicated times. Supernatants were collected for cytokine analysis and cells were biotinylated by the membrane-impermeable biotinylation reagent where indicated; cells lysates, total membranes, or surface biotinylated proteins were isolated for SDS-PAGE and immunoblot analysis as previously described (26, 43). The following mouse monoclonal antibodies were used: HA (Biolegend clone 16B12 #901502; 1:1,000), pIKBα (Cell Signaling Technology #9246; 1:500), IKBα (Cell Signaling Technology #4814; 1:500), E-cadherin (E-cad) (BD Biosciences #610182, 1:2,500 dilution). The following polyclonal antibodies were used: FXYD5 (Sigma-Aldrich #HPA010817, 1:1,000 and M178 from Santa Cruz Biotechnology #98247, 1:200), and β-actin (Cell Signaling Technology #4967, 1:1,000). Immunoblots were quantified by densitometry using Image J 1.46r (National Institutes of Health, Bethesda, MD). Where indicated, surface biotinylated proteins were treated with *O*-glycosidase and Neuraminidase Bundle according to the manufacturer's instructions (New England Biolabs, Inc.) prior to loading on SDS-PAGE as we previously described (26).

Interferon α (IFN-α, Biolegend) and TNF-α (Biolegend) were added to A549 cells for up to 24 and 2 h, respectively, as described for LPS treatment.

Flow Cytometry and Cell Sorting

Myeloid populations from whole lung were isolated and defined as previously described (51). Briefly, perfused lungs were inflated with digestion buffer (1 mg/ml of Collagenase D and 0.1 mg/ml DNase I, both from Roche) and coarsely minced with scissors before processing in C-tubes (Miltenyi) with a GentleMACS dissociator (Miltenyi), according to the manufacturer's instructions. Homogenate was passed through 40-µm nylon mesh to obtain a single-cell suspension and subjected to red blood cell lysis (BD Pharm Lyse, BD Biosciences). Live cells were counted using a Countess cell counter (Invitrogen) by trypan blue exclusion.

Cells were then stained with the following cocktail: CD45-FITC (eBioscience #11-0451-81, 0.1 µg/µl), MHCII-PerCPCy5.5 (Biolegend #107626, 0.01 µg/µl), Ly6C-eFluor450 (eBioscience #348-5932-80, 0.02 µg/µl), CD24-APC (eBioscience #317-0242-80, 0.01 µg/µl), Ly6G-Alexa700 (BD Bioscience #561236, 0.04 µg/µl), NK1.1-Alexa700 (BD Bioscience #560515, 0.06 µg/µl), CD11b-APCcy7 (Biolegend #101225, 0.02 µg/µl), CD64-PE (Biolegend #139303, 0.02 µg/µl), SiglecF-PECF594 (BD Bioscience #562757, 0.02 µg/µl), CD11c-PEcy7 (BD Bioscience #561022, 0.02 µg/µl). Multicolor flow cytometry was performed with an LSR Fortessa using DIVA software (BD Biosciences) and the following gating outlined below and in **Figure 5**. FlowJo software version 10.0.8 (FlowJo, LLC) was used for all compensation and data analysis.

After excluding doublets and dead cells, myeloid cells were identified using the pan-hematopoietic marker CD45 (51). As shown in **Figure 5**, the CD45$^+$ population was then separated into Ly6G/NK1.1$^-$ and Ly6G/NK1.1$^+$ populations using a shared channel to pull out NK cells (NK1.1$^+$ CD11b^{hi} CD24^{hi}) and neutrophils (Ly6G CD11b^{int} CD24^{int}). The Ly6G/NK1.1$^-$ population was further divided based on SiglecF and CD11c expression to identify alveolar macrophages (SiglecF^{hi} CD11c^{hi}) and eosinophils (SiglecF^{hi} CD11c^{low}). From the remaining SiglecF^{low} CD11c^{low} group, a CD11b^{hi} population was then selected and segregated based on MHCII expression. Within the MHCII^{low} cluster, cells could be defined as classical monocytes (Ly6C^{hi}) or non-classical monocytes (Ly6C^{low}). Alternatively, interstitial macrophages (IMs) were identified as MHCII^{hi} CD64^{hi} CD24^{low}.

Mouse ATII cells (mATII) were isolated and defined as previously described (52). Briefly, whole lung was subjected to enzymatic and manual digestion to obtain a single cell suspension. Cells were then stained with Epcam (eBioscience #17-5791-80, 0.1 µg/ml), CD45, CD31-PE (eBioscience #12-0311-81, 0.1 µg/ml), and MHCII-eFlour450 (eBioscience #48-5321-82, 0.1 µg/ml). mATII cells were identified as CD45$^-$ EpCAM$^+$, CD31$^-$ and sorted on a BD FACSAria 5-laser.

Fluorescent Staining and Confocal Microscopy

Isolated mATII cells were plated on glass-bottom dishes (MatTek corporation), fixed by incubation with 3.75% formaldehyde in PBS for 15 min at 37°C, and actin filaments were visualized using fluorescein phalloidin (Thermo Fisher Scientific) as described previously (53). Confocal microscopy images of mCherry-tagged FXYD5 and stained actin filaments were acquired using a Zeiss LSM 510 laser scanning confocal microscope and ZEN 2009 software (Carl Zeiss MicroImaging GmbH).

Anti-CCR2 Antibody Treatment

Mice were injected retro-orbitally with 6 μg/100 μl of anti-CCR2 monoclonal antibody (clone MC-21) in PBS (49) 48 h after adenoviral instillation. Mice were sacrificed after 24 h and BALF was obtained as described above.

Statistical Analysis

Data are expressed as mean ± SD. For comparisons between two groups, significance was evaluated by Student's *t*-test, and when more than two groups were compared, one-way ANOVA was used followed by the Dunnett's or Sidak test using GraphPad Prism 7.02 software.

RESULTS

The Increase in FXYD5 Is Required for the Secretion of Inflammatory Mediators by the AEC in Response to LPS

Alveolar epithelial cells produce the first wave of cytokines, which trigger local and systemic inflammatory responses (54). We have reported that overexpression of FXYD5 in normal kidney epithelial cells increases the inflammatory response to LPS (40) and that overexpression of FXYD5 in the mouse alveolar epithelium increases alveolar epithelial permeability (26). Moreover, treatment of mice with LPS increased the level of FXYD5 in lungs (30). However, whether endogenous FXYD5 plays a role in the generation of an alveolar epithelial inflammatory response to LPS has not been studied. To determine whether LPS modulates FXYD5 levels in AEC, MLE-12 cells were treated with LPS for up to 24 h and cell culture media, cell lysates, and surface biotinylated plasma membrane (PM) proteins were collected. In the PM fraction of MLE-12 cells, FXYD5 was detected as a 60–70 kDa band (**Figure 1A**), suggesting that the plasmalemma-located FXYD5 is heavily *O*-glycosylated in these cells similar to that found in A549 cells (26). An additional 25 kDa band was seen in MLE-12 cell lysates (not shown) that represents the intracellular immature unglycosylated or less glycosylated fraction of FXYD5. LPS time dependently increased PM level of FXYD5 in MLE-12 cells with a peak at 6 h (**Figure 1A**). CCL2, which is abundantly produced by ATII cells, plays an important role in the local regulation of inflammatory processes (55). As expected, treatment of MLE-12 cells for 6 h with LPS strongly stimulated CCL2 mRNA synthesis as well as the secretion of CCL2 and IL-6 into the culture media compared with untreated controls (**Figures 1B–D**). Silencing of FXYD5 in those cells with a specific siRNA prevented the LPS-stimulated increase in the transcription of CCL2 and secretion of both cytokines as compared with a control siRNA (**Figures 1B–D**). In isolated primary mouse ATII, infection with lentivirus coding for specific shRNA FXYD5 prevented the LPS-stimulated increase in FXYD5 and CCL2 mRNA by 62 and 30%, respectively (**Figure 1E**).

Next, we investigated the signaling pathway by which the increase in FXYD5 regulates cytokine production. The dominant pathway triggered by PRR activation is the canonical NF-κB pathway. The NF-κB complex comprises IκB (inhibitor of NF-κB) bound to two proteins, p50 and p65; when not stimulated, the complex resides in the cytoplasm. Different stimuli lead to phosphorylation and degradation of IκB removing inhibitory effects and allowing the translocation of active NF-κB, the p50-p65 heterodimer, to the nuclei (56). Nuclear translocation triggers the expression of over 150 genes, including those encoding cytokines (57). We analyzed whether FXYD5 promotes the secretion of cytokines *via* the NF-κB signaling pathway by assessing the phosphorylation of one of IκB proteins, IκBα. In A549 cells, 6 h of LPS treatment led to an increase in FXYD5 at the PM similar to the one observed in MLE-12 (**Figure 1F**). A549 cells were transfected with siRNA specific for FXYD5, 24 h later stimulated with LPS for 6 h, and phosphorylation of IκBα was assessed in cell lysates. LPS treatment increased the IκBα phosphorylation, which was prevented by FXYD5 silencing (**Figure 1F**). Taken together, the results indicate that FXYD5 is required for the NF-κB-dependent secretion of inflammatory cytokines and chemokines induced by LPS, suggesting that FXYD5 is an important mediator of the pro-inflammatory response of AEC to LPS.

Increased FXYD5 Is Sufficient to Induce AEC Secretion of Inflammatory Mediators

Previous studies in breast cancer cells have demonstrated that FXYD5 knockdown decreases, while FXYD5 overexpression increases, both the NF-κB-responsive promoter activity and CCL2 production in cancer cells (41). To determine whether the increase in FXYD5 in AEC induces the NF-κB-dependent secretion of pro-inflammatory mediators, we infected cells with Ad-FXYD5, and 40 h after infection, determined the expression of FXYD5 (**Figure 2A**), phosphorylation of IκBα (**Figure 2B**), cellular levels of CCL2 mRNA (**Figure 2C**), and CCL2 and IL-6 levels in the culture media (**Figures 2D,E**). Expression of exogenous FXYD5 stimulated IκBα phosphorylation, the synthesis of CCL2 mRNA, and the release of CCL2 and IL-6 by AEC, suggesting that the increase in FXYD5 is sufficient to induce the inflammatory response in AEC.

Increased FXYD5 in AEC Contributes to the Inflammatory Response to LPS *In Vivo*

To determine whether FXYD5, which is abundantly expressed in ATII cells (26), contributes to the inflammatory response in LPS-induced acute lung injury, we performed intratracheal instillation of LPS into mice and measured FXYD5 mRNA and protein levels in lung peripheral tissue after 2, 4, 6, and 24 h. Administration of LPS time dependently increased the level of FXYD5 mRNA with a peak after 6 h of instillation (**Figure 3A**). In mouse lung peripheral tissue lysates, FXYD5 was detected by Western blot

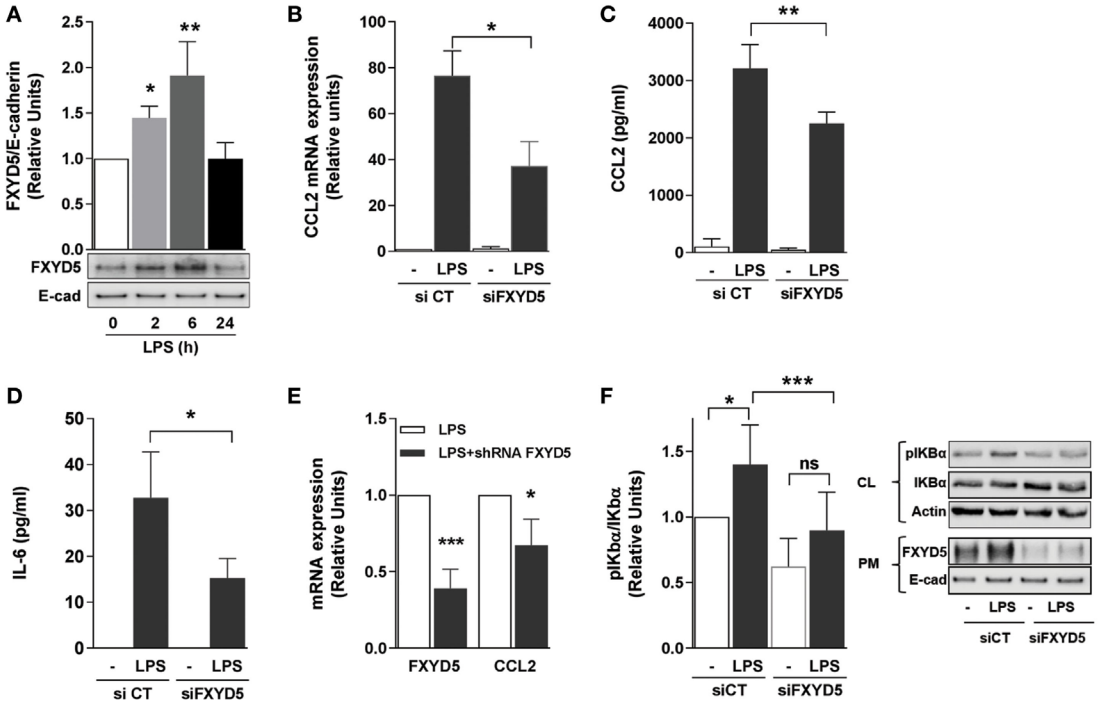

FIGURE 1 | FXYD5 plays a role in LPS-induced inflammatory response by activating the NF-κB signaling pathway in alveolar epithelial cells. (A) MLE-12 cells were treated with 100 ng/ml LPS for the indicated period of time, PM proteins were isolated after cell surface labeling with biotin and characterized by immunoblot with an anti-FXYD5 specific antibody. Densitometric quantification of immunoblots of FXYD5 in relation to E-cad is shown ($n = 3$). **(B)** MLE-12 were cells transfected with a FXYD5-specific siRNA and 24 h later treated with LPS for 6 h. CCL2 mRNA was measured by RT-qPCR $n = 4$. **(C,D)** MLE-12 cells were treated like in B, culture media was collected, and CCL2 **(C)** and IL-6 **(D)** were determined by ELISA ($n = 5$). **(E)** FXYD5 was silenced in isolated mATII cells with shFXYD5 for 72 h and then treated with LPS for 6 h. FXYD5 and CCL2 mRNA were measured by RT-qPCR ($n = 3$). **(F)** A549 cells were treated as in B and cell lysate (CL) and PM proteins were isolated after cell surface labeling with biotin. Densitometric quantification (left panel) of immunoblots (right panel) of pIκBα in relation to total IκBα is shown ($n = 6$). Values of PBS-treated controls were normalized to 1. Bars represent means ± SD. Statistical significance was analyzed by one way ANOVA and Dunnetts's **(A)** or Sidak's multiple comparison test **(B–D,F)** or Student's *t*-test **(E)**. $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$; ns, non-significant.

in two bands, a major band at 60–70 kDa and a minor band at 25 kDa (**Figure 3B**). Only the 60–70 kDa fraction of FXYD5 is seen in surface biotinylated fraction in MLE-12 cells (**Figure 1B**), indicating that the 60–70 kDa in mouse lung lysates corresponds to the mature heavily glycosylated FXYD5 residing at the PM. LPS increased the abundance of both forms in a time-dependent manner. To assess whether the inflammatory response elicited by LPS in the lung is dependent on the presence of FXYD5, we silenced FXYD5 by instillation of lentiviral particles coding for shFXYD5, which decreased FXYD5 expression in the peripheral tissue by ~70% (**Figures 3C,D**). Treatment with LPS increased the concentration of proteins in the BALF (a measure of the permeability of the alveolo-capillary barrier) and total cell count in BALF (a measure of inflammatory cell recruitment to the lung) (**Figures 3E,F**). A decrease in FXYD5 in the lung peripheral tissue lowered the concentration of proteins in the BALF after LPS treatment as compared with control mice exposed to LPS (**Figure 3E**). Also, BALF from mice with silenced FXYD5 contained fewer inflammatory cells and reduced CCL2 after LPS treatment than that obtained from sh-control-treated mice (**Figures 3F,G**), suggesting that FXYD5 contributes to the LPS-induced production of CCL2 and the recruitment of inflammatory cells into the lung.

To determine whether the relationship between elevated FXYD5 and inflammation is causal, we studied the effects of intratracheal administration of an endotoxin-free adenoviral construct coding for mouse mCherry-HA-FXYD5 (Ad-FXYD5) or an empty adenovirus (Ad-Null) to mice (26). ATII cells from infected mice were isolated by flow-cytometry as $CD45^{-}$ $CD31^{-}$ $Ep\text{-}Cam^{+}$ cells. The expression of exogenous FXYD5 in ATII cells was evident from the red fluorescence of the mCherry tag present in this construct (**Figure 4A**). Instillation of Ad-FXYD5 increased total cell number in BALF (**Figure 4B**) as compared with mice infected with Ad-Null. Moreover, in agreement with our *in vitro* data, FXYD5 overexpression in mice increased the levels of CCL2 mRNA (**Figure 4C**) and the secretion of CCL2, TNF-α, and IL-6 into the alveolar space (**Figures 4D–F**).

FXYD5 Induces the Recruitment of Different Subsets of Myeloid Cells to the Lung

Together, the results in **Figures 3** and **4** suggest that FXYD5 is required for LPS-induced cellular infiltration into the alveolar space. To evaluate whether the increased level of FXYD5 leads

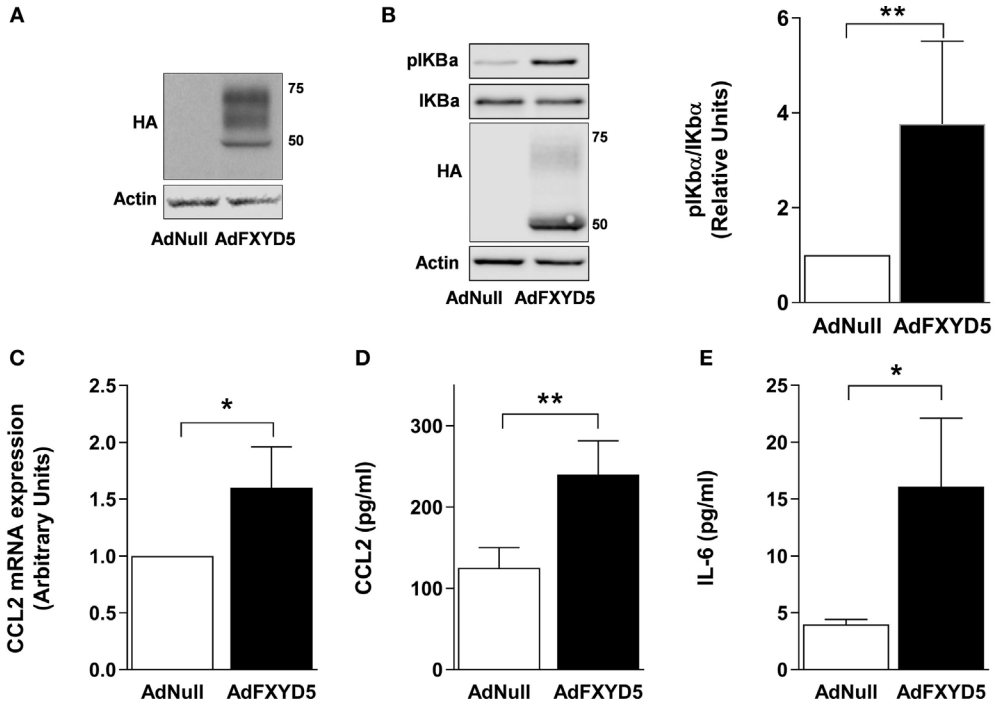

FIGURE 2 | Overexpression of FXYD5 promotes the secretion of inflammatory cytokines by activating the NF-κB signaling in alveolar epithelial cells. **(A)** MLE-12 cells were incubated with Ad-Null or Ad-FXYD5 as described in Section "Materials and Methods." The expression of FXYD5 was determined in the CL by Western blot with an HA antibody. **(B)** A549 cells were treated as in **(A)** and CL was isolated. Left panel: representative immunoblots. Right panel: densitometric quantification of pIκBα in relation to total IκBα. The expression of FXYD5 was determined with an HA antibody ($n = 4$). **(C)** MLE-12 cells were treated as in **(A)** and CCL2 mRNA was quantified by RT-qPCR ($n = 3$). **(D–E)** MLE-12 cells were treated as in **(A)**, culture media was collected, and CCL2, $n = 5$ **(D)** and IL-6, $n = 5$ **(E)** were determined by ELISA. Values of AdNull-treated controls were normalized to 1. Bars represent means ± SD. Statistical significance was analyzed by unpaired Student's *t*-test. $^*p \leq 0.05$; $^{**}p \leq 0.01$.

to enhanced recruitment of specific subcellular myeloid populations into the lung, mice were instilled with Ad-FXYD5 72 h prior to treatment with LPS for 24 h, and changes in leukocyte populations within the lung were analyzed by flow cytometry. After excluding doublets and dead cells, myeloid cells were identified using pan-hematopoietic marker CD45. Using the gating strategy described in the Section "Materials and Methods" and **Figure 5A**, no significant differences were detected in the recruitment of $Ly6G^+CD11b^{int}CD24^{int}$ neutrophils (**Figure 5B**), $NK1.1^+CD11b^{hi}CD24^{hi}$ NK cells (**Figure 5C**), or $SiglecF^{hi}CD11c^{hi}$ alveolar macrophages (**Figure 5D**) while $SiglecF^{hi}CD11c^{low}$ eosinophils (**Figure 5E**) were increased after infection with AdFXYD5. Additionally, we observed increased recruitment of $CD11b^{hi}MHCII^{hi}$ IMs (**Figure 5F**) and $CD11b^{hi}$ $MHCII^{low}Ly6C^{hi}$ classical monocytes (**Figure 5G**) in the presence of higher levels of FXYD5 post-LPS challenge.

CCR2⁺ Classical Monocytes Are Involved in FXYD5-Mediated Inflammation

In mice, expression of Ly6C and CD11b identifies a subset of monocytes that expresses high levels of CCR2 (58). CCL2 and its receptor CCR2 are critical determinants for recruitment of monocytes to the lungs (4, 6, 59, 60), where they have key roles in amplifying lung injury by orchestrating an overly exuberant inflammatory response (1, 14, 61). To determine whether classical monocytes contribute to the FXYD5-induced inflammatory response, we infected mice with Ad-FXYD5 or Ad-Null, and 48 h after the infection depleted monocytes by the injection of an anti-CCR2 antibody. The presence of the antibody decreased the cellular infiltration into the lung, stimulated by infection with Ad-FXYD5 (**Figure 6A**). As an alternative approach, $CCR2^{-/-}$ mice, which lack CCR2, and WT mice were infected with Ad-FXYD5, and BALF was collected after 72 h. The absence of CCR2 decreased the cellular infiltrates in the lungs (**Figure 6B**), suggesting, again, that classical monocytes play a role in FXYD5-induced inflammation. The levels of CCL2 were significantly increased in the CCR2 KO infected with Ad-FXYD5 as compared with the WT-infected mouse (**Figure 6C**).

Taken together, the results demonstrate that the FXYD5 abundance in AEC is increased in response to LPS, and the prevention of this increase by silencing FXYD5 partially abolishes pro-inflammatory effects of LPS. The increased levels of FXYD5 activate the production of CCL2 by AEC, which, in turn, leads to the recruitment of $CCR2^+$ monocytes cells into the alveolar spaces to worsen lung injury.

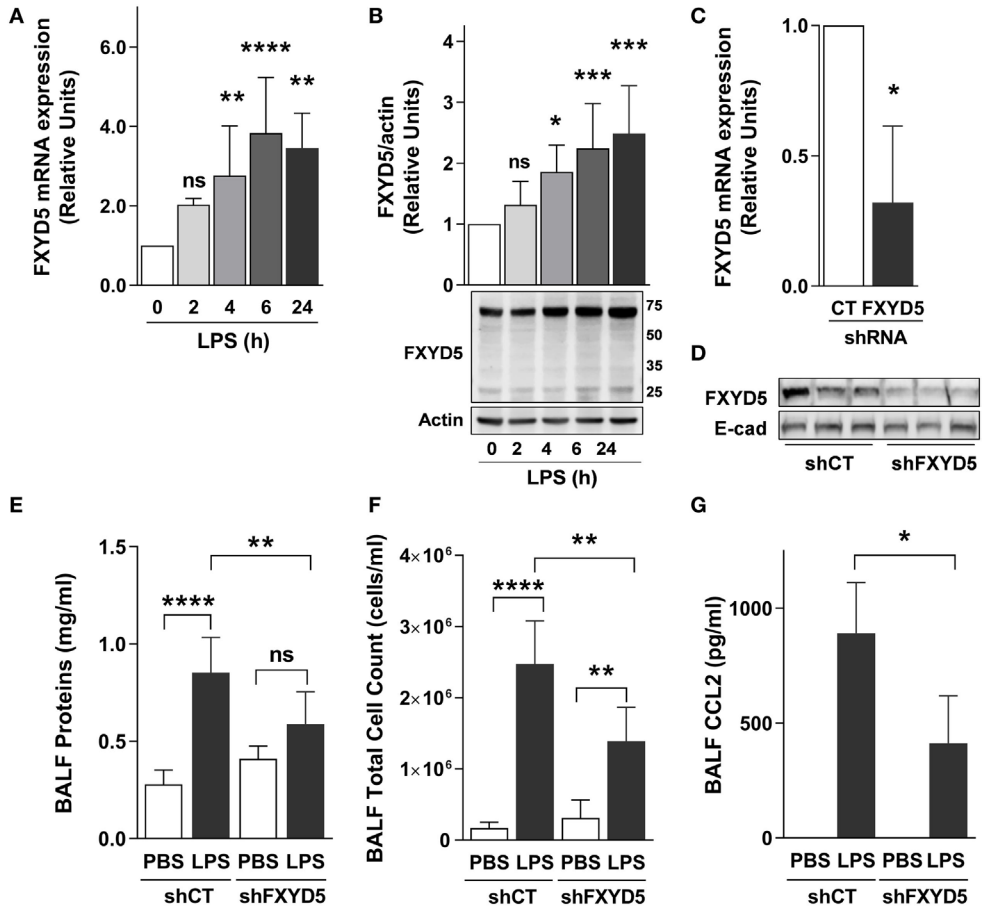

FIGURE 3 | **Increased levels of FXYD5 are required for LPS-induced lung inflammation**. **(A)** LPS was instilled to mice for the indicated times and the levels of FXYD5 mRNA were determined by RT-qPCR in lung peripheral tissue ($n = 8$). **(B)** Mice were treated as in A and FXYD5 was determined in lung peripheral tissue cell lysates by Western blot. Bars indicate densitometric quantification of plasma membrane FXYD5 (top band) in relation to actin ($n = 8$). **(C,D)** Control (CT) or shFXYD5 lentiviral constructs were instilled into mice. Silencing was assessed by measuring FXYD5 mRNA by RT-qPCR **(C)**. $n = 4$ or protein abundance in lung peripheral tissue total membranes **(D)**. Representative immunoblot showing the abundance of FXYD5, E-cadherin was used as a loading control $n = 6$. **(E–G)** Mice treated as in C were given LPS for 6 h and BALF was obtained. Proteins **(E)**, total cells **(F)**, and CCL2 **(G)** were determined as described in Section "Materials and Methods." Values of PBS-treated controls were normalized to 1. Bars represent means ± SD. Statistical significance was analyzed by one way ANOVA and Dunnetts's **(A,B)** unpaired Student's *t*-test **(D)** or Sidak's multiple comparison test **(E,F)**. $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$.

FXYD5 Is Required for NF-κB Activation Downstream of Several Cytokine Receptors

Since expression of exogenous FXYD5 induces the inflammatory response even in the absence of LPS, we studied whether FXYD5 contributes to pro-inflammatory pathways downstream of receptors other than TLR4. To address this question, we measured the activation of NF-κB and the production of cytokines after stimulating A549 cells in the presence or absence of FXYD5 with IFN-α (100 U/ml) or TNF-α (50 ng/ml). IFN-α signals through the type I interferon receptor (IFNAR) (62), while the effects of TNF-α are initiated by its binding to the ubiquitously expressed TNF receptor 1 (TNFR1) or to the TNF receptor 2 that is mainly expressed in lymphocytes and endothelial cells (63). Treatment with IFN-α induced the phosphorylation of IκBα that was detected after 15 min and reached its maximum after 1 h (**Figure 7A**). The knockdown of FXYD5 prevented the activation of NF-κB and significantly inhibited the increase in cytokine secretion in response to IFN-α (**Figures 7A–C**).

Treatment of epithelial cells with TNF-α led to the phosphorylation of IκB and a dramatic decrease in its total amount after 5 min of treatment (**Figure 7D**), suggesting a rapid degradation of IκBα in these conditions. The loss of IκBα was followed by its partial recovery after 1 and 2 h of treatment (**Figure 7D**), which is consistent with previously published data on rapid re-synthesis of IκBα after its TNF-α-induced degradation (64). FXYD5 silencing prevented the TNF-α-induced phosphorylation of IκBα and the concomitant production of IL-6 and CCL2 (**Figures 7E,F**). Collectively, these results suggest that FXYD5 is a required mediator of the inflammatory response in epithelial cells.

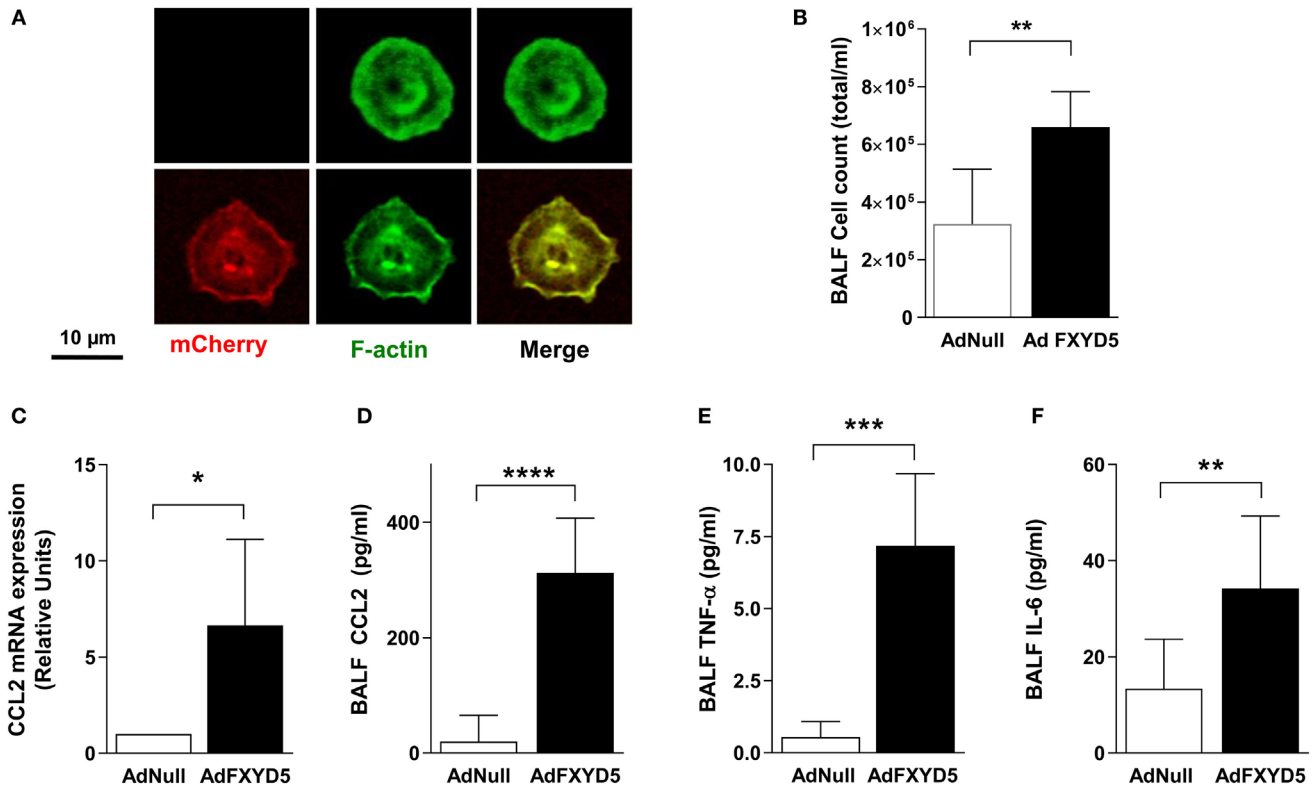

FIGURE 4 | Increased levels of FXYD5 are sufficient to induce lung inflammation. Mice were instilled with adenoviruses encoding m-Cherry-HA-FXYD5 (AdFXYD5) or an empty control (AdNull) and 72 h later ATII cells, BALF or lung peripheral tissue were isolated. **(A)** Confocal microscopy analyses of FACS-sorted mice ATII cells. Red m-cherry fluorescence reflects the expression of FXYD5. Green fluorescence shows actin filaments used to visualize cells. **(B)** Total cell count in BALF. **(C)** CCL2 mRNA was determined by RT-qPCR in lung peripheral tissue. **(D–F)** CCL2, TNF-α, and IL-6 were determined by ELISA in BALF. Values of Ad-Null-treated controls were normalized to 1. Bars represent means ± SD $n \leq 5$. Statistical significance was analyzed by unpaired Student's *t*-test. $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$.

DISCUSSION

The respiratory epithelium is constantly exposed to invading particles and potential pathogens. In addition to creating a barrier for pathogens, AEC secrete inflammatory mediators that recruit innate and adaptive immune cells to the alveolar space (1, 4, 7, 65–68). The mechanisms regulating the extent of epithelial inflammatory responses to infection and tissue injury are not fully understood. The data presented here demonstrate that in AEC, FXYD5, acting upstream of NF-κB, is necessary and sufficient for the secretion of pro-inflammatory cytokines *in vivo* and *in vitro*. Under our experimental conditions, LPS rapidly increases FXYD5 in AEC resulting in the secretion of CCL2 and the recruitment of $CCR2^{+}$ monocytes to the alveolar space. These monocytes, often referred as inflammatory monocytes, are responsible for the secretion of a large number of soluble mediators that regulate the activity of other inflammatory cells.

Lipopolysaccharide-induced acute lung injury is an animal model that replicates several key pathologic processes of acute respiratory distress syndrome, including cytokine release, inflammatory cell influx, and lung capillary permeability, which results in pulmonary edema (69). In our study, LPS stimulation of isolated mouse ATII cells, as well as mouse and human alveolar epithelial cell lines, resulted in a rapid and substantial increase in the secretion of several cytokines. These effects were prevented by silencing FXYD5 using different silencing RNA, which strongly suggests a role for FXYD5 in the production of inflammatory mediators by AEC. Moreover, acute overexpression of FXYD5 in AEC increased secretion of CCL2 and IL-6. In conjunction with our previous data showing that FXYD5 overexpression produces a disruptive effect on alveolar–epithelial barrier (26), these results suggest that FXYD5 impairs the integrity of the barrier not only by directly disrupting epithelial junctions formed by Na,K-ATPase β1 subunits (26) but also by secreting cytokines that recruit immune cells into alveolar spaces, which further enhance the impairment of alveolar–epithelial barrier.

In agreement with our previous report (30), we found that inhalation of LPS results in a time-dependent increase in FXYD5 expression in the lung. This increase temporally correlated with the secretion of cytokines into the BALF and recruitment of immune cells to the lung. Either after LPS instillation or FXYD5 overexpression, we observed that a significant portion of FXYD5 is localized at the PM and heavily *O*-glycosylated (26), which contrasts with previous studies that reported that in normal tissues, including the lung, FXYD5 is expressed only as a low molecular mass protein with no or very minimal glycosylation (24, 29).

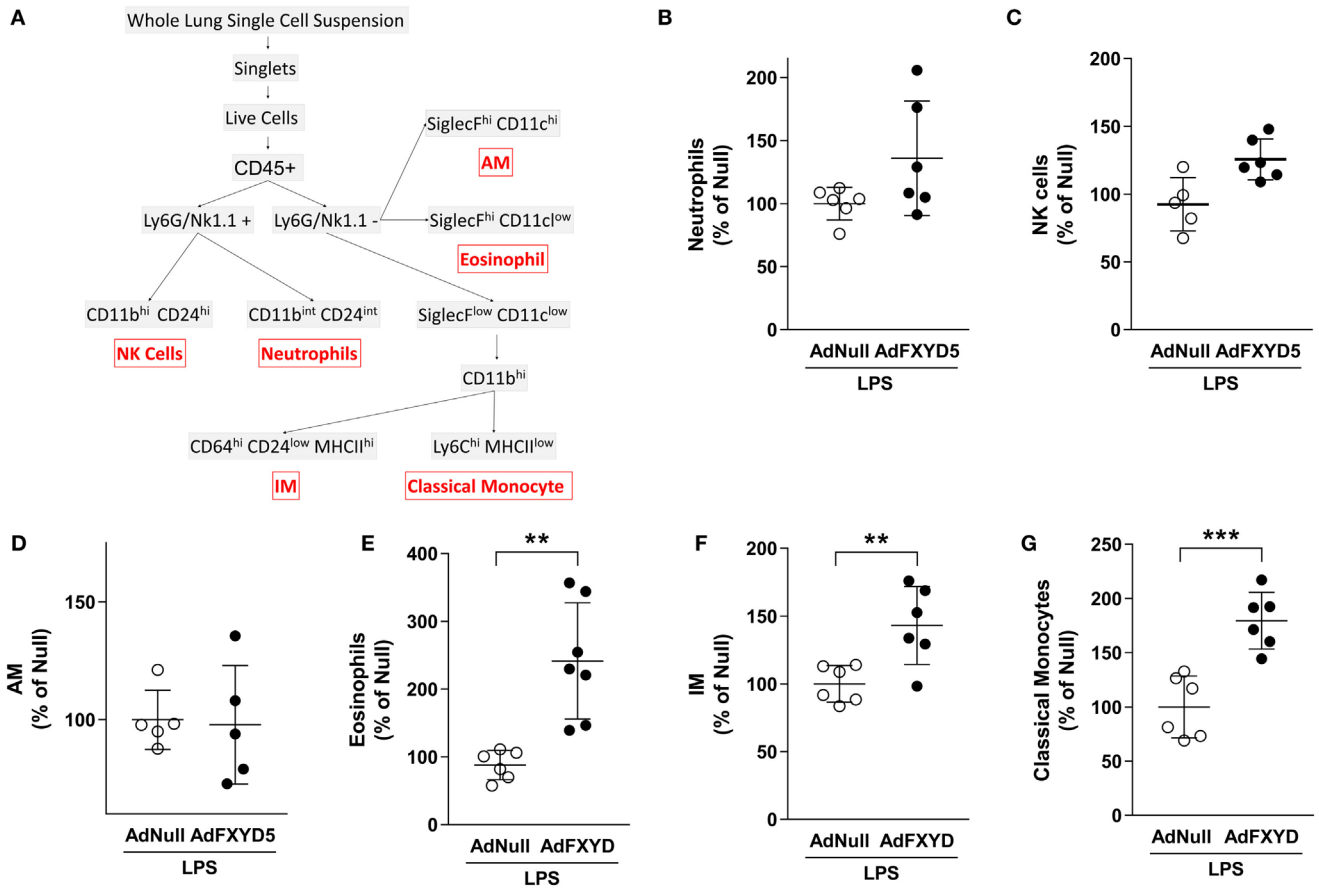

FIGURE 5 | FXYD5-induced changes in myeloid-cell subsets in mouse lungs during LPS-induced lung injury. **(A)** Gating strategy used to identify myeloid-cell subsets in the normal mouse lung. **(B–G)** Mice were instilled with Ad-FXYD5 or Ad-Null and treated with LPS for 12 h. Cells were isolated from enzymatically digested mouse lungs. Changes of myeloid-cell subsets in Ad-FXYD5-infected mice relative to Ad-Null-infected control identified as described in **(A)** are shown. Values of Ad-Null-treated controls were normalized to 100%. Values represent means ± SD $n = 6$. Differences between groups were compared using unpaired Student's *t*-test. $^{**}p \leq 0.01$; $^{***}p \leq 0.001$.

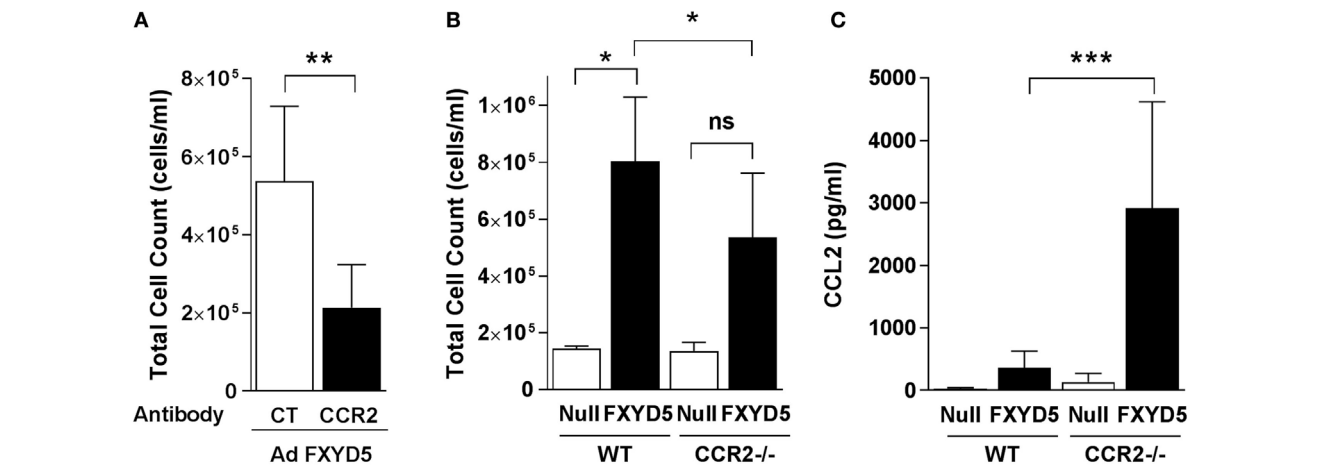

FIGURE 6 | Increased levels of FXYD5 are sufficient to recruit CCR2^{+} classical monocytes to the lung. **(A)** Mice were instilled with Ad-FXYD5 and treated with the anti-CCR2 antibody for 24 h prior to the measurements. Total cell count in BALF was determined ($n = 5$). **(B,C)** WT and CCR2$^{-/-}$ mice were instilled like in **(A)**. Total cell counts $n = 3$ **(B)** and CCL2 $n = 4$ **(C)** were determined in BALF. Values represent means ± SD. Differences between groups were compared using unpaired Student's *t*-test or one way ANOVA Sidak's multiple comparison test. $^{*}p \leq 0.05$ $^{**}p \leq 0.01$; $^{***}p \leq 0.001$.

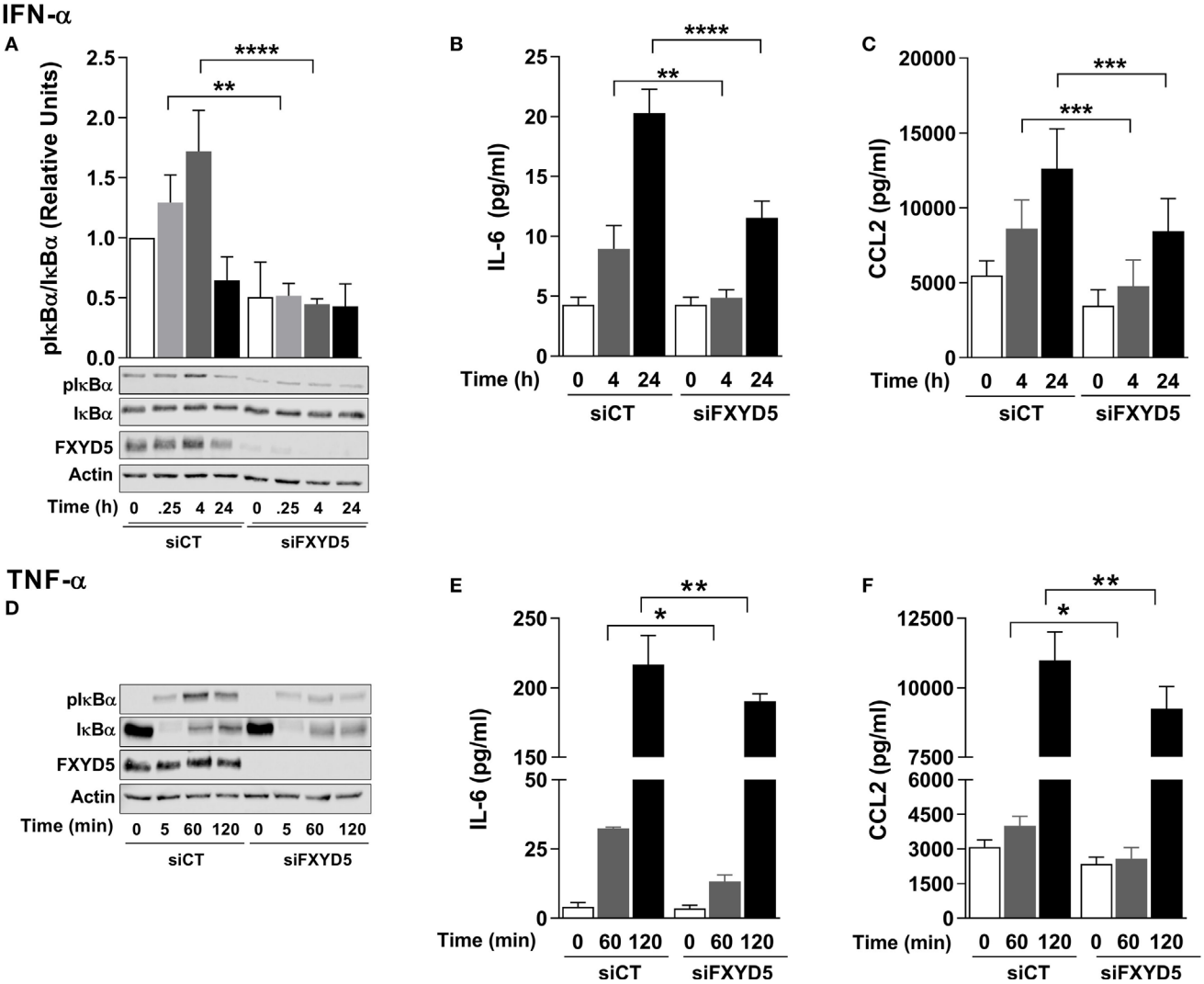

FIGURE 7 | **FXYD5 is required for the NF-κB-mediated secretion of cytokines in response to IFN-α and TNF-α by alveolar epithelial cells**. **(A)** A549 cells were transfected with a control (siCT) or FXYD5 specific (siFXYD5) siRNA, 24 h later treated with 100 U/ml IFN-α for the indicated period of time, and cell lysates were analyzed by immunoblot using specific antibodies as indicated. Bottom panel: representative immunoblots. Top panel: densitometric quantification of pIκBα in relation to total IκBα $n = 4$. **(B,C)** A549 cells were treated as in **(A)**, culture media was collected, and IL-6 **(B)** and CCL2 **(C)** were determined by ELISA $n = 3$. **(D)**. A549 cells were transfected with siCT or siFXYD5, 24 h later treated with 50 ng/ml TNF-α for the indicated period of time, and cell lysates were analyzed by immunoblot using specific antibodies as indicated. Representative immunoblots $n = 4$. **(E,F)** A549 cells were treated as in **(D)**, culture media was collected, and IL-6 **(E)** and CCL2 **(F)** were determined by ELISA $n = 4$. Values of PBS-treated controls were normalized to 1. Bars represent means ± SD. Statistical significance was analyzed by one way ANOVA and Sidak's multiple comparison test. $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$; $^{****}p \leq 0.0001$, ns, non-significant.

Moreover, we showed that endogenous expression of FXYD5 in the lung epithelium is required for the epithelial inflammatory response, as silencing of FXYD5 decreased the number of cells in BALF after LPS treatment. This is consistent with previous reports suggesting that leukocyte recruitment during bacterial infection is due to the response of the alveolar epithelium rather than resident alveolar macrophages (8) and that the profile of cytokines released by ATII cells determines specific leukocyte recruitment (70). The data presented here demonstrate that FXYD5 overexpression in the absence of LPS or other stimuli is sufficient to activate cytokine secretion in AEC and to increase the number of cells in BALF, suggesting that the increase in FXYD5 alone, by stimulating cytokine secretion, leads to the recruitment of immune cells into the lung. The recruitment of cells by FXYD5 overexpression was decreased by treating mice with the antibody against CCR2, and the same effect was observed in mice lacking CCR2, indicating that FXYD5-induced secretion of CCL2 causes chemotaxis of CCR2-positive monocytes to the alveolar spaces.

The overexpression of FXYD5 in conjunction with LPS treatment significantly increased the recruitment of interstitial and monocyte-derived macrophages to the lung. Tissue resident alveolar macrophages are the predominant immune cells found

within the alveolar airspaces during steady-state conditions, while classical inflammatory Ly6C^{hi} monocytes and IM represent a very low proportion of circulating white blood cells in an uninfected mouse and are rapidly recruited to sites of infection and inflammation (58, 71, 72). Upon stimulation, Ly6C^{hi} monocytes exit the bone marrow in a CC-chemokine receptor 2 (CCR2)-dependent manner and are recruited to inflamed tissues (58). In agreement with our data, it has been described that IM expand more rapidly in response to foreign stimuli compared with alveolar macrophages as IM are preferentially replenished from blood monocytes (72) and CCR2^{+} monocytes emigration from the bone marrow is normal during early-stage of bacterial infection of mice (58). In addition, overexpression of FXYD5 increased the recruitment of eosinophils to the lung in response to LPS. These data suggest that FXYD5 induces secretion of other cytokines/chemokines because CCL2 is not among the major chemoattractants of eosinophils such as IL-5, RANTES (CCL5), eotaxin, and others (73–76).

Further, we demonstrated that the presence of FXYD5 in AEC is required for NF-κB activation induced by LPS, TNF-α, or IFN-α as FXYD5 silencing prevented IκBα phosphorylation and reduced cytokine secretion in response to these stimuli. Moreover, overexpression of FXYD5 in the absence of any stimuli induced both IκBα phosphorylation and cytokine secretion. Taken together, these results indicate that FXYD5 is an important component of NF-κB signaling pathway. This conclusion is consistent with previously published data showing that FXYD5 overexpression in breast cancer cells induces the phosphorylation of AKT (38), which promotes the transcriptional activity of NF-κB-responsive promoter elements and increases levels of CCL2 mRNA (38, 41). Taken together, these data suggest that FXYD5 increases CCL2 transcription by inducing AKT-dependent activation of NF-κB signaling. In support of a role of an FXYD5/AKT dependent activation of NF-κB, binding of IFN-α to IFNAR activates PI3K *via* STAT5, which in turn, activates NF-κB (77–79). Activation of PI3K has been also described downstream of TLR4 and TNFR1 (80, 81). Our recent data in kidney cells, stably transfected with FXYD5, suggested that FXYD5 modulates NF-κB signaling by regulating the location of TNF-α receptor, TNFR1 (30). It is possible that the plasmalemma-located FXYD5, by interacting with the PM receptor complexes, modulates their association with other proteins as well as their location and mobility in the membrane. Such a possibility is consistent with the data showing that the efficiency of LPS/TLR4 signaling is affected by receptor mobility in the lipid bilayer that permits its clustering and binding to other proteins (82–85). However, considering significant differences in the composition of these receptor complexes as well as the fact that FXYD5 activates NF-κB even in the absence of other stimuli, a possibility that the intracellular forms of FXYD5 contribute to NF-κB signaling downstream of the PM receptors but upstream IκBα phosphorylation cannot be excluded. Taken together, our results suggest that the presence of FXYD5 in the alveolar epithelium is required for stimuli-induced pulmonary inflammation and injury. The deleterious effects of enhanced FXYD5 may be twofold: (1) the impairment of the function of the epithelial barrier through the disruption of adherens junctions (26) and (2), as shown here, the activation of the NF-κB pathway to recruit CCR2^{+} monocytes and IMs.

In conclusion, FXYD5 is a pro-inflammatory protein, which activates NF-κB-dependent cytokine secretion and infiltration of immune cells to the alveolar spaces. A better understanding of the mechanism by which alveolar epithelial FXYD5 modulates the expression of CCL2 and other cytokines may help to develop new therapies for the treatment of pulmonary inflammation following exposure to various Gram-negative bacteria commonly found in hospital settings.

ETHICS STATEMENT

Mice were provided with food and water *ad libitum*, maintained on a 14-h-light–10-h-dark cycle, and handled according to National Institutes of Health guidelines and an experimental protocol approved by the Northwestern University Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

PB, PS, ET, AY, and NM performed experiments; PB assisted with the research design and data analysis; KR, HP, and JS provided reagents; PB, HP, KR, and JS discussed and edited the manuscript; OV and LD designed the research, performed experiments, analyzed data, and wrote the manuscript.

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Inhibition of TNF Receptor p55 By a Domain Antibody Attenuates the Initial Phase of Acid-Induced Lung Injury in Mice

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Background: Tumor necrosis factor-α (TNF) is strongly implicated in the development of acute respiratory distress syndrome (ARDS), but its potential as a therapeutic target has been hampered by its complex biology. TNF signals through two receptors, p55 and p75, which play differential roles in pulmonary edema formation during ARDS. We have recently shown that inhibition of p55 by a novel domain antibody (dAb™) attenuated ventilator-induced lung injury. In the current study, we explored the efficacy of this antibody in mouse models of acid-induced lung injury to investigate the longer consequences of treatment.

Methods: We employed two acid-induced injury models, an acute ventilated model and a resolving spontaneously breathing model. C57BL/6 mice were pretreated intratracheally or intranasally with p55-targeting dAb or non-targeting "dummy" dAb, 1 or 4 h before acid instillation.

Results: Acid instillation in the dummy dAb group caused hypoxemia, increased respiratory system elastance, pulmonary inflammation, and edema in both the ventilated and resolving models. Pretreatment with p55-targeting dAb significantly attenuated physiological markers of ARDS in both models. p55-targeting dAb also attenuated pulmonary inflammation in the ventilated model, with signs that altered cytokine production and leukocyte recruitment persisted beyond the very acute phase.

Conclusion: These results demonstrate that the p55-targeting dAb attenuates lung injury and edema formation in models of ARDS induced by acid aspiration, with protection from a single dose lasting up to 24 h. Together with our previous data, the current study lends support toward the clinical targeting of p55 for patients with, or at risk of ARDS.

Keywords: CD120a, TNFRSF1a, acid aspiration, inflammation, respiratory mechanics

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INTRODUCTION

Acute respiratory distress syndrome (ARDS) is a major cause of patient morbidity and mortality within the ICU, constituting ~10% of ICU admissions worldwide with an associated mortality of 30–50% (1). ARDS can result from various insults, of which aspiration of acidic gastric contents is a major contributor both within the community and in the operating theater during anesthesia

(2). Inflammation has been considered to be key in developing ARDS, but attempts to minimize "global" inflammation, e.g., by use of corticosteroids have delivered limited mortality benefits (3–7), suggesting the need for a more targeted approach. Among all potential targets, tumor necrosis factor-α (TNF) is one of the strongest candidates for such interventions—it is one of the earliest expressed "gate-keeper" cytokines in response to almost any potentially damaging situation, modulating subsequent inflammatory responses, and has been repeatedly implicated in the development and progression of ARDS (8), including direct effects on pulmonary edema formation and clearance. Despite this, previous clinical trials of anti-TNF therapy have shown little beneficial impact within the ICU setting (9). This may be partly attributed to the biophysical properties of the inhibitors used (e.g., affinity and tissue penetration), and also likely related to the complex nature of TNF signaling.

Tumor necrosis factor signals through two cell surface receptors, TNF receptor (TNFR) type I (p55) and TNFR type II (p75). Historically, p75 signaling was considered to be adjunct to the p55 pathway (10, 11), but it is now becoming clear that each TNFR subtype has signaling capabilities of its own, which in particular circumstances may lead to directly opposing consequences (12–16). In line with this, we have previously shown using genetically modified mice that specific absence of p55 is protective in the very acute phase of ARDS induced by mechanical ventilation (17), while absence of p75 seems to be detrimental. We have also found that p55 signaling triggers alveolar epithelial cell dysfunction in the early phase of ARDS, promoting lung permeability as well as impairing alveolar fluid reabsorption (18). These findings suggest that while total TNF signaling blockade would likely be counterproductive, specific therapeutic strategies targeting the individual TNFRs could be effective in ARDS.

We have recently demonstrated efficacy of selective pharmacological blockade of p55 signaling in an acute model of mouse ventilator-induced lung injury (VILI), using a novel IgG fragment known as a domain antibody (Biopharmaceuticals R&D, GlaxoSmithKline, Stevenage, UK) (19). Domain antibodies (dAb™) offer multiple advantages over conventional antibody technology (20)—for example, they have fewer off-target effects and can be delivered at much higher concentrations per unit mass compared to conventional antibodies due to the lack of an Fc region and can be manufactured to be suitable for local delivery such as inhalation. In the current study, we tested the efficacy of a dAb antagonist of murine p55, in mouse models of acute and resolving acid aspiration-induced lung injury (21). This enabled us to both evaluate the acute benefits of pharmacological p55 blockade in a highly clinically relevant model and explore whether any beneficial effects persisted beyond the acute stage. A similar dAb antagonist of human p55 has recently entered early clinical development for lung injury (22).

MATERIALS AND METHODS

All protocols were approved by the Ethical Review Board of Imperial College London and carried out under the authority of the UK Home Office in accordance with the Animals (Scientific Procedures) Act 1986, UK. Male C57BL/6 mice (Charles River, Margate, UK) aged 9–12 weeks old, weighing 25–30 g were used throughout.

The p55-targeting dAb sequence was identified from a phage display library. To determine binding kinetics, murine p55 or p75 was immobilized on an IgG surface, varying concentrations of dAb (from 0.25 to 16 nM) were passed over the surface, and interactions were evaluated via surface plasmon resonance using a Biacore T200 system. Association constant (ka) of the dAb for p55 was determined as 3.455×10^7 (1/Ms) and dissociation constant (kd) was 0.0014 (1/s), indicating high affinity of binding (KD) of 4.05×10^{-11}M, assuming a 1:1 binding stoichiometry. In contrast, no specific binding was observed to p75.

Ventilated Acid Aspiration Model

We have previously shown that genetic absence of TNFR p55 signaling attenuates pulmonary edema formation during the first 2–3 h after acid instillation in mice (18). Therefore, initial experiments were carried out to determine whether p55 dAb administration would have a similar influence during the acute phase of acid aspiration-induced lung injury.

Mice were anesthetized (intraperitoneal ketamine 80 mg/kg and xylazine 8 mg/kg), tracheostomized, and connected to a custom-made ventilator/pulmonary function testing system as described previously (23). Animals had a cannula placed in the carotid artery for monitoring of blood pressure, blood gas analysis, and fluid replacement. All animals were ventilated using 7 ml/kg tidal volume, 2.5 cmH_2O positive end-expiratory pressure (PEEP), and respiratory rate of 120/min, with 100% O_2. Following instrumentation, either specific murine TNFR p55 blocking dAb (Dom-1m-15-12, GSK) or non-targeting control dAb ("dummy") was intratracheally delivered in bolus form (25 μg of antibody in a volume of 50 μl) via a fine cannula passed through the endotracheal tube. The lungs were then immediately recruited with four sustained inflation maneuvers (35 cmH_2O, 5 s) to maximize distribution of the antibody into the lungs. Mice were ventilated for 1 h to allow respiratory mechanics to return toward normal, and then 65 μl of 0.075M hydrochloric acid (HCl) was instilled into the trachea through the endotracheal tube. Lungs were again recruited by sustained inflations, and mice were ventilated for a further 3 h. Anesthesia was maintained by bolus administrations of intraperitoneal ketamine (40 mg/kg) and xylazine (4 mg/kg) every 20–25 min.

Airway pressure and arterial blood pressure were monitored continuously. Plateau pressure, and respiratory system elastance and resistance were determined every 20 min by the end-inflation occlusion technique, followed each time by sustained inflation (35 cmH_2O for 5 s) to avoid the development of atelectasis (23). Arterial blood gases were assessed at predetermined points throughout the protocol (immediately before, 60, 120, and 180 min after acid instillation). At the end of the experiments, animals were exsanguinated, lung lavage was performed using 750 μl of saline, and lung tissue samples were taken for further analysis. Each animal was experimented on a separate day, so each observation reflects an independent experiment.

Spontaneously Breathing Model of Acid Aspiration

While the use of mechanical ventilation provides many advantages, including real-time cardiorespiratory monitoring and an ability to directly compare findings with those of our previous studies in genetically modified animals (18), by definition the model is limited to investigation within the acute phase. We have recently developed a spontaneously resolving model of acid aspiration-induced lung injury (21), which, unlike a number of other models, mimics most of the features of clinical ARDS over up to 5–10 days (24–26). We therefore employed this resolving model to investigate the impact of intranasally delivered p55-targeting dAb on the later phases of injury/start of the repair process.

All animals were anesthetized briefly with inhalational isoflurane (2%) and intranasally dosed with 100 μg (50 μl total volume, divided into two nostrils) of either p55-targeting dAb or dummy dAb. Four hours after dosing (a period designed to allow distribution of the antibody and full recovery of respiratory mechanics from the nasal dosing procedure under non-ventilated conditions), animals were reanesthetized either for physiological analysis (see below—0 h mice) or for acid instillation according to our previously published protocols (21). In brief, mice were suspended vertically for orotracheal instillation, and a fine catheter passed through the vocal cords. A total of 75 μl of an isoosmolar solution of 0.1M hydrochloric acid (pH 1.0) was instilled, and mice received an intraperitoneal bolus of 0.9% saline for fluid resuscitation. Mice were maintained in a humidified chamber containing supplemental oxygen (decreasing from FiO_2 1.0–0.4) over the next 4 h and were then returned to individually ventilated cages. Due to the nature of the dosing technique and postdosing care requirements, a maximum of three animals were dosed with acid/antibody on a single day. Each set of data shown in the Section "Results," of any given combination of treatment (dummy versus p55-targeting dAb) and time point (0, 24, 48, and 72 h), therefore represents observations from 5 to 8 mice, obtained from 3 to 4 independent experiments.

Physiological analysis was carried out at predetermined end points, i.e., 0, 24, 48, or 72 h post acid. Mice were anesthetized (ketamine 80 mg/kg, xylazine 8 mg/kg) and instrumented as described for the acute ventilated model. Immediately after completing surgical preparation, lungs were recruited by sustained inflation (35 cmH_2O for 5 s). Mice were then ventilated with 7 ml/kg tidal volume, 2.5 cmH_2O PEEP, and respiratory rate of 120/min using 100% O_2 for 30 min, in order to standardize the volume history of the lung and determine the PaO_2/FiO_2 ratio from carotid blood samples (21). At the end of 30 min ventilation, respiratory mechanics and blood gases were evaluated, and animals were terminated by exsanguination. The right lung was tied off at the hilum, weighed, and placed in an oven at 60°C for determination of wet:dry weight ratio. The left lung was lavaged using 400 μl saline and dissected out for further analysis.

Evaluation of Injury and Inflammation in Lavage Samples

Total protein concentration in lung lavage fluid was measured as an indicator of alveolar-capillary permeability (Bio-Rad, Hertfordshire, UK). Concentrations of interleukin 6 (IL-6), the neutrophil chemoattractants CXCL1 and CXCL2, and the monocyte chemoattractant CCL2 were measured by ELISA (R&D Systems, Abingdon, UK). The number of neutrophils in lavage fluid was determined by microscopic cytology using hemocytometer and Cytospin-prepared slides.

Lung Tissue Flow Cytometry

The identification and quantification of leukocytes within lung tissue was performed by flow cytometry using methods described and validated previously. For the acute ventilated model, lung samples were removed, minced, and passed through a 40 μm filter (19, 27). Samples were resuspended in a washing buffer (PBS with 2% FCS, 0.1% sodium azide, and 5 mM EDTA) and stained for 30 min in the dark at 4°C with fluorophore-conjugated anti-mouse antibodies for CD11b (clone M1/70), Gr-1 (RB6-8C5) (both BD BioSciences), and F4/80 (CI:A3-1) (Biolegend). For the spontaneously breathing resolution model, the potential for influx of other cell types over time led us to modify this approach for further identification of leukocyte subpopulations (18). Lung tissues were excised and fixed with Cytofix/Cytoperm (BD Biosciences, Oxford, UK), mechanically disrupted by gentleMACS Dissociator (Miltenyi Biotech, Surrey, UK) and passed through a 40 μm filter to prepare single-cell suspensions. Samples were then stained for 30 min in the dark at 4°C with fluorophore-conjugated anti-mouse antibodies for CD11b, Gr-1, Ly6C (AL-21), and NK1.1 (PK136) (BD BioSciences). Importantly, we showed previously (28) that although the Gr-1 antibody used (clone RB6-8C5) binds both Ly6G and Ly6C epitopes under non-fixed conditions (i.e., for the acute ventilated model), the use of Cytofix/Cytoperm results in loss of Ly6C recognition by the antibody. Therefore, in the context of the resolution model, Gr-1 staining is representative solely of cell Ly6G expression. Cell samples were analyzed by a CyAn flow cytometer with Summit software (Beckman Coulter, High Wycombe, UK), and further data analysis was performed by FlowJo software (Tree Star, Ashland, OR). Absolute leukocyte counts were determined using microsphere beads (Invitrogen, Paisley, UK).

Statistical Analysis

Statistical analyses were performed using SPSS version 22 (IBM, Portsmouth, UK). The normality of model residuals was assessed by QQ plot and Shapiro–Wilk test. Data that were not normally distributed were transformed (see legends for details), and subsequent parametric distribution confirmed before analyses were carried out. Data that could not be normalized by transformation were analyzed using non-parametric tests. Time-course data in the acute ventilated model were analyzed by repeated measures analysis of variance (ANOVA) followed by pairwise analysis of individual time points, while end-point analyses were carried out by Student's *t*-test. In the resolving injury model, differences between treatment groups were evaluated on each day, using either Student's *t*-test or Mann–Whitney *U*-test. A value of $p < 0.05$ was considered significant.

RESULTS

Acute Model

We first carried out experiments to determine the influence of p55-targeting dAb administration on the acute phase of acid aspiration-induced lung injury. As part of the model development, experiments were performed comparing the physiological consequences of acid instillation versus those of saline instillation. Initial administration of dummy dAb led to a transient increase in peak inspiratory pressure (**Figure 1A**), which returned toward normal as fluid was distributed and absorbed within the lungs (the same pattern was apparent following administration of p55-targeting dAb; data not shown). Instillation of either saline or acid 60 min later caused similar increases in airway pressure. In animals receiving saline, peak inspiratory pressure decreased and plateaued. In contrast, animals that received acid instillation showed an initial improvement in airway pressure, which then deteriorated over the final 60 min. Intratracheal administrations also caused small, transient decreases in blood pressure, most likely secondary to the sustained inflation maneuvers carried out to distribute instilled fluids (**Figure 1B**). Blood pressure was otherwise well maintained throughout the experiments until the final 60 min during which it deteriorated somewhat, particularly in acid-treated animals.

The physiological consequences of acid instillation were then compared between animals pretreated with either the dummy non-targeting dAb or the p55-targeting dAb ($N = 6$ independent experiments/group). The initial mechanics response to acid was similar between the two groups (**Figure 2A**), consisting of a transient spike in elastance followed by a return toward pre-instillation levels. In the dummy dAb group, elastance then increased from ~100 min until the end, whereas in contrast, the p55-targeting dAb-treated mice showed little increase, resulting in a significantly attenuated elastance change (p value for interaction <0.01, although pairwise analysis did not detect significant differences at individual time points). Respiratory system resistance showed similar patterns in both groups, consisting of a decrease following acid instillation that remained relatively stable thereafter (**Figure 2B**). There was also a decrease in arterial pO_2 and an increase in pCO_2 during the final hour of ventilation in dummy-treated animals (**Figures 2C,D**). These impairments in gas exchange were significantly attenuated by treatment with the p55-targeting dAb (p-value for interaction <0.05, with pairwise analysis showing significant difference at the 180 min time point).

Lung lavage fluid in the dummy-treated group contained a substantial amount of protein, indicating an increase in epithelial/endothelial barrier permeability (**Figure 3A**, dotted line represents data from saline-treated animals for visual comparison). This was somewhat, though not significantly, reduced by p55-targeting dAb. Evaluation of pro-inflammatory cytokines in lung lavage fluid showed that IL-6 and CXCL1 showed a tendency to be reduced, while CXCL2 and CCL2 were significantly attenuated in p55-targeting dAb-treated mice (**Figures 3B–E**).

Finally, lung leukocyte recruitment was determined in each group of mice. Neutrophil infiltration into the alveolar space (evaluated by microscopic cytology of lung lavage fluid) was significantly attenuated by p55-targeting dAb (**Figures 4A,B**). Total lung tissue neutrophil and monocyte recruitment were evaluated by flow cytometry (**Figure 4C**). Neutrophil recruitment was somewhat (though not significantly) reduced following p55-targeting dAb, while inflammatory Ly6C^{hi} monocyte numbers were significantly attenuated (**Figures 4D,E**).

Resolving Model

While the data from the acute model indicate that intratracheal inhibition of p55 using the dAb attenuates both pulmonary edema and inflammation during the early phase of ALI/VILI, it does not necessarily follow that this would translate to a prolonged benefit. We therefore carried out experiments to investigate the consequences of p55-targeting dAb on the later phase of ALI using the resolving model.

Acid instillation caused a clear physiological lung injury in dummy dAb-treated animals, consistent with previous reports using this model (21, 29). Respiratory system elastance (determined after 30 min of ventilation) was increased at 24 h after acid, and subsequently returned toward baseline although did not reach normal values by day 3 (**Figure 5A**). Similarly, arterial pO_2 was substantially decreased (**Figure 5B**) and pCO_2 increased

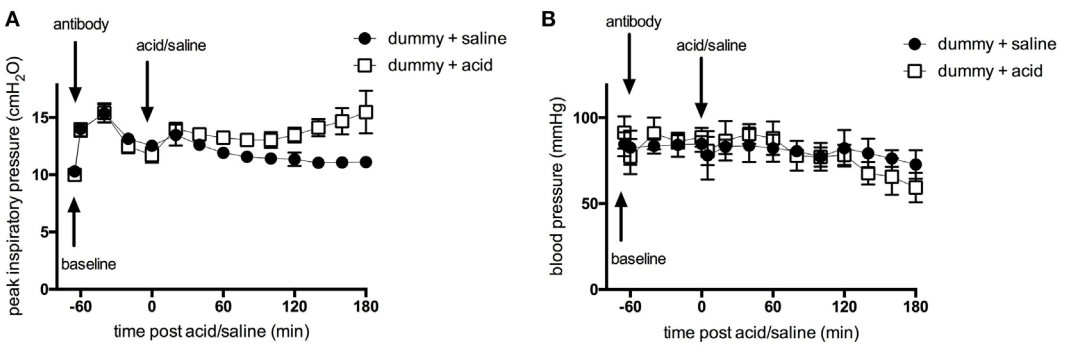

FIGURE 1 | Peak inspiratory pressure (A) and arterial blood pressure (B) in ventilated animals following intratracheal treatment with dummy dAb, followed by challenge with either intratracheal saline or hydrochloric acid. $N = 4$ (dummy + saline), or 6 (dummy + acid) at each time point. Data are expressed as mean ± SD.

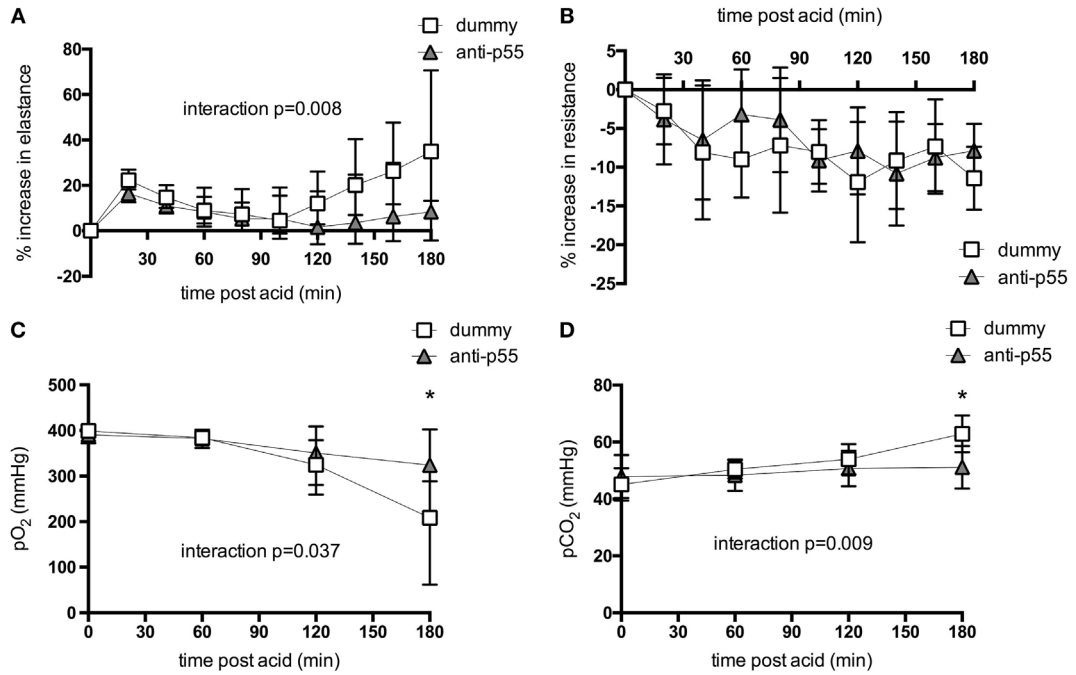

FIGURE 2 | Respiratory system elastance (A) and resistance (B), and arterial pO_2 (C) and pCO_2 (D) following acid instillation in animals treated with dummy or p55-targeting dAb. Mechanics data are expressed as % increase following acid. Elastance data **(A)** were log-transformed to achieve normal distribution, while pO_2 **(C)** was normalized by raising to the power of 2. These data are displayed as back-transformed mean with error bars representing 90% confidence intervals. Resistance data and pCO_2 were normally distributed and thus displayed as mean ± SD. Repeated measures analysis of variance revealed significant interactions between treatment and time for elastance change ($p < 0.01$), pO_2 ($p < 0.05$), and pCO_2 ($p < 0.01$). *$p < 0.05$ between dummy and dAb-treated animals at 180 min after acid. $N = 5–6$ observations from independent experiments at each time point.

(**Figure 5C**) 1 day after acid. pO_2 remained low at day 2 and then started to recover at day 3, while pCO_2 showed some signs of return toward normal at day 2. The single pretreatment with p55-targeting dAb led to a significant attenuation in each of these parameters at 24 h. It was clear however that by 48 h most of the protection afforded by the dAb was lost, with animals appearing as injured as dummy dAb-treated mice. Acid instillation also caused increases in pulmonary edema/permeability, assessed by lavage fluid protein (**Figure 5D**) and lung wet:dry weight ratio (**Figure 5E**). Both of these markers peaked around days 1–2 before returning toward (but not achieving) baseline levels by day 3. Pretreatment with the p55-targeting dAb reduced lavage fluid protein levels (again, only at day 1) and lung wet:dry ratio (at days 1 and 2).

Finally, the effect of p55-targeting dAb on inflammation within the lungs was evaluated after acid instillation. Lavage fluid CXCL1 (**Figure 6A**) was increased to a variable degree in dummy dAb-treated animals, but still significantly attenuated at days 1 and 2 following p55-targeting dAb. In contrast, levels of CCL2 (**Figure 6B**) were similar between the treatment groups on all days, and unlike CXCL1, did not return to baseline by day 3. Neutrophil infiltration into the alveolar space (by differential cytology) was highly variable and showed little difference between treatment groups (**Figures 6C,D**), apart from a small reduction in neutrophil percentage at day 2 following p55-targeting dAb. Lung tissue recruitment of neutrophils and $Ly6C^{hi}$ monocytes, evaluated by flow cytometry (**Figure 6E**), showed large increases between 24 and 48 h after acid in dummy dAb-treated animals (**Figures 6F,G**), consistent with previously published kinetics in this model of injury (30). However, there was no significant difference in cell recruitment with p55-targeting dAb, apart from a reduction in $Ly6C^{hi}$ monocytes at day 3.

DISCUSSION

Despite much research, and major advances in our understanding of the pathophysiology, effective pharmacological therapies for ARDS patients remain elusive. It is generally accepted that inflammation plays an important role in ARDS, and numerous drug targets have been identified within preclinical settings, but none of these have translated into clinical treatment. The reasons for this have been widely discussed, including the recent debate regarding the similarity of inflammatory responses between rodents and humans (31, 32), and the possible different responses of ARDS subphenotypes (33). Perhaps more to the point is that animal models of ARDS generally replicate a limited number of the features seen in patients (24, 25). Many models are either too severe/invasive to explore longer consequences (i.e., animals cannot be studied beyond the acute phase) or too mild to replicate the complex pathology. Thus, these models often have very limited predictive power, particularly when they are considered in isolation, in terms of the consequences of inhibiting pathways.

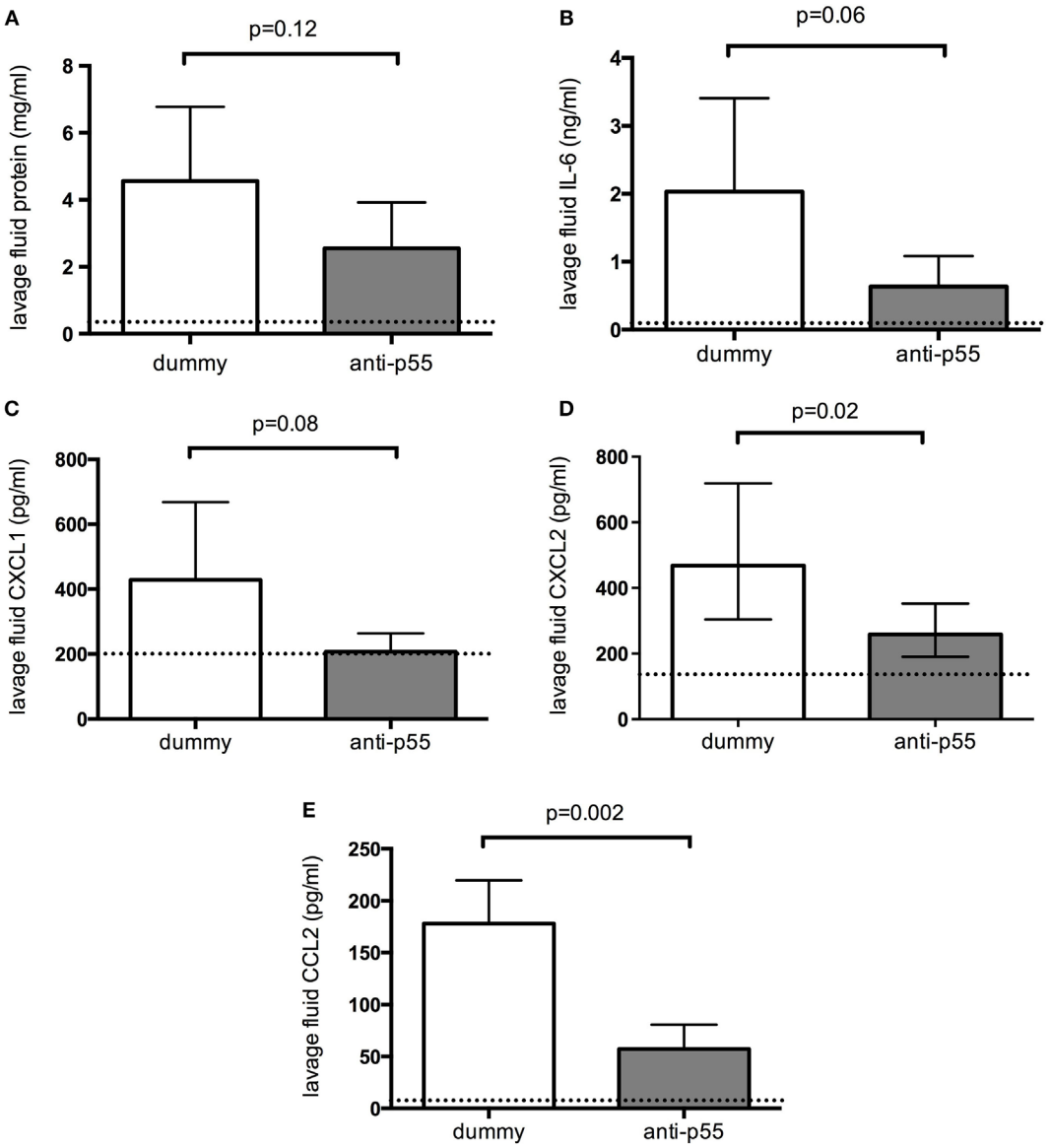

FIGURE 3 | Lavage fluid levels of total protein (A), interleukin 6 (B), CXCL1 (C), CXCL2 (D), and CCL2 (E) 180 min after acid instillation in animals treated with dummy or p55-targeting domain antibody. Dotted lines represent data from saline-treated animals for visual comparison. Lavage fluid CXCL2 (D) required log-transformation to normal distribution and is therefore displayed as geometric mean with error bars representing 90% confidence intervals. All other data were normally distributed and thus are displayed as arithmetic mean ± SD. *T*-tests were used to evaluate differences between treatments. $N = 5$ observations from independent experiments for each group (corresponding to the total number of mice assessed for these parameters).

For this reason, in the current study, we explored the impact of a domain antibody (dAb™) targeting the p55 TNFR, which we previously showed to be efficacious in attenuating VILI in mice (19), in more clinically relevant acute and resolving models of acid aspiration-induced lung injury.

In the acute ventilated model, the data demonstrate a very clear attenuation of injury in the animals receiving p55-targeting dAb. Specifically, respiratory mechanics and blood gases were significantly preserved. While pairwise analysis of individual time points indicated that only pO_2 and pCO_2 showed significant differences, and then only at the final time point, repeated measures ANOVA demonstrated clear interaction effects for elastance, pO_2 and pCO_2. Given that the repeated measures ANOVA both maximizes the data being utilized within any given analysis and decreases the chance of type II error by reducing within group variability, these data show that the development of "physiological injury" over time was attenuated by the administration of p55-targeting dAb. We did not evaluate lung histology in these animals, although respiratory system mechanics and blood gasses are clinically important parameters crucial for the diagnosis of ARDS, which are not always evaluated within animal models. We have previously evaluated histological changes

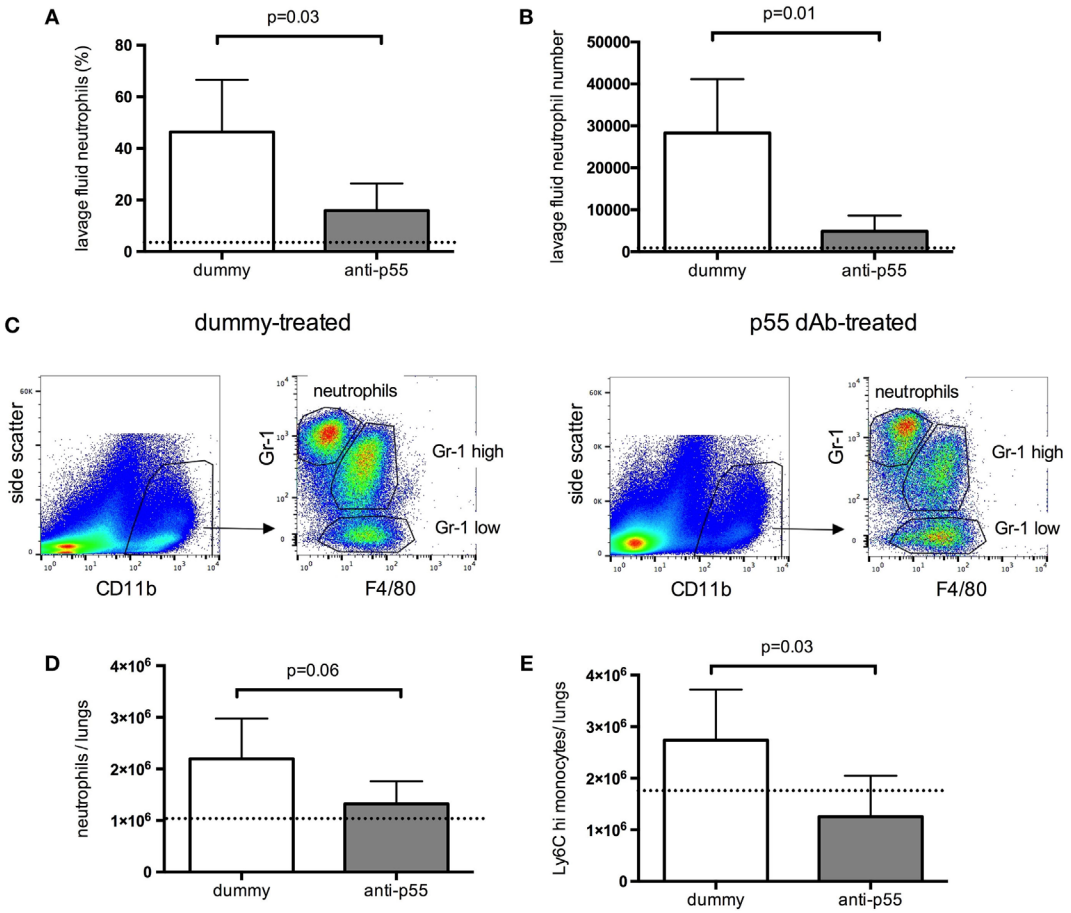

FIGURE 4 | Recruitment of leukocytes into the alveolar space (A,B), and lung tissue (C–E), 180 min after acid instillation in animals treated with dummy or p55-targeting domain antibody. For the acute ventilated model, tissue neutrophils were identified as CD11b^{high}, Gr-1^{high}, F4/80 negative events, while monocytes were identified as CD11b^{high}, F4/80 positive events, and differentiated by their expression of Gr-1. Under the non-fixed conditions used for these experiments, the Gr-1 antibody used (clone RB6-8C5) binds both Ly6G and Ly6C epitopes. Panel **(C)** shows side-by-side representative flow cytometric plots for lungs from dummy dAb and p55-targeting dAb-treated animals. Cell numbers were determined by use of fluorescent counting beads and expressed as total cell counts in both lungs **(D,E)**. Data are displayed as mean ± SD, with *t*-tests used to evaluate differences. *N* = 4–5 observations from independent experiments for each group (corresponding to the total number of mice assessed for these parameters).

within the acute and resolving acid-induced injury models (18, 21) and showed that it correlates well with these other markers. In addition to the changes observed, lung permeability showed a tendency toward protection. Overall, these data are consistent with our previous investigations into acid-induced lung injury in genetically modified animals (18), confirming the importance of the p55 TNFR pathway in this model of pulmonary injury and edema formation. Interestingly, in that previous study, we found that "classic" inflammatory mediators (IL-6, CXCL1, CCL2) and leukocyte recruitment were unaffected by the absence of p55 signaling (18), while here we found quite clearly that the acute intratracheal inhibition of p55 using the dAb led to significant attenuation of alveolar cytokine/chemokine levels, and reduced recruitment of neutrophils and inflammatory Ly6C^{hi} monocytes. The reasons for this apparent difference may relate to consequences of compartmentalized inhibition of p55 signaling versus whole body absence of p55, or the effects of chronic compensation of signaling pathways in genetically modified animals. A similar phenomenon is apparent when comparing genetic modification versus acute inhibition of p55 in models of VILI (17, 19) so although the underlying reasons are unclear, they are not model specific. An alternative explanation could be that the dAb has some additional "off-target" effects. We believe this is unlikely, as binding data demonstrated that dAb has high affinity binding to the p55 TNFR and no specific binding to the closely related p75 receptor. This could be clarified in future experiments by exploring the consequences of p55 dAb administration into p55 knockout mice.

A major aspect of the current study was to explore the influence of p55 inhibition beyond the very acute (3–4 h) phase; it may be dangerous to assume that any early consequences of p55 inhibition will continue to be beneficial into the later stages of disease progression, without understanding the knock-on effects of interfering with this pathway. For this reason, we

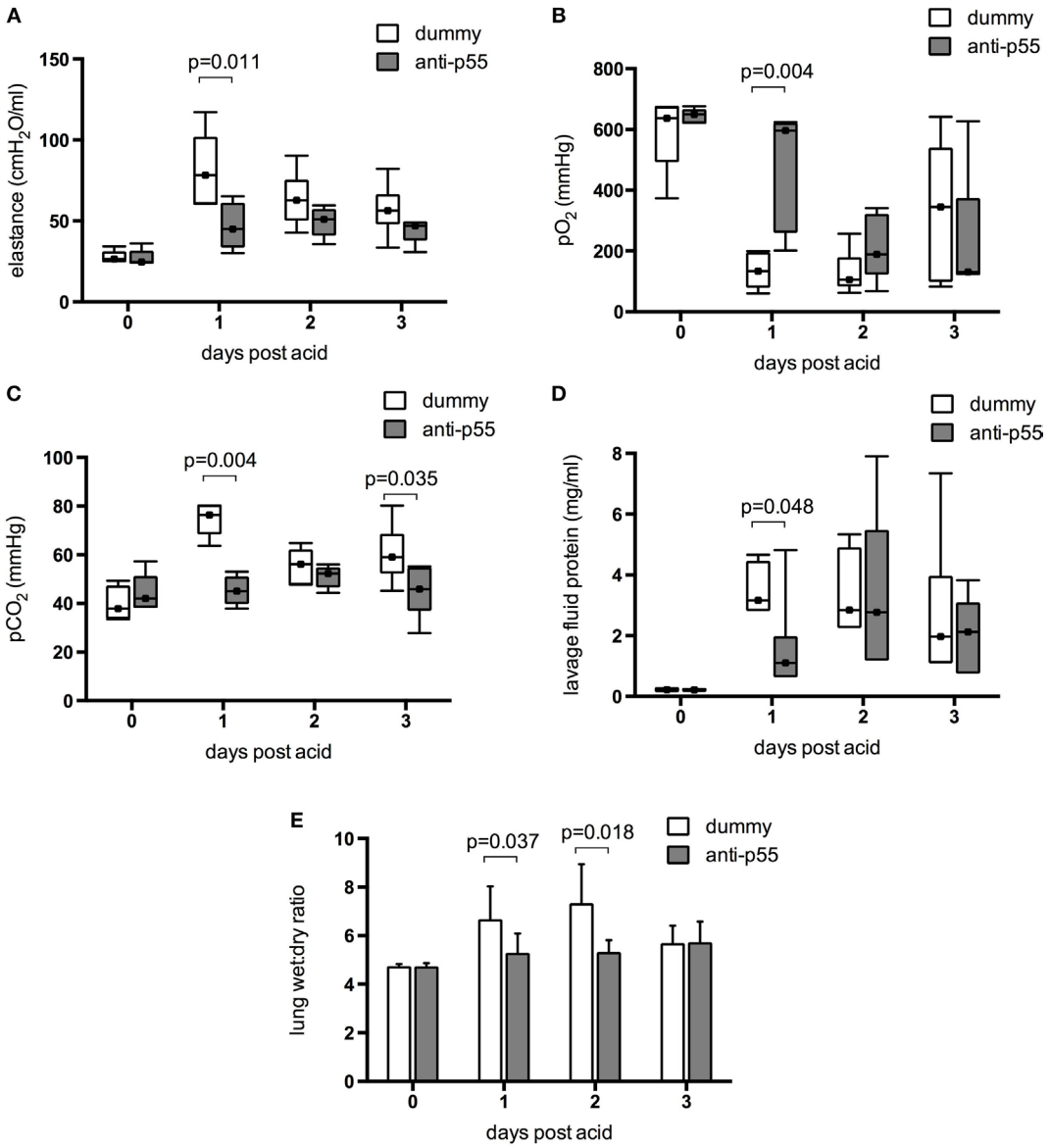

FIGURE 5 | Physiological indications of injury in the resolution model were determined in terms of elastance (A), arterial pO_2 (B), and pCO_2 (C) measured after 30 min ventilation with 100% O_2. Data were evaluated for differences due to treatment on each day. Data did not achieve normal distribution even with transformation, and thus are displayed as box-whisker plots and analyzed using Mann–Whitney *U*-test. Error bars extend to maximum and minimum values, while markers within boxes show the median. Physiological parameters of injury were significantly attenuated by p55-targeting dAb treatment at day 1, but this was mostly lost by day 2. $N = 5–8$ for elastance and 5–7 for blood gases at each time point. Permeability/edema was evaluated by lavage fluid protein **(D)** and wet:dry weight ratio **(E)**. Lavage fluid protein is displayed as box-whisker plots and was analyzed using Mann–Whitney *U*-test, while wet:dry weight is displayed as mean ± SD and was analyzed using Student's *t*-test. $N = 5–7$ for lavage protein and 5–8 for wet:dry ratio at each time point.

utilized a more chronic model of acid aspiration-induced injury developed within our research group (21). For the purposes of this study, we investigated the first 72 h after injury, a time frame that from our previous work encompasses the peak of injury and beginnings of a return toward homeostasis. In order to avoid frequent airway manipulation, we chose to use the intranasal route for delivery of domain antibody, followed by intratracheal acid instillation. Studies have estimated that the intranasal administration technique achieves approximately 50% delivery into the airspaces (34). This, combined with the fact that we were looking for consequences of domain antibody administration over a much longer time period, led us to increase the antibody dose from 25 μg in the acute model to 100 μg. Within these experiments, we found that markers of respiratory system mechanics, blood gases, and edema/permeability were significantly improved by the single pretreatment with p55-targeting dAb. However, for a number of these markers, most notably arterial pO_2 and lavage fluid protein, the injury induced by acid

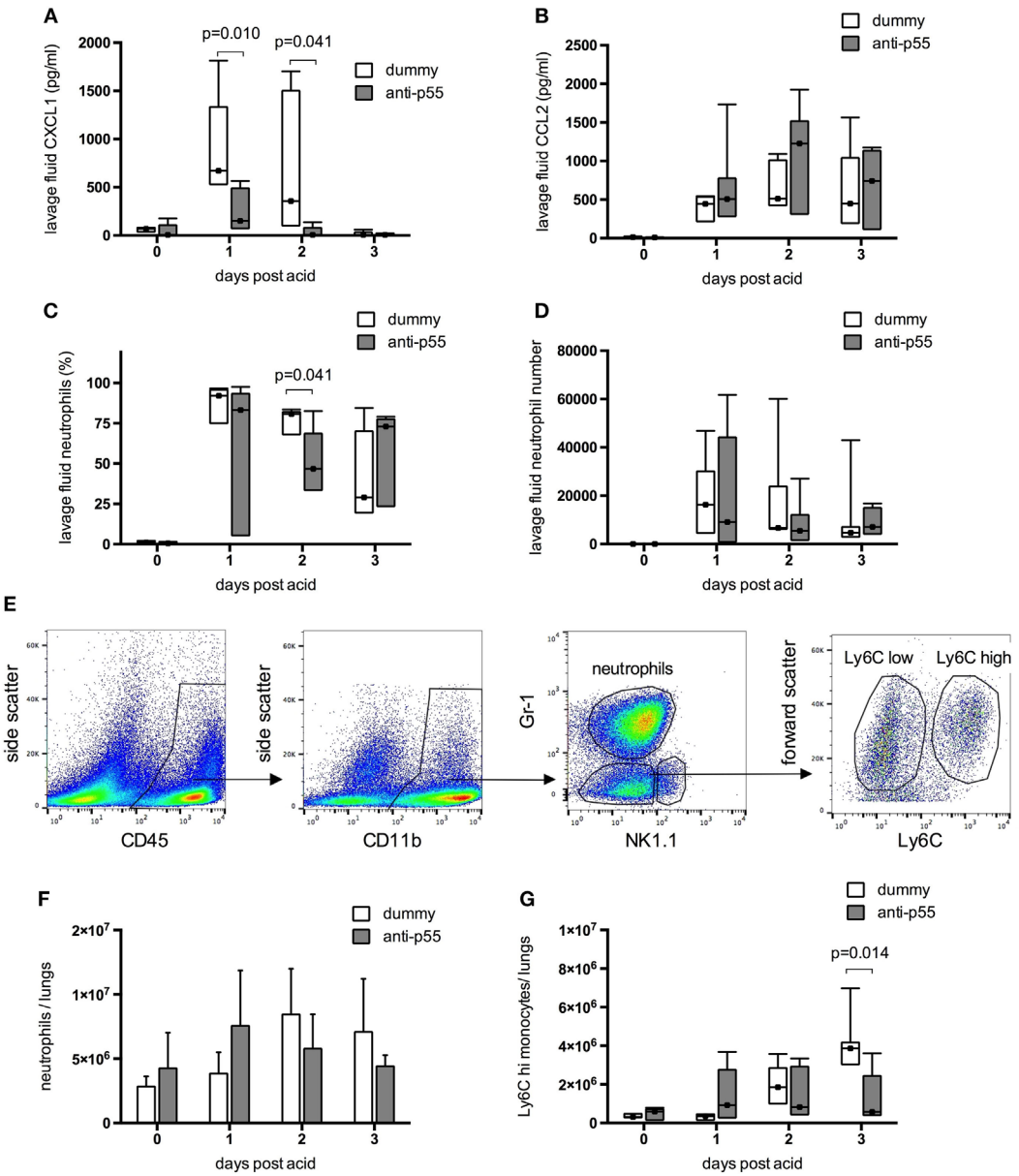

FIGURE 6 | Inflammation within the resolution model was evaluated in terms of lavage fluid cytokines and leukocyte recruitment. Data were evaluated for differences due to treatment on each day. Lavage fluid CXCL1 **(A)** and CCL2 **(B)** could not be normalized and are displayed as box-whisker plots with analysis by Mann–Whitney *U*-test ($N = 4–7$ at each time point). CXCL1 levels were significantly attenuated following pretreatment with p55-targeting dAb, while CCL2 was unaffected. Neutrophil infiltration into the alveolar space **(C,D)** was highly variable. Neutrophil percentage was reduced at day 2 by dAb treatment, although numbers recruited were not significantly attenuated. For the resolution model neutrophils, monocytes, and NK cells within lung tissue were identified as CD45 positive, $CD11b^{high}$ events using flow cytometry, and differentiated by their expression of Gr-1 and NK1.1 [representative plots from dummy-treated mouse at 24 h after acid shown in panel **(E)**]. NK cells were identified as NK1.1 positive events. We showed previously (28) that although the Gr-1 antibody used (clone RB6-8C5) binds both Ly6G and Ly6C epitopes under non-fixed conditions, the use of Cytofix/Cytoperm results in loss of Ly6C recognition by the antibody. Therefore, in the current context, Gr-1 staining is representative of cell Ly6G expression. Within the NK1.1 negative events, neutrophils were thus identified as Gr-1 (Ly6G) high events, while Gr-1 (Ly6G) low events were designated as monocytes. These were further subcategorized based on expression of Ly6C. Total lung tissue recruitment of neutrophils **(F)** and $Ly6C^{hi}$ monocytes **(G)** were not different following dummy or p55-targeting dAb treatment, apart from a reduction in $Ly6C^{hi}$ monocytes at day 3. Neutrophil numbers in lung tissue **(F)** are displayed as mean ± SD, while $Ly6C^{hi}$ monocyte numbers **(G)** and neutrophils in lavage fluid **(C,D)** could not be normalized and are displayed as box-whisker plots (evaluated using Mann–Whitney *U*-test). $N = 5–7$ for each time point.

was delayed rather than prevented. Thus, in general, most of the protective effects of a single dose of p55-targeting dAb treatment in terms of physiology were lost after 24 h.

p55-targeting dAb treatment also influenced inflammatory markers within the chronic model, although these were less pronounced than the physiological findings. Levels of CXCL1

and lavage fluid neutrophils (percentage of alveolar cells) were attenuated at days 1–2, but CCL2 levels were not. Interestingly, there was no clear difference in numbers of lung tissue leukocytes on day 1 (or 2) after acid, at which point physiological injury was clearly attenuated following p55-targeting dAb. Ly6C^{hi} monocyte recruitment was attenuated at day 3, and while these cells have been reported as being injurious in the acute phase of injury (27, 35, 36), the physiological relevance of the current finding is unclear. It is possible that this later phase of monocyte recruitment represents transmigration of a reparative subset (37), although this remains speculation.

Tumor necrosis factor-α, as a highly pleiotropic cytokine, plays a multitude of roles during the pathogenesis of ARDS, and these roles may themselves change during the progression of the syndrome from acute exudative to chronic resolution phases. The data from this study and our previous investigations (17–19) indicate that while TNF p55 signaling is involved in both physiological injury/pulmonary edema formation and lung leukocyte recruitment, there is a clear lack of correlation between these two processes. Other pathophysiological mechanisms mediated by TNF may be more important in determining alveolar epithelial barrier function and fluid balance during ARDS than its role in recruiting leukocytes, which may involve more redundant pathways. Specifically, TNF has a complicated involvement in clearance of pulmonary edema fluid (38). We have previously shown that inhibition of p55 signaling was able to prevent caspase-8 activation within epithelial cells and thus allow maintenance of barrier function and alveolar fluid clearance (18). Inhibition of TNF may also aid fluid clearance by reduction of downstream CXCL1 expression (seen in the current study), the human homolog of which (CXCL8) depresses fluid transport (39). However, TNF has also been shown in opposition to this, to promote clearance of water from the lung via direct activation of epithelial sodium channels (40), prevention of which could be highly damaging. We did not evaluate alveolar fluid clearance in this study, but there was no clear evidence that our treatment regime had adverse effects on physiological parameters, possibly because the fluid clearance promoting activity of TNF seemingly occurs mainly through a receptor-independent pathway (41). Within our acute experiments, the earliest detectable consequence of p55 inhibition occurred around 120 min after acid injury, at which point elastance started to diverge from dummy dAb-treated animals. Future study of such early time points may therefore yield important information regarding the links between TNF, leukocytes, and physiological injury.

In the current study, we chose to use a prophylactic strategy of dosing animals before induction of injury to identify the true potential of p55 inhibition using the dAb. Once their lungs are significantly injured by acid to the level comparable to clinical ARDS, mice do not tolerate anesthesia and intra-airway drug delivery (either intratracheal or intranasal) very well. For this reason, it was not possible to give repeated dosings of antibody, which may have enhanced or prolonged the protective effects observed. The domain antibody used within this study has been formulated specifically for airway administration and has a very short half-life in the circulation, so unfortunately systemic dosing to explore later consequences was also not possible here. Future work may include testing of a recently characterized p55-targeting dAb (DMS5540, GSK), which has been modified to provide an extended circulating half-life (42), although it remains unclear whether this would be able to penetrate into the alveolar space (or indeed whether this would be necessary). While it could be argued that prophylactic dosing in preclinical models of disease may not represent the most clinically relevant scenario, it is perhaps not so unreasonable in the case of ARDS. Recent studies report that up to 75% of patients suffering from ARDS acquired or developed it after entering hospital or during their ICU stay (43, 44). Identification of treatments that work safely when delivered prophylactically to "at risk" patients, or very early during the onset of disease, may therefore be a useful strategy to prevent progression to ARDS, although the same interventions should not be expected to be therapeutically efficacious in patients with established disease.

In conclusion, our data show clearly that targeting of the p55 TNFR with use of an intrapulmonary domain antibody attenuates many physiological indications of acid aspiration-induced lung injury, including respiratory mechanics, blood gases, and markers of edema/permeability. These data show the persistent importance of p55 TNF signaling in both the very acute and later phases of lung injury, and thus strongly support a role for p55 inhibition in patients with or at risk of lung injury, an approach that is currently being developed clinically. Although in the current study the beneficial effects were mainly lost after 24 h of injury, the protection up to this point was achieved with just a single intranasal dosing. Subsequent experiments will be necessary to determine the potential for modified dosing regimes to prolong the attenuation in injury observed.

ETHICS STATEMENT

All animal protocols were approved by the Ethical Review Board of Imperial College London and carried out under the authority of the UK Home Office in accordance with the Animals (Scientific Procedures) Act 1986, UK, and EU Directive 2010/63/EU.

AUTHOR CONTRIBUTIONS

MW, MT, PM, and AB were involved in the initial design of the study. MW, KW, SB, CO, BP, KO, and JC were involved in the acquisition and analysis of data. MW, KW, and MT prepared the manuscript. MW, KW, SB, CO, BP, KO, JC, PM, AB, and MT were involved in revising the manuscript and approving it for publication.

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REVIEW
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The Role of Transient Receptor Potential Channel 6 Channels in the Pulmonary Vasculature

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Canonical or classical transient receptor potential channel 6 (TRPC6) is a Ca^{2+}-permeable non-selective cation channel that is widely expressed in the heart, lung, and vascular tissues. The use of TRPC6-deficient ("knockout") mice has provided important insights into the role of TRPC6 in normal physiology and disease states of the pulmonary vasculature. Evidence indicates that TRPC6 is a key regulator of acute hypoxic pulmonary vasoconstriction. Moreover, several studies implicated TRPC6 in the pathogenesis of pulmonary hypertension. Furthermore, a unique genetic variation in the TRPC6 gene promoter has been identified, which might link the inflammatory response to the upregulation of TRPC6 expression and ultimate development of pulmonary vascular abnormalities in idiopathic pulmonary arterial hypertension. Additionally, TRPC6 is critically involved in the regulation of pulmonary vascular permeability and lung edema formation during endotoxin or ischemia/reperfusion-induced acute lung injury. In this review, we will summarize latest findings on the role of TRPC6 in the pulmonary vasculature.

Keywords: transient receptor potential channels, transient receptor potential channel 6, hypoxic pulmonary vasoconstriction, pulmonary hypertension, vascular permeability

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INTRODUCTION

Regulation of the intracellular Ca^{2+} ($[Ca^{2+}]_i$) homeostasis is a crucial factor in many physiological processes (1). Altered Ca^{2+} homeostasis in both vascular endothelium and smooth muscle has been documented for a majority of pathophysiological conditions in the pulmonary vasculature. Changes in $[Ca^{2+}]_i$ play a pivotal role in the regulation of contraction, migration, and proliferation of vascular smooth muscle cells (2). Furthermore, Ca^{2+} signaling in endothelial cells (ECs) is essential for the maintenance of the endothelial barrier integrity (3).

Non-selective cation channels (NSCCs) play an important role in the regulation of vascular tone and vascular smooth muscle cell proliferation by mediating the entry of cations (4). Among the ion channels located in the pulmonary vasculature, members of the canonical or classical transient receptor potential (TRPC) channels subfamily allow for the entry of Na^+ and Ca^{2+}. There is growing evidence that transient receptor potential channel 6 (TRPC6) mediates receptor-operated cation entry and is critically involved in numerous physiological processes. Recent studies have provided important insights into the role of TRPC6 in normal physiology and disease states of the pulmonary vasculature. We provide an overview on current knowledge regarding the role of TRPC6 channels in pulmonary vasculature and potential therapeutic strategies.

REGULATION OF CALCIUM HOMEOSTASIS

In general, Ca^{2+} enters cells from extracellular fluid through L-type voltage-dependent calcium channels or NSCCs, which can be divided into store-operated calcium channels (SOCCs) and receptor-operated calcium channels (ROCCs) (**Figure 1**). Stimulation of G-protein-coupled receptors initiates signaling mechanisms leading to activation of ROCC. These signaling pathways include phospholipase C (PLC) activation resulting in production of diacylglycerol (DAG) along with inositol 1,4,5-trisphosphate (IP_3) from phosphatidylinositol 4,5-bisphosphate (PIP_2). DAG regulates the activity of ROCC to induce receptor-operated Ca^{2+} entry, whereas IP_3 generation induces depletion of the intracellular Ca^{2+} stores in the endoplasmic reticulum, leading to induction of store-operated Ca^{2+} entry. Ca^{2+} entry through SOCCs plays a very important role in Ca^{2+} stores replenishment in the endoplasmic/sarcoplasmatic reticulum and maintaining Ca^{2+} homeostasis.

CLASSICAL TRANSIENT RECEPTOR POTENTIAL CHANNEL 6

Transient receptor potential (TRP) channels play a prominent role in the regulation of the cation homeostasis (5). TRP channels belong to a large and diverse family of mostly NSCCs. In this regard, they are non-selectively permeable to cations, including potassium (K^+), sodium (Na^+), calcium (Ca^{2+}), and magnesium (Mg^{2+}) (6). Based on amino acid sequence homology, the 28 mammalian TRP channels are grouped into six subfamilies, one of which is the TRPC (for classical or canonical) subfamily (6). The TRPC subfamily includes seven members, TRPC1 to TRPC7, and can be further divided into subfamilies on the basis of their structural and functional similarities. All TRPC proteins have a common structure. Mainly, they are composed of four N-terminal ankyrin repeats, six transmembrane domains with a putative pore between domains 5 and 6, and several protein-binding domains (4). TRPC proteins can form homomeric or heteromeric channels consisting of four monomers.

Transient receptor potential channel 6 is a NSCC, which is about six times more permeable for Ca^{2+} than for Na^+ (7). It belongs to the subfamily of ROCC and there is good evidence that TRPC6 is directly activated by DAG (8). TRPC6 is ubiquitously expressed in the whole vasculature (9). In the pulmonary circulation, TRPC6 is most prominent in pulmonary artery smooth muscle cells (PASMCs) and ECs (10). TRPC6 mRNA and protein were identified in PASMCs isolated from both proximal and distal pulmonary arteries (11–13). However, TRPC6 expression is higher in PASMCs isolated from distal pulmonary arteries than in those isolated from proximal vessels (14). Recently, expression of TRPC6 in pulmonary venous smooth muscle cells has also been demonstrated (15).

PULMONARY HYPERTENSION

Pulmonary hypertension (PH) is a pathophysiological disorder that may involve various clinical conditions and can complicate

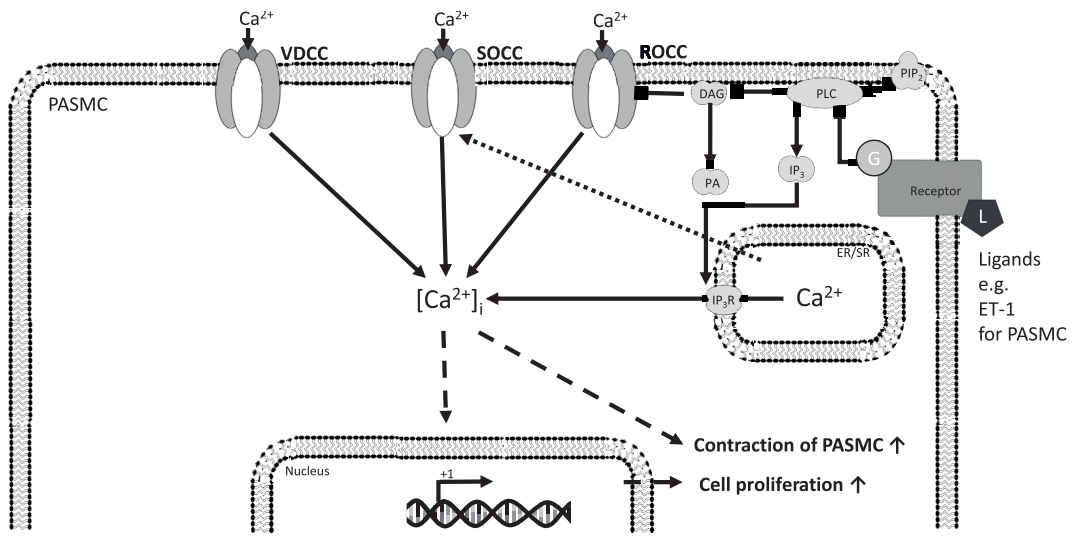

FIGURE 1 | $[Ca^{2+}]_i$ homeostasis regulation in precapillary pulmonary arterial smooth muscle cells (PASMCs) and ECs. Ca^{2+} enters cells from extracellular fluid through L-type voltage-dependent calcium channels or non-selective cation channels, which can be divided into SOCCs and ROCCs. The initiation of ROCC-mediated Ca^{2+}-influx from the extracellular space is thought to be induced by ligand-activated G-protein coupled receptors, starting a PLC-mediated hydrolyzation of PIP_2 to IP_3 and DAG. DAG regulates the activity of ROCC to induce receptor-operated Ca^{2+} entry, whereas IP_3 generation induces depletion of the intracellular Ca^{2+} stores in the endoplasmic reticulum, leading to induction of store-operated Ca^{2+} entry. The increased $[Ca^{2+}]_i$ drives different cellular responses. Ca^{2+}, calcium ion; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; ROCC, receptor-operated calcium channel; SOCC, store-operated calcium channel; VDCC, L-type voltage-dependent calcium channel; DAG, diacylglycerol; DAGK, DAG kinase; EC, endothelial cell; ER/SR, endoplasmic/sarcoplasmic reticulum; IP_3, inositol trisphosphate; IP_3R, inositol trisphosphate receptor; L, ligand; PA, phosphatidic acid; PASMC, precapillary pulmonary arterial smooth muscle cells; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; VEGF, vascular endothelial growth factor; solid arrows indicate direct interactions; dotted arrows illustrate indirect interactions.

cardiovascular and respiratory diseases (16). PH is characterized by remodeling of the pulmonary vessels, leading to a progressive increase in pulmonary vascular resistance (PVR), right ventricular failure, and premature death. PH is defined as a resting mean pulmonary artery pressure ≥25 mmHg (17). The disorder has been classified into five clinical groups based on their similarities in clinical presentation, pathophysiological mechanisms, and therapeutic options: pulmonary arterial hypertension (PAH) (Group 1); PH due to left heart disease (Group 2); PH due to chronic lung disease and/or hypoxia (Group 3); chronic thromboembolic PH (Group 4); and PH due to unclear and/or multifactorial mechanisms (Group 5) (18).

Depending on the pulmonary artery wedge pressure values, PH is divided into precapillary and postcapillary forms. Pulmonary artery wedge pressure provides an indirect estimate of left atrial pressure and its elevation >15 mmHg in patients with PH indicates presence of postcapillary PH due to left heart disease (Group 2) (17). Precapillary PH is defined by the presence of PH and a pulmonary artery wedge pressure ≤15 mmHg and includes the clinical groups 1, 3, 4, and 5 (17).

Pulmonary arterial hypertension is a progressive disease characterized by the presence of precapillary PH and a PVR > 3 Wood units in the absence of other causes of precapillary PH (17). It includes idiopathic PAH (IPAH), hereditary PAH, and PAH associated with diseases, drugs, and toxins (APAH) (18). Sustained pulmonary vasoconstriction, *in situ* thrombosis, and pathological pulmonary vascular remodeling due to excessive vascular cell growth leading to intimal narrowing and vascular occlusion are the main causes for the increased PVR and pulmonary arterial pressure in IPAH patients. In addition, pulmonary vascular remodeling with increased muscularization contributes to elevated PVR as well as hyperreactivity of pulmonary vessels to various vasoconstrictor agents. Neointimal and medial hypertrophy in small and medium-sized pulmonary arteries is a key aspect of pulmonary vascular remodeling in IPAH patients.

Role of TRPC6 in Hypoxic Pulmonary Vasoconstriction (HPV)

Acute HPV is an adaptive response of the pulmonary circulation to a local alveolar hypoxia, by which local lung perfusion is matched to ventilation resulting in optimization of ventilation–perfusion ratio and thus gas exchange (19, 20). This dynamic mechanism is also known as von Euler–Liljestrand mechanism (21) and can be found in fish, reptiles, birds, and mammals. Acute HPV occurs throughout the pulmonary vascular bed, including arterioles, capillaries, and veins, but is most pronounced in small pulmonary arterioles (22, 23). In isolated pulmonary arteries and isolated perfused lungs, the HPV response is typically biphasic (24–26). The first phase is characterized by a fast but mostly transient vasoconstrictor response that starts within seconds and reaches a maximum within minutes. The following second phase is characterized by a sustained pulmonary vasoconstriction. Acute HPV in local alveolar hypoxia is limited to the affected lung segments and is not accompanied by an increase in pulmonary artery pressure.

A rise of $[Ca^{2+}]_i$ in PASMCs is a key element in HPV (27, 28). We have demonstrated that TRPC6 plays an essential role in acute HPV (29). We have shown that the first acute phase of HPV (<20 min of hypoxic exposure) was completely abolished in isolated, ventilated, and buffer-perfused lungs from TRPC6-deficient mice. However, the vasoconstrictor response during the second sustained phase (60–160 min of hypoxic exposure) in TRPC6$^{-/-}$ mice was not significantly different from that in wild-type mice (29). During hypoxia, DAG is accumulated in PASMCs and leads to activation of TRPC6 (29). Accumulation of DAG can result from PLC activation or from ROS-mediated DAG kinase (DAGK) inhibition (30, 31). Along these lines, inhibition of DAG synthesis by the PLC inhibitor U73122 inhibited acute HPV in wild-type mouse lungs (32). Blocking DAG degradation to phosphatidic acid through DAGKs or activation of TRPC6 with a membrane-permeable DAG analog 1-oleoyl-2-acetyl-sn-glycerol (OAG) resulted in normoxic vasoconstriction in wild-type but not in TRPC6$^{-/-}$ mice (32). Recently, the cystic fibrosis transmembrane conductance regulator and sphingolipids have been demonstrated to regulate TRPC6 activity in HPV, as both translocate TRPC6 channels to the caveolae and activate the PLC–DAG–TRPC6 pathway (33). Cytochrome P-450 epoxygenase-derived epoxyeicosatrienoic acids also induced translocation of TRPC6 to the caveolae during acute hypoxia (34). Consistent with these data, 11,12-epoxyeicosatrienoic acids increased pulmonary artery pressure in a concentration-dependent manner and potentiated HPV in heterozygous but not in TRPC6-deficient lungs (34). As the constriction of the pulmonary vessels in response to the thromboxane mimetic U46619 is not altered in TRPC6$^{-/-}$ mice, TRPC6 channels appear to be a key regulator of acute HPV. These studies are summarized in **Figure 2**.

In PASMCs isolated from small precapillary arteries of TRPC6-deficient mice, cation influx and currents induced by severe hypoxia (1% O_2) were completely absent (29). The rise of $[Ca^{2+}]_i$ in response to hypoxia was not dependent on Ca^{2+} release from internal stores, because, in the absence of extracellular Ca^{2+}, no hypoxia-induced increases in $[Ca^{2+}]_i$ were detected (29). Interestingly, blocking voltage-gated Ca^{2+} channels almost completely inhibited acute HPV in isolated wild-type mouse lungs and Ca^{2+} influx in wild-type PASMCs (29), suggesting that Na^+ influx through TRPC6 channels leads to membrane depolarization and activation of voltage-gated L-type Ca^{2+} channels mediating the bulk of the Ca^{2+} influx and contraction of smooth muscle cells (35). Importantly, the lack of acute HPV in TRPC6 KO mice has profound physiological relevance, because partial occlusion of alveolar ventilation provoked severe hypoxemia in TRPC6$^{-/-}$ but not in wild-type mice (29). These data provide compelling evidence that different molecular mechanisms regulate pulmonary vascular responses to acute and sustained hypoxia. TRPC6 channels may thus represent a potential therapeutic target for the control of pulmonary hemodynamics and gas exchange in hypoxic conditions.

Role of TRPC6 in Experimental PH

A variety of animal models are currently used to study PH. These models have provided a plethora of scientific information and

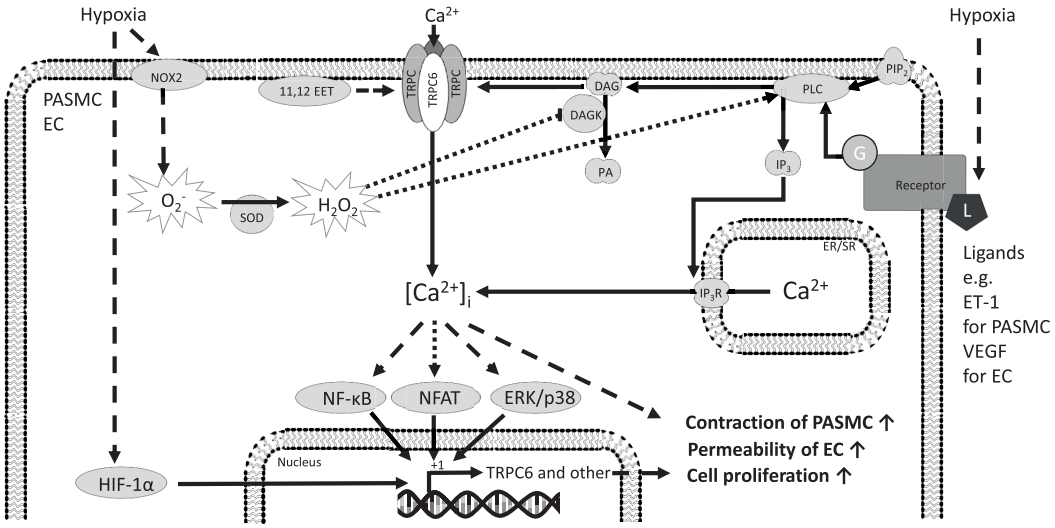

FIGURE 2 | Mechanisms of TRPC6 regulation and function in precapillary pulmonary arterial smooth muscle cells (PASMCs) and ECs in response to hypoxia. The TRPC6 protein forms homomeric and heteromeric channels composed of TRPC6 alone or TRPC6 and other TRPC proteins. TRPC6 is expressed in PASMCs from mice, rat, as well as humans and is suggested to play a significant role in human idiopathic PAH. The initiation of TRPC6-mediated Ca^{2+} influx from the extracellular space is thought to be induced by ligand-activated G-protein coupled receptors, starting a PLC-mediated hydrolyzation of PIP_2 to IP_3 and DAG. It has been already shown that DAG activates TRPC6-containing channels to induce Ca^{2+} influx from the extracellular space. Ca^{2+} entry through TRPC6 might be triggered by hypoxia-induced O_2^- production or hypoxia-induced DAG accumulation and that the increased $[Ca^{2+}]_i$ drives different cellular responses through ERK and p38, NFAT, and NF-κB downstream signaling. These pathways might be involved in the induction of TRPC6 expression and contribute to the modulated cellular response associated with hypoxia. Moreover, hypoxia leads to acute stabilization of HIF-1α, which might induce TRPC6 expression among other proteins. 11,12 EET, 11,12-epoxyeicosatrienoic acid; Ca^{2+}, calcium ion; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; DAG, diacylglycerol; DAGK, DAG kinase; EC, endothelial cell; ER/SR, endoplasmic/sarcoplasmic reticulum; ERK, extracellular signal-regulated kinase; ET-1, endothelin-1; G, G-protein; H_2O_2, hydrogen peroxide; HIF-1α, hypoxia-inducible factor 1 alpha; IP_3, inositol trisphosphate; IP_3R, inositol trisphosphate receptor; L, ligand; NF-κB, nuclear factor kappa-light-chain enhancer of activated B-cells; NFAT, nuclear factor of activated T-cells; NOX2, NADPH (nicotinamide adenine dinucleotide phosphate) oxidase 2; O_2^-, superoxide; PA, phosphatidic acid; p38, p38 mitogen-activated protein kinase; PASMC, precapillary pulmonary arterial smooth muscle cells; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; SOD, superoxide dismutase; TRPC, classical transient receptor potential channel; TRPC6, classical transient receptor potential channel 6; VEGF, vascular endothelial growth factor; solid arrows indicate direct interactions; dotted arrows illustrate indirect interactions. Not all interaction partners have been identified.

made significant contribution to our understanding of molecular mechanisms in PH. In animals, PH can be induced by pharmacologic/toxic substances, genetic manipulations, exposure to environmental factors, or surgical interventions (36).

Exposure to chronic hypoxia is the most commonly used animal model of PH in biomedical research. In global alveolar hypoxia, which occurs at high altitude and chronic respiratory diseases, HPV involves the entire pulmonary vascular bed leading to elevation of pulmonary artery pressure. Chronic global alveolar hypoxia induces structural remodeling of pulmonary vessels due to smooth muscle cell proliferation and migration characterized by increased muscularization of smaller arteries with extension of smooth muscle cells into previously non-muscularized arterioles (37). This vascular remodeling has previously been thought to be a major determinant of the persistent elevation of PVR in chronic hypoxia-induced PH (38–40). However, recent studies have provided evidence that sustained vasoconstriction is an important contributor to chronic hypoxia-induced PH (41).

Although TRPC6 is important in the acute phase of HPV in mouse lungs, the data regarding its role in chronic hypoxic PH are controversial. We have previously shown that, despite disrupted acute HPV, TRPC6-deficient mice display sustained HPV and chronic hypoxia-induced PH with pulmonary vascular remodeling and RV hypertrophy after 3 weeks of hypoxia (10% O_2), which are indistinguishable from those in wild-type mice (29). Slightly but significantly lower right ventricular systolic pressure was observed in TRPC6$^{-/-}$ mice exposed to 1 week of hypoxia when compared to wild-type mice (42). Nevertheless, this difference was not significant after 3 weeks of exposure to hypoxia (42). In contrast, other authors have demonstrated attenuation of PH and pulmonary vascular remodeling in TRPC6 KO mice after 4 weeks of hypoxia (43). Although the exact reason is not clear, differences in age (44, 45), gender (46), strain, and substrain (47, 48) of mice can account for most of the discrepancies.

Excessive proliferation of PASMCs is the main cause of pulmonary arterial medial hypertrophy, which narrows the intraluminal diameter, increases the resistance to blood flow, and eventually leads to PH. Proliferation of PASMCs is regulated by $[Ca^{2+}]_i$. There is increasing evidence that elevated TRPC6 expression might be responsible for the elevated $[Ca^{2+}]_i$. Interestingly, it has been shown that enhanced expression of TRPC6, STIM2, and Orai2 as proteins of the store-operated Ca^{2+} influx underlies the change of the phenotype of PASMCs from the contractile to the proliferative (49). Furthermore, deletion of TRPC6 significantly

attenuated Ca^{2+} currents in the proliferative phenotype of PASMCs (49).

Transient receptor potential channel 6 upregulation in PASMCs has been demonstrated to be dependent on hypoxia-inducible transcription factor 1 (HIF-1) (50). Overexpression of HIF-1 led to TRPC6 upregulation under normoxic conditions while partial deficiency in HIF-1 resulted in hypoxia-induced Ca^{2+} influx in PASMCs, suggesting an important role of HIF-1 for sustained expression of TRPC6 channels (50). Although short-term hypoxia (1% O_2 for 72 h) did not produce any changes in TRPC6 mRNA expression in isolated murine PASMCs (51), increased expression of TRPC6 on mRNA and protein level was detected in pulmonary arteries and PASMCs isolated from pulmonary arteries of mice and rats exposed to chronic hypoxia (42, 50, 52). Moreover, a Notch-dependent upregulation of TRPC6 channels in PASMCs in response to chronic hypoxia has recently been reported (43). It has been shown that TRPC6 is induced by BMP4 in rat PASMCs *via* the p38MAPK and ERK1/2 pathways (53, 54). Additionally, BMP4 may increase TRPC6 expression by elevating NOX4-mediated ROS levels in PASMCs (55). Interestingly, BMP4 expression has been shown to be dependent on HIF-1 as well (15).

Chronic lung diseases including chronic obstructive pulmonary disease (COPD) are often complicated by PH (56). Growing evidence implicates cigarette smoke (CS) products in the initiation of pulmonary vascular alterations in COPD (57). Recently, we have demonstrated the formation of PH in mice chronically exposed to tobacco smoke (58). Similarly, development of PH has been documented in rats chronically exposed to CS (59, 60). CS is an inflammatory stimulus, which upregulates Ca^{2+}-regulatory molecules. In this regard, TRPC6 was upregulated in rat lungs and isolated rat PASMCs after 4, 12, and 20 weeks of CS exposure (59). In another study, expression of TRPC1 and TRPC6 was increased in PASMCs isolated from distal pulmonary arteries of rats after 1, 3, and 6 months of CS exposure (60). Furthermore, PASMCs in rats exposed to CS for 3 and 6 months showed a higher basal $[Ca^{2+}]_i$ and an increased Ca^{2+} entry (60).

The role of TRPC6 in other models of PH has not been investigated in detail. Increased expression of TRPC6 protein in distal pulmonary arteries was observed in the monocrotaline-induced rat model of PH (61). Chronic thromboembolic PH in a rat model is associated with upregulation of TRPC1 and TRPC6 in PASMCs isolated from distal pulmonary arteries, elevated basal $[Ca^{2+}]_i$, and an increased Ca^{2+} entry (62).

There is growing evidence that in addition to TRPC6, other members of the TRPC family also contribute to the pulmonary vascular remodeling in PH. Culture of isolated PASMCs under hypoxic conditions led to upregulation of TRPC1 mRNA (50, 51, 63). Furthermore, enhanced expression of TRPC1 and TRPC4 mRNA and protein has been documented in pulmonary arteries and PASMCs isolated from mice and rats with PH induced by various stimuli (50, 59, 60, 62, 64, 65). Treatment of murine PASMCs with TRPC1-specific small interfering RNA resulted in significant attenuation of hypoxia-induced proliferation of cells (51). Consistent with this, PASMCs isolated from $TRPC1^{-/-}$ mice showed diminished proliferation under hypoxic conditions (51). Additionally, $TRPC1^{-/-}$ mice exposed to chronic hypoxia were protected from development of PH, which was associated with attenuated pulmonary vascular remodeling (51). In line with our data, reduced chronic hypoxic vascular remodeling in $TRPC1^{-/-}$ mice has been demonstrated by an independent research group (42). Moreover, downregulation of TRPC1 expression by small interfering RNA attenuated PH and pulmonary vascular remodeling in a murine model of hypoxia-induced PH (66). Interestingly, in mice deficient for both TRPC1 and TRPC6, chronic hypoxia-induced changes in pulmonary arterial pressure, right ventricular hypertrophy, and pulmonary vascular remodeling are even more inhibited compared to those in mice with a deficiency for a single gene (42). In a recent study, deficiency for TRPC4 has been shown to confer a survival benefit, which was associated with diminished vasculopathy in a rat model of severe PAH (67).

Involvement of TRPC6 in IPAH

Pulmonary arterial hypertension is characterized by progressive adverse structural changes in the resistance pulmonary arteries driven mainly by excessive vascular cell growth (68). Vascular remodeling in PAH is mediated by multiple stimuli. It is widely recognized that PASMCs in IPAH patients have a hyperproliferative phenotype and contribute to the pro-proliferative microenvironment in the vascular wall of their pulmonary arteries (68, 69). The enhanced $[Ca^{2+}]_i$ plays an key role in PASMC growth (70). Furthermore, increased $[Ca^{2+}]_i$ levels have been observed in PASMCs from IPAH patients (71). Expression studies revealed that c-jun/STAT3-induced upregulation of TRPC6 expression underlies PDGF-mediated proliferation of PASMCs (72). The mRNA and protein expression of TRPC6 in lung tissues and PASMCs from IPAH patients has been shown to be much higher than in those from normotensive patients (73). Furthermore, inhibition of TRPC6 gene expression by small interfering RNA significantly diminished proliferation of PASMCs from IPAH patients suggesting that the abnormally increased PASMC proliferation in these patients may be due to enhanced expression of TRPC6 (73).

Mounting evidence implicates inflammatory mechanisms in the development of PAH (74, 75). A unique genetic variant of the TRPC6 gene promoter has been identified, which might link inflammatory responses to the upregulation of TRPC6 expression and ultimate development of pulmonary vascular abnormality in IPAH (76). Sequencing TRPC6 regulatory regions of 268 patients with IPAH revealed three biallelic single-nucleotide polymorphisms (SNPs): −361(A>T), −254(C>G), and −218(C>T) (76). Among these three SNPs, only the −254(C>G) SNP was associated with IPAH by increasing basal TRPC6 gene promoter activity. Furthermore, the −254(C>G) SNP introduces a new binding site for the inflammatory transcription factor nuclear factor κB (NF-κB) in the promoter region of the TRPC6 gene and thus enhances NF-κB-mediated promoter activity and stimulates TRPC6 expression in PASMCs (76). In addition, this SNP has functional relevance as it also affects TRPC6 channel activity. In PASMCs from IPAH patients with the −254(C>G) SNP, TNF-α-induced activation of NF-κB significantly increased TRPC6 expression, elevated the resting $[Ca^{2+}]_i$, and enhanced OAG-induced Ca^{2+}

influx (76). In contrast, inhibition of nuclear translocation of NF-κB by overexpression of an IκBα super-repressor significantly diminished TNF-α-mediated enhancement of TRPC6 expression, resting $[Ca^{2+}]_i$, and agonist-induced elevation of $[Ca^{2+}]_i$. The importance of NF-κB has been demonstrated in experimental models of PAH (77–79). More importantly, activation of NF-κB has recently been observed in the pulmonary vessels of patients with end-stage IPAH (80). Thus, in the presence of inflammatory triggers, individuals carrying the −254(C>G) SNP may have an increased risk of developing IPAH (81). Although the functional significance of the two other SNPs, −361(A>T) and −218(C>T), is not clear, it has been shown that patients with IPAH and APAH carrying all three SNPs develop a more severe disease (82).

TRPC6 As a Therapeutic Target in PH

Transient receptor potential channel 6 is predominantly expressed in tissues harboring smooth muscle cells including the lungs (83, 84). However, $TRPC6^{-/-}$ mice do not have any major pathological phenotypes probably because TRPC6 channels have little basal activity and modest importance under physiological conditions (85). Moreover, loss of TRPC6 is compensated by the activity of closely related TRPC3 channels in the systemic vasculature (86) and airway smooth muscle (87). Nevertheless, TRPC6 channels are specifically activated in various disease conditions suggesting their pathophysiological relevance and thus represent attractive therapeutic targets. Importantly, systemic application of TRPC inhibitors in mice was not associated with any serious side effects (88, 89).

A number of non-selective small molecule inhibitors of TRPC6 channel activities including 2-APB and SKF-96365 have become available during recent years (85, 90). Also, antagonists including synthetic gestagen norgestimate and compound 8009-5364 with IC50 values in a low micromolar range and with higher selectivity for TRPC6 have been identified (91, 92). As the members of the TRPC3/6/7 subfamily have very similar biochemical and biophysical properties, most of the TRPC6-selective blockers exhibit poor selectivity between the subfamily members (85, 90). A continuous search for selectively acting pharmacological TRPC6 has recently identified new highly potent TRPC6 inhibitors with subtype selectivity, SAR7334, and larixyl acetate (93, 94). Most importantly, these drugs effectively blocked acute HPV in isolated mouse lungs (92–94). However, the only inhibitor that has been tested in experimental PH is the non-specific TRPC blocker 2-APB, which prevented development of PH in mice exposed to chronic hypoxia (43).

Evidence supporting the role of TRPC6 in the pathogenesis of IPAH suggests that it might serve as a pharmacologic target. Although the selective TRPC6 inhibitors represent promising drug candidates for the treatment of PH, they have not yet been tested in experimental models of PH. It would be highly desirable to confirm the therapeutic efficacy and safety of the new potent and selective TRPC6 blockers in animal models of PH with the ultimate goal of development of new therapeutic strategies for patients with PH.

Recent studies suggest that specific drugs approved for PAH treatments can also target TRPC6 expression and activity. In a small number of PAH patients with a positive response to acute vasodilator testing, initial therapy includes high doses of calcium channel blockers. However, most of the PAH patients do not react to calcium channel blockers, and they are treated with drugs approved for PAH therapy. Currently, established clinical practice treatments of PAH target three signaling pathways that are involved in the pathogenesis of PH: endothelin, nitric oxide, and prostacyclin (95). These therapies include endothelin receptor antagonists, phosphodiesterase type 5 inhibitors, soluble guanylate cyclase stimulators, prostacyclin receptor agonists, and epoprostenol. Bosentan has been found to directly downregulate TRPC6 expression in addition to its well-known blockade of endothelin receptors (96).

In PASMCs from chronically hypoxic rats, the potent phosphodiesterase type 5 inhibitor sildenafil decreased acutely basal $[Ca^{2+}]_i$ (97). Chronic treatment of rats exposed to 10% O_2 for 21 days with sildenafil showed a decreased right ventricular pressure and right ventricular hypertrophy, which is related to decreased TRPC6 mRNA and protein expression in pulmonary arteries (63). Furthermore, knockdown of TRPC6 gene by small interference RNA diminished the hypoxic increases of basal $[Ca^{2+}]_i$ and Ca^{2+} influx in PASMCs exposed to hypoxia for 60 h (63). It has been shown that inhibition of the Ca^{2+}/NFAT pathway is involved in the antiproliferative effect of sildenafil on PASMCs (98). More recent studies have revealed that sildenafil inhibits hypoxia-induced TRPC6 protein expression in PASMCs *via* the cGMP–PKG–PPARγ axis (99).

TRPC6 IN ACUTE LUNG INJURY (ALI)

Acute lung injury is characterized by lung edema due to increased lung vascular permeability of the alveolar-capillary barrier and subsequent impairment of arterial oxygenation. Ca^{2+} homeostasis has been shown to be essential in the mechanism of barrier disruption and endothelial contraction (3). Elevated $[Ca^{2+}]_i$ leads to changes in EC morphology and increased endothelial permeability. Recent studies have shown that Ca^{2+} entry through TRPC6 is essential for increased endothelial permeability and compromised barrier function in pulmonary vasculature (100).

In ALI, lung vascular barrier disruption usually coincides with the invasion of immune cells and activation of inflammatory signaling pathways (101). Various mediators, including platelet-activating factor (PAF), vascular endothelial growth factor (VEGF), thrombin, tumor necrosis factor-α (TNF-α), and others, induce changes in EC shape and consequently an increase in endothelial permeability (3, 102). PAF, a critical mediator in numerous experimental models of ALI, has been shown to increase lung vascular permeability by activation of acid sphingomyelinase (ASM) (103). In an extension of that study, the authors provided evidence that ASM activation by PAF causes rapid recruitment of TRPC6 channels into caveolae of lung ECs, thus facilitating endothelial Ca^{2+} entry and subsequent increases in endothelial permeability (104). Translocation of the TRPC6 to caveolin-rich areas in the plasma membrane in response to bradykinin has also been shown to be facilitated by 11,12-epoxyeicosatrienoic acids (105). TRPC6 has also been implicated in the VEGF-mediated increase in $[Ca^{2+}]_i$ and subsequent downstream signaling in microvascular ECs (106–108). In human

pulmonary ECs, interaction of a protein called phosphatase and tensin homolog with TRPC6 enables cell surface expression of the channel in ECs and OAG-induced Ca^{2+} entry through TRPC6 as well as a subsequent increase in monolayer permeability (109). Thrombin-mediated Ca^{2+} entry through TRPC6 in human pulmonary artery ECs activated RhoA in a protein kinase C-α-dependent manner and thereby induced EC shape change and an increase in endothelial permeability (100).

A novel function for TRPC6 in pulmonary ECs in ALI induced by the endotoxin lipopolysaccharide (LPS) has also been indentified (110). In that study, LPS induced generation of DAG by binding to toll-like receptor 4 (TLR4), and DAG in turn directly activated TRPC6 and increased Ca^{2+} entry in ECs resulting in enhanced lung vascular permeability. Most interestingly, TRPC6 signaling was also important for the LPS/TLR4-mediated NF-κB activation and lung inflammation (110).

Lung edema and endothelial injury are accompanied by an influx of neutrophils into the interstitium and alveolar space (111). Therefore, activation and recruitment of polymorphonuclear neutrophils are thought to play key roles in the progression of ALI. When neutrophils are recruited to inflamed tissue, they become migratory and traverse the walls of blood vessels. It is known that the stimulation of CXC-type Gq-protein-coupled chemokine receptors activates PLC and induces a sustained increase in $[Ca^{2+}]_i$ (112). An important role of TRPC6 signaling was demonstrated in CXCR2-induced intermediary chemotaxis (113). A deficiency for TRPC6 in neutrophil granulocytes negatively affects macrophage inflammatory protein-2 and OAG-induced cell migration (114). It has also been shown that TRPC6 expressed in ECs promotes leukocyte transendothelial migration by mediating trafficking of the lateral border recycling compartment membrane (115).

Recently, we have investigated the role of TRPC6 in lung ischemia-reperfusion edema (LIRE) formation in mice (31). Remarkably, global $TRPC6^{-/-}$ mice were fully protected from LIRE, whereas global TRPC1- and TRPC4-deficient mice showed no protection. Bone marrow transplantation experiments using TRPC6 KO and wild-type mice allowed us to exclude the involvement of TRPC6 in immune cells. In line with our *in vivo* findings, pulmonary ECs isolated from TRPC6 KO mice displayed reduced permeability in response to hypoxia. A detailed analysis of signaling pathways underlying TRPC6 activation showed that mice lacking NOX2, but not NOX1 and NOX4, were also protected from LIRE. Moreover, mice deficient for NOX2 specifically in pulmonary arterial ECs displayed protection from LIRE. Consistent with our *in vivo* findings, we observed enhanced O_2^- production by endothelial NOX2 during the ischemic (hypoxic) phase. We have shown that after extracellular conversion to hydrogen peroxide (H_2O_2), H_2O_2 penetrates into the cell, where it inhibits DAGK $\eta_{1/2}$ activity and activates PLCγ, resulting in DAG accumulation and activation of TRPC6. Furthermore, elevation in $[Ca^{2+}]_i$ was diminished in ECs lacking either NOX2 or TRPC6, indicating that NOX2 influences TRPC6-dependent Ca^{2+} homeostasis. Our studies provided a unique mechanistic insight into the pathogenesis of LIRE involving production of superoxide by endothelial Nox2, activation of PLCγ, inhibition of DAGK, and DAG-mediated activation of TRPC6 (31). These studies are summarized in **Figure 3**.

CONCLUDING REMARKS

In summary, TRPC6 channels are involved in various physiological and pathophysiological processes in the pulmonary vasculature. There is a clear evidence for the importance of

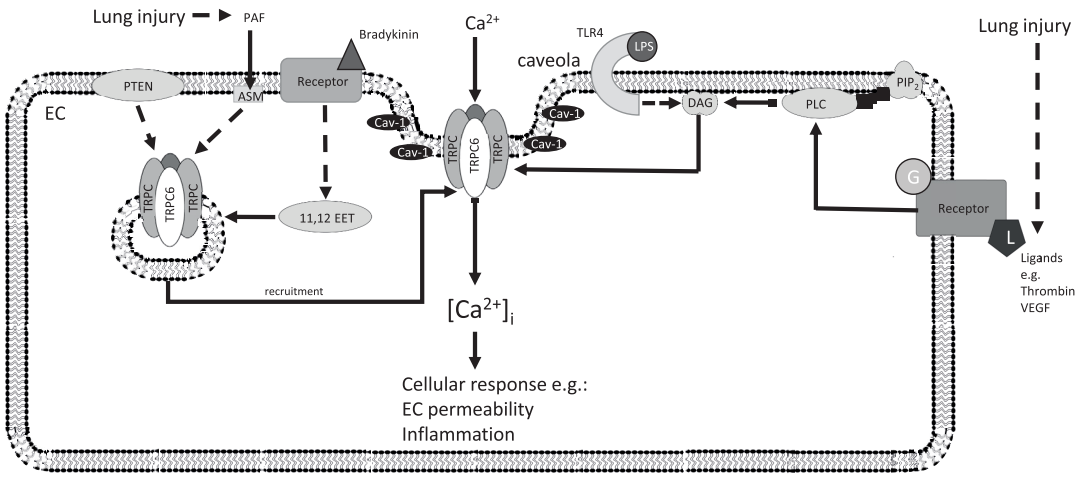

FIGURE 3 | Additional TRPC6 signaling pathways in ECs after lung injury. Recruitment of TRPC6 by the indicated factors increases the density of TRPC6 channels at the plasma membrane (left), which open after activation of endothelial receptors (right) and increase endothelial permeability and inflammatory processes inducing endothelial dysfunction. 11,12 EET, 11,12-epoxyeicosatrienoic acid; ASM, acid sphingomyelinase; Ca^{2+}, calcium ion; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; Cav-1, caveolin-1; DAG, diacylglycerol; EC, endothelial cell; G, G-protein; HIF-1α, hypoxia-inducible factor 1 alpha; L, ligand; LPS, lipopolysaccharide; PAF, platelet-activating factor; PTEN, phosphatase and tensin homolog; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; TLR4, toll-like receptor 4; TRPC, classical transient receptor potential channel; TRPC6, classical transient receptor potential channel 6; VEGF, vascular endothelial growth factor; solid arrows indicate direct interactions; dotted arrows illustrate indirect interactions. Not all interaction partners have been identified.

TRPC6 in the mechanism of acute HPV. Although the role of TRPC6 in chronic hypoxia-induced PH is controversial, there is evolving evidence for an important function of TRPC6 in pulmonary vascular remodeling in IPAH and endothelial barrier disruption in ALI. Therefore, TRPC6 is a promising target for pharmacological interventions. In physiological processes like acute HPV, TRPC6 activators may be useful to redirect blood flow from non-ventilated regions to oxygen-rich regions of the lungs to avoid life-threatening arterial hypoxemia. In pathophysiological processes like excessive vascular remodeling, PH, or enhanced endothelial permeability, inhibitors of TRPC6 channels might represent a valuable approach. Thus, specific drugs designed to target TRPC6 channels have to be identified as a prerequisite to develop new therapeutic strategies in diseases coupled to physiological and pathological functions of TRPC6 channels.

AUTHOR CONTRIBUTIONS

MM, AE, CV, and AS drafted the manuscript. MM, AE, CV, HG, RS, TG, AD, NW, and AS revised the manuscript critically for important intellectual content and approved the final version of the manuscript submitted.

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Inhibition of the NOD-Like Receptor Protein 3 Inflammasome Is Protective in Juvenile Influenza A Virus Infection

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Influenza A virus (IAV) is a significant cause of life-threatening lower respiratory tract infections in children. Antiviral therapy is the mainstay of treatment, but its effectiveness in this age group has been questioned. In addition, damage inflicted on the lungs by the immune response to the virus may be as important to the development of severe lung injury during IAV infection as the cytotoxic effects of the virus itself. A crucial step in the immune response to IAV is activation of the NOD-like receptor protein 3 (NLRP3) inflammasome and the subsequent secretion of the inflammatory cytokines, interleukin-1β (IL-1β), and interleukin-18 (IL-18). The IAV matrix 2 proton channel (M2) has been shown to be an important activator of the NLRP3 inflammasome during IAV infection. We sought to interrupt this ion channel-mediated activation of the NLRP3 inflammasome through inhibition of NLRP3 or the cytokine downstream from its activation, IL-1β. Using our juvenile mouse model of IAV infection, we show that inhibition of the NLRP3 inflammasome with the small molecule inhibitor, MCC950, beginning 3 days after infection with IAV, improves survival in juvenile mice. Treatment with MCC950 reduces NLRP3 levels in lung homogenates, decreases IL-18 secretion into the alveolar space, and inhibits NLRP3 inflammasome activation in alveolar macrophages. Importantly, inhibition of the NLRP3 inflammasome with MCC950 does not impair viral clearance. In contrast, inhibition of IL-1β signaling with the IL-1 receptor antagonist, anakinra, is insufficient to protect juvenile mice from IAV. Our findings suggest that targeting the NLRP3 inflammasome in juvenile IAV infection may improve disease outcomes in this age group.

Keywords: children, influenza, inflammasome, inflammation, MCC950, acute lung injury

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INTRODUCTION

Influenza A virus (IAV) is a significant respiratory pathogen in the pediatric age group. Despite widespread vaccination efforts, ~80 per 100,000 children in the United States are hospitalized each year with seasonal IAV (1), and up to 24% of hospitalizations require intensive care unit admission for life-threatening disease (2). Underlying medical conditions increase the risk of severe IAV infection, but a considerable amount of morbidity and mortality occurs in healthy children. The effectiveness of antiviral drugs, which target IAV proteins, is hindered by the need to administer

them early in the course of infection and the increasing resistance of seasonal IAV to these compounds (3). Consequently, therapy for children with severe IAV infection largely consists of supportive care. Hence, there is an urgent need to develop new therapeutic strategies to reduce the fatal pathology observed in children hospitalized with severe IAV infection.

The host immune response to IAV plays an important role in reducing morbidity and mortality as well as promoting viral clearance. However, IAV infections in pediatric patients can be associated with aberrant or dysregulated cytokine and cellular inflammatory responses [reviewed in Ref. (4)]. Among the potentially injurious cytokines produced during IAV infection are interleukin-1β (IL-1β) and interleukin-18 (IL-18), which are secreted following activation of the NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasome. The NLRP3 inflammasome is tightly regulated, requiring two signals for activation. Signal 1 occurs through pathogen detection by pattern recognition receptors that act through the transcription factor, NF-κB, to increase the expression of pro-IL-1β, as well as inflammasome components, including NLRP3 and pro-caspase-1. A second signal is then required for NRLP3 inflammasome complex assembly and activation. Several IAV-specific products have been identified as potent Signal 2 activators, including IAV viral RNA and IAV matrix 2 (M2) protein (5–7). The IAV M2 protein, a proton channel involved in viral replication (8), has been shown to activate the NLRP3 inflammasome in an IAV strain-independent manner (7). In bone marrow derived macrophages primed with lipopolysaccharide, which activates Signal 1, lentivirus expression of the M2 protein from a number of seasonal and pandemic strains of IAV resulted in IL-1β secretion. The IAV M2 protein activates the inflammasome by promoting proton efflux following M2 localization to the acidified Golgi apparatus. Inhibition of M2 protein function *via* the introduction of M2 mutants, or the use of amantadine or rimantadine, which block movement of protons through the M2 channel, has also been shown to inhibit IL-1β maturation and secretion (7). Unfortunately, the development of resistance to these medications has diminished their efficacy in the treatment of seasonal IAV, and they are no longer considered standard of care in the United States.

The role of NLRP3 inflammasome in IAV infection was first explored in mice deficient in its three components, NLRP3, caspase-1, or ASC. Decreased survival was consistently seen in mice lacking caspase-1 or ASC when challenged with IAV (9–11). However, the role of the NLRP3 protein itself appeared to be dependent on the inoculating dose of IAV, as NLRP3 deficiency did not impact mortality when a low dose of IAV was used (11), but did lead to worse survival after infection with higher doses (9, 10). The authors reasoned that the increased mortality seen in mice deficient in NLRP3 inflammasome components was due to impaired viral clearance, as viral titers remained elevated late in infection (9, 11). Conversely, more recent studies have demonstrated that excessive NLRP3 inflammasome activity can contribute to IAV-induced lung injury and death (6, 12). Therefore, NLRP3 inflammasome activity must be carefully controlled to achieve IAV clearance without causing unnecessary damage to surrounding tissues. This pathway may be of particular importance in the pathogenesis of severe IAV infection in children (4, 13). Therefore, using our mouse model of pediatric IAV infection, which has been shown to mimic human disease, we investigated how modulation of the inflammatory response might change outcomes in life-threatening IAV infection. Using a small molecule inhibitor of the NLRP3 inflammasome (MCC950) and an antagonist of the receptor for IL-1β (anakinra), we found that inhibition of the NLRP3 inflammasome could ameliorate life-threatening IAV infection in juvenile mice, but inhibition of IL-1β signaling alone could not.

MATERIALS AND METHODS

Animals

129S wild-type mice were provided by Jackson Laboratories and bred in house. Mice were provided with food and water *ad libitum*, maintained on a 14 h light, 10 h dark cycle, and handled according to the National Institutes of Health guidelines. All procedures complied with federal guidelines and were approved by The Institutional Animal Care and Use Committee at Northwestern University.

Virus

Influenza virus strain A/WSN/1933 (WSN) was grown for 48 h at 37.5°C and 50% humidity in the allantoic cavities of 10- to 11-day-old fertile chicken eggs. Viral titers were measured by plaque assay in Madin–Darby canine kidney (MDCK) epithelial cells. Virus aliquots were stored in liquid nitrogen, and freeze/thaw cycles were avoided.

In Vitro Influenza Virus Infection of THP-1 Cells

THP-1 cells were plated in 6-well plates at a density of 0.5×10^6 per well. They were differentiated with phorbol myristate acetate (5 nM) for 48 h and cultured in complete RPMI medium for 72 h. Cells were infected with IAV WSN at a multiplicity of infection (MOI) of 1, 2, or 3 for 2 h. Cells were then washed with phosphate-buffered saline (PBS) and cultured in complete RPMI medium for 24 h. The cell-free supernatant was collected for ELISA. For drug-therapy experiments, differentiated THP-1 cells were treated with MCC950 (1 μM, Adipogen), anakinra (0.5 μg/mL, Kineret™), or vehicle control (PBS) for 1 h. Immediately after the drug treatment, cells were infected with IAV WSN (MOI 2) for 2 h. The infected cells were washed with PBS and cultured in complete RPMI medium containing MCC950 (1 μM), anakinra (0.5 μg/mL), or vehicle control for 24 h. The cell-free supernatant was collected for ELISA. ELISA was done for IL-1β (eBioscience, San Diego, CA, USA) and Caspase-1 (R&D Systems, Minneapolis, MN, USA).

Cell Imaging

Human THP-1 monocytes were plated on sterilized 18CIR-1 coverglasses in a 12-well plate at a density of 0.25–0.5 million cells per well and differentiated. Cells were then treated with an MOI of 2 of IAV for 24 h and probed for active caspase-1 by means of FAM-YVAD-FMK (FAM-FLICA caspase-1 assay kit #97, ImmunoChemistry, Bloomington, MN, USA) according to the

manufacturer's instructions. Nuclei were labeled with Hoechst 33342, and then cells were fixed in 2.7% paraformaldehyde for 5 min at room temperature. Images were acquired by means of a Nikon A1R laser scanning confocal microscope.

In Vivo Influenza Virus Infection

Juvenile (4-week-old) mice were anesthetized with isoflurane and infected intratracheally with WSN [12.5 plaque forming units (PFU) in 50 µL PBS] or an equal volume of PBS.

Inflammasome Inhibition *In Vivo*

MCC950 (Adipogen) reconstituted in sterile PBS was administered intraperitoneally in juvenile mice at a dose of 10 mg/kg daily beginning on day 3 postinfection (p.i.) until tissue harvest, death, or recovery. Anakinra (Kineret™) was administered intraperitoneally in juvenile mice at a dose of 100 mg/kg on day 3 p.i. until tissue harvest, death, or recovery.

Bronchoalveolar Lavage Fluid (BALF) Harvest

A 20-gauge angiocatheter was ligated into the trachea, and the lungs were lavaged twice with sterile PBS (700 µL). The lavage fluid was centrifuged at 1,000 *g* for 10 min. The pellet was resuspended, and the cells were counted using the Invitrogen Countess Automated Cell Counter (Invitrogen, Grand Island, NY, USA). Protein levels in the supernatant were measured by Bradford Assay (BioRad), and cytokine levels were measured using ELISA. Interleukin (IL)-18 was measured using the mouse IL-18 ELISA Kit (MBL International Corporation, Woburn, MA, USA) according to the manufacturer's instructions. Interleukin-6 (IL-6) was measured using the mouse IL-6 Ready-Set-GO ELISA Kits (eBioscience, San Diego, CA, USA). Interferon (IFN)-α was measured using the mouse IFN Alpha ELISA Kit (PBL Assay Science, Piscataway, NJ, USA).

Wet-to-Dry Weight Ratios

Mice were anesthetized and lungs were surgically removed *en bloc*. Lungs were weighed in a tared container. The lungs were then dried at 45°C in a Speed-Vac SC100 evaporator (Thermo Scientific, Waltham, MA, USA) until a constant weight was obtained, and the wet-to-dry weight ratio was calculated.

Histology

Mice were anesthetized and lungs were perfused *via* the right ventricle with 10 mL HBSS with calcium and magnesium. A 22-gauge angiocatheter was sutured into the trachea, heart and lungs were removed en bloc, and then lungs were inflated with 0.7 mL of 4% paraformaldehyde at a pressure not exceeding 16 cm H_2O. Tissue was fixed in 4% paraformaldehyde overnight at 4°C, then processed, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). Images were acquired by means of a TissueGnostics automated slide imaging system (TissueGnostics, Vienna, Austria).

Lung Harvest and Homogenization

For plaque assay, lungs were homogenized in PBS (20 µL/mg lung). For western blot, lungs were homogenized in RIPA buffer with protease inhibitor (20 mM Tris–HCl, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, Roche complete ULTRA Tablet). Homogenized lungs were centrifuged at 1,000 *g*. The supernatant was frozen at 80°C.

Western Blot

The presence of indicated proteins in lung homogenates from day 7 p.i. was assessed by western blotting using the following antibodies: NLRP3 (Adipogen), Caspase-1 (14F468) (Santa Cruz sc-56036), ASC (Adipogen), IL-18 (Biovision, 5180R-10), and Actin (Santa Cruz).

Flow Cytometry for Intracellular Staining of NLRP3 Inflammasome Components

Mice were anesthetized and lungs were perfused *via* the right ventricle with 10 mL HBSS with Ca^{2+} and Mg^{2+}. The lung lobes were removed and inflated with enzyme solution (5 mL of 0.2 mg/mL DNase I and 2 mg/mL Collagenase D in HBSS with Ca^{2+} and Mg^{2+}) using a 30G needle. The tissue was minced and then processed in GentleMACS dissociator (Miltenyi) according to the manufacturer's instructions. Processed lungs were passed through a 40 µm cell strainer, and red blood cells were lysed with BD Pharm Lyse (BD Biosciences, San Jose, CA, USA). Remaining cells were counted with a Countess Cell Counter (Invitrogen, Grand Island, NY, USA). CD45 microbeads were added, and cells were eluted according to the Miltenyi manufacturer's instructions. Cells were stained with viability dye Aqua (Invitrogen) and stained with a mixture of fluorochrome-conjugated antibodies (see **Table 1** for lists of fluorochromes, antibodies, manufacturers, and clones). Data were acquired on a BD LSR II flow cytometer using BD FACSDiva software (BD Biosciences), and data analyses were performed with FlowJo software (TreeStar, Ashland, OR, USA). Cell populations were identified using sequential gating strategy, and the percentage of cells in the live/singlets gate was multiplied by the number of live cells to obtain an absolute live-cell count. The expression of activation markers is presented as median fluorescence intensity (MFI).

TABLE 1 | Fluorochrome-conjugated antibodies used for flow cytometry.

Fluorochrome	Antibody	Manufacturer	Clone
FITC	CD45	eBioscience	30-F11
PerCPCy5.5	MHCII	BioLegend	M5/114.15.2
eFluor450	CD11b	eBioscience	M1/70
Alexa700	Ly6G	BD Pharmingen	1A8
APCCy7	Ly6C	eBioscience	HK1.4
PE	CD64	BioLegend	X54-5/7.1
PECF594	Siglec F	BD Horizon	E50-2440
PECy7	CD11c	BD Pharmingen	HL3

Additional antibodies used for intracellular staining:

Fluorochrome	Antibody	Manufacturer
APC	mNLRP3/NALP3	R&D
Biotin	Caspase-1	NOVUS
APC	Streptavidin	eBioscience

Plaque Assay

Confluent monolayers of MDCK cells were infected with stock virus or lung homogenate serially diluted in 1% bovine serum albumin Dulbecco's Modified Eagle Medium (DMEM) for 2 h at 37°C. Plates were washed with PBS and an overlay of 50% 2× Replacement Media (2× DMEM, 0.12 M $NaHCO_3$, 2% Penn–Strep, and 1% HEPES), 50% avecil (2.35%), and *N*-acetyl trypsin (1.5 μg/mL) remained on the cells for 72 h at 37°C. Overlay was removed, and the monolayers were then stained with Naphthalene Blue-Black and plaques counted.

Statistical Analysis

Data are expressed as means ± SD. Differences between two groups were assessed by using a Student's *t*-test. Differences between three or more groups were assessed using one-way analysis of variance with a Bonferroni multiple comparisons test. Values of $P < 0.05$ were considered to be significant. The log rank test was used in the analysis of the Kaplan–Meier curve. All analyses were performed using GraphPad Prism software version 6.0 for Windows (GraphPad Software, San Diego, CA, USA).

RESULTS

MCC950 and Anakinra Decrease NLRP3 Inflammasome Activity *In Vitro* in Macrophages Infected with IAV

MCC950 is a small molecule inhibitor of the NLRP3 inflammasome. Although its exact mechanism of action is unknown, it has been shown to be specific to NLRP3 and to prevent the activation of caspase-1 and the maturation and secretion of IL-1β and IL-18 in response to multiple NLRP3 inflammasome stimuli (14–16). Anakinra is a synthetic version of the naturally occurring IL-1β receptor antagonist. It prevents the downstream signaling of the IL-1β receptor. We tested the ability of MCC950 and anakinra to inhibit the NLRP3 inflammasome *in vitro*.

To determine the IAV inoculation dose necessary for NLRP3 inflammasome activation, we infected THP-1 cells (a human monocyte cell line derived from a 1-year-old patient) with IAV at an MOI of 1, 2, and 3 for 24 h. As shown in **Figures 1A,B**, there was a dose dependent increase in caspase-1 and IL-1β levels in the supernatant from IAV-infected macrophage cells. Based on these results we chose to test the ability of MCC950 and anakinra to inhibit NLRP3 inflammasome activation in response IAV at an MOI of 2.

THP-1 cells were pretreated with MCC950 (1 μM) or anakinra (0.5 μg/mL) and then infected with IAV (A/WSN/2009) at an MOI of 2. As shown in **Figures 1C,D**, cells infected with IAV had a robust increase in caspase-1 and IL-1β in the supernatant. In contrast, when the cells were treated with the NLRP3 inhibitor, MCC950, or the IL-1β receptor antagonist, anakinra, detection of caspase-1 and IL-1β during IAV infection was greatly reduced. Caspase-1 activation was also assessed using a specific fluorescent probe, FAM-YVAD-FMK (17). Cells infected with IAV showed robust caspase-1 activation following treatment with IAV, with caspase-1 forming aggregates throughout the cytoplasm. However, caspase-1 activation was severely reduced in cells treated with MCC950 prior to IAV infection. No caspase-1 activation was observed in uninfected THP-1 cells (**Figure 1E**).

MCC950 Improves Survival of Juvenile Mice Infected with IAV

The induction of IL-1β by IAV has been shown to be NLRP3 inflammasome dependent (8–10). We assessed the ability of MCC950 to prevent NLRP3 inflammasome activation and alter the young host's inflammatory response to IAV infection. Juvenile mice were infected with IAV [A/WSN/2009 12.5 PFU intratracheal (i.t.)] to achieve infection of the lower respiratory tract. Starting on day 3 p.i., we administered MCC950 [10 mg/kg intraperitoneal (i.p.), once daily (q.d.)] or an equal volume of vehicle control (i.p., q.d.) until recovery or death. We observed that the median survival of IAV-infected, vehicle-treated juvenile mice was day 11 p.i, with only 18% of PBS-treated mice surviving infection (**Figure 2A**). In contrast, 75% of IAV-infected, MCC950-treated juvenile mice were alive on day 11 p.i. IAV infection is typically associated with significant weight loss, which was observed in both IAV-infected, PBS-treated and IAV-infected, MCC950-treated mice. Importantly, the majority of the IAV-infected, MCC950-treated mice began to regain weight between days 8 and 9 p.i. (**Figure 2B**). Surviving IAV-infected, MCC950-treated juvenile mice exhibited coat ruffling, febrile shaking, and mild lethargy, but the majority of animals recovered. At 7 days p.i., indices of lung injury were not different between IAV-infected, PBS-treated and IAV-infected, MCC950-treated mice. Both groups displayed elevated levels of cellular infiltration (**Figure 2C**) and protein leakage (**Figure 2D**) into the BALF, and both groups had increased wet-to-dry weight ratios (**Figure 2E**). In accordance with this, histological examination of IAV-infected, PBS-treated and IAV-infected, MCC950-treated mice on day 7 p.i. demonstrated a similar degree of lung injury at this time point (**Figures 2F,G**).

MCC950 Inhibits the NLRP3 Inflammasome in Juvenile Lungs

We next sought to compare NLRP3 inflammasome activation in IAV-infected, PBS-treated and IAV-infected, MCC950-treated juvenile mice. IL-18 was elevated in the BALF from IAV-infected, PBS-treated mice, but was significantly attenuated in IAV-infected, MCC950-treated mice (**Figure 3A**). Importantly, the IAV-induced increase in NLRP3 protein expression observed in PBS-treated mice was absent in MCC950-treated mice (**Figure 3B**). Additional western blot analysis of inflammasome components in lung homogenates showed a similar increase in ASC in response to IAV infection in both PBS-treated and MCC950-treated mice (**Figure 3C**). Mature caspase-1 was increased in the BALF from IAV-infected, PBS-treated mice, compared to uninfected controls (**Figure 3D**). In contrast, caspase-1 secretion was inhibited in IAV-infected, MCC950-treated mice (**Figure 3D**). IL-6 and tumor necrosis-α (TNF-α), which are inflammatory cytokines that are not dependent on NLRP3 inflammasome activation, were not different between the two treatment groups (**Figures 3E,F**).

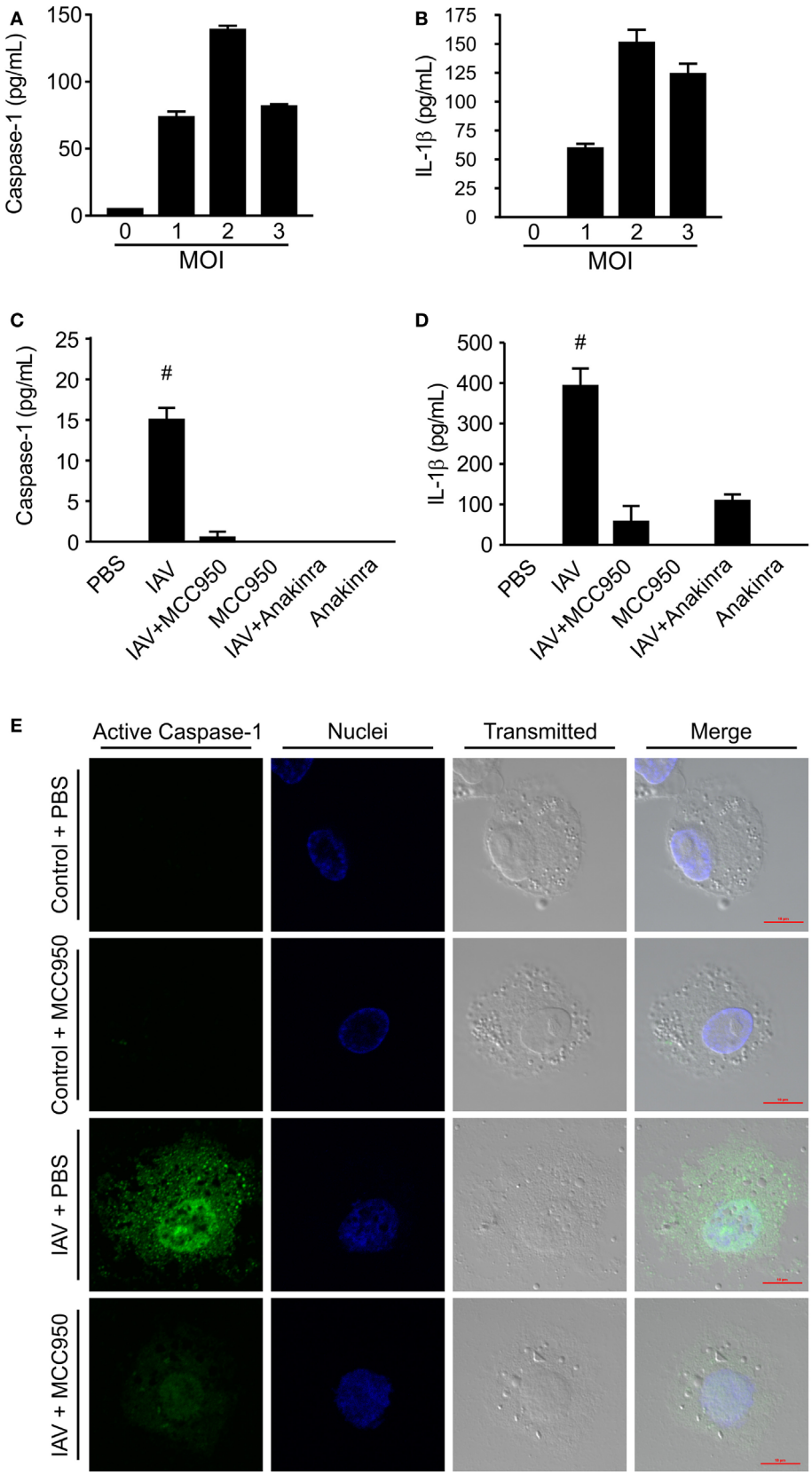

FIGURE 1 | Continued

FIGURE 1 | Continued
MCC950 and anakinra inhibit influenza A virus (IAV)-induced NOD-like receptor protein 3 inflammasome activation in THP-1 macrophages. Differentiated human THP-1 macrophages were infected with IAV (WSN) at a multiplicity of infection (MOI) of 1, 2, or 3 for 2 h. Infected cells were cultured for 24 h, and supernatant was evaluated by ELISA for **(A)** caspase-1 or **(B)** interleukin-1β (IL-1β). Differentiated human THP-1 macrophages were treated with MCC950, anakinra, or vehicle control. Cells were either infected with IAV (WSN, MOI 2) for 2 h or sham infected and treated with drug therapy alone. Cells were then washed and cultured in media containing MCC950, anakinra, or vehicle control. Supernatant was collected 24 h after IAV infection and evaluated by ELISA for **(C)** caspase-1 or **(D)** IL-1β. # indicates significant elevation over all other conditions. **(E)** MCC590-treated or vehicle-treated cells were fixed 24 h following IAV infection (WSN MOI 2) and fluorescently labeled to show active caspase-1 (green) and nuclei (blue). Scale bars, 10 μm.

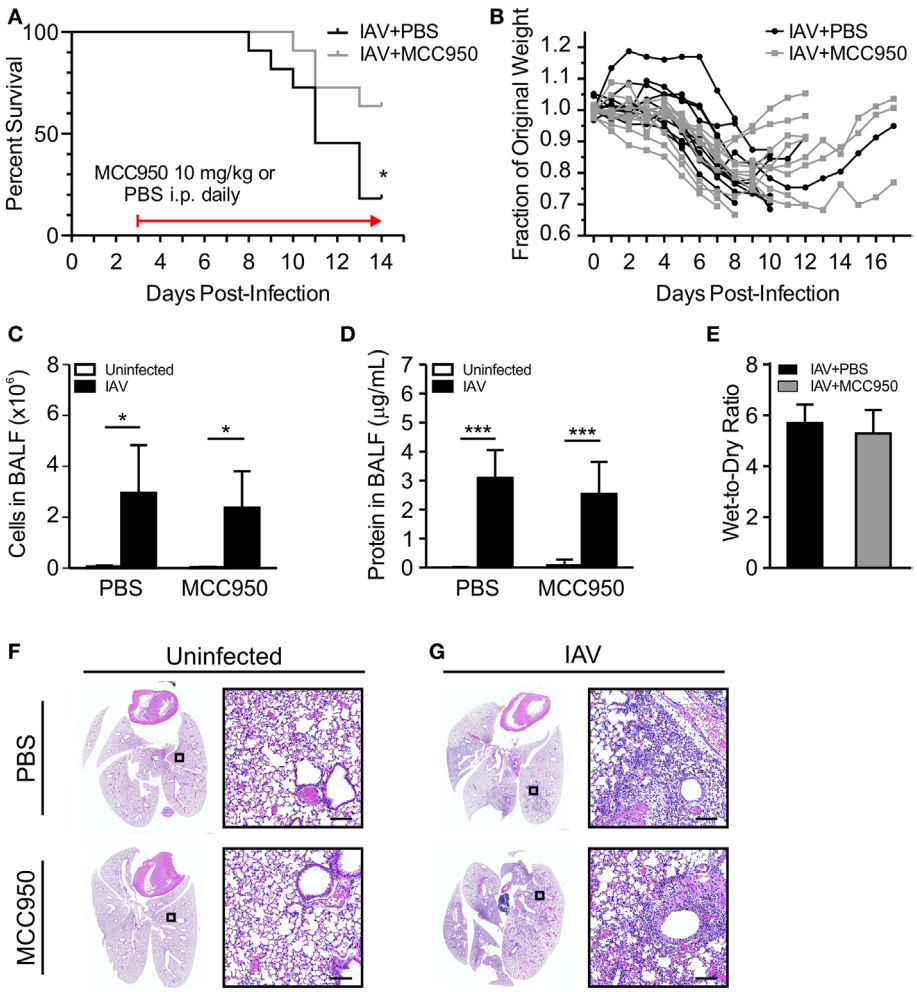

FIGURE 2 | MCC950 improves survival in juvenile mice infected with influenza A virus (IAV). Juvenile mice were infected with IAV [WSN 12.5 plaque forming units (PFU) intratracheal] and treated with MCC950 [10 mg/kg intraperitoneal (i.p.) daily] or phosphate-buffered saline (PBS) control beginning on day 3 postinfection (p.i.). **(A)** Mortality. **(B)** Weight loss. Bronchoalveolar lavage fluid (BALF) or whole lungs were collected from IAV-infected, MCC950-treated mice and IAV-infected, PBS-treated mice 7 days p.i. **(C)** Total number of cells in BALF. **(D)** Protein in BALF. **(E)** Wet-to-dry weight ratio. *$p < 0.05$, ***$p < 0.001$. **(F,G)** Hematoxylin and eosin stained lung sections from juvenile mice 7 days p.i. with 12.5 PFU of IAV and treatment with 10 mg/kg MCC950 or PBS control. Images shown are representative of three mice for each condition. Scale bars, 100 μm.

MCC950 Does Not Prevent Monocyte Recruitment to the Lungs but Does Inhibit NLRP3 Inflammasome Activation in Alveolar Macrophages

Macrophages are a main source of NLRP3 inflammasome activation during IAV infection (18). To investigate the impact of MCC950 treatment on NLRP3 inflammasome activation in these cells, we isolated alveolar macrophages (CD45+, CD64+, CD11c+, Siglec F+) and monocyte-derived cells (CD45+, CD11b+, Ly6C+, CD64+) from the lungs of IAV-infected, PBS-treated and IAV-infected, MCC950-treated mice using 10-color flow cytometry (19). In addition, we assessed the expression levels of NLRP3, caspase-1, and IL-1β with intracellular staining.

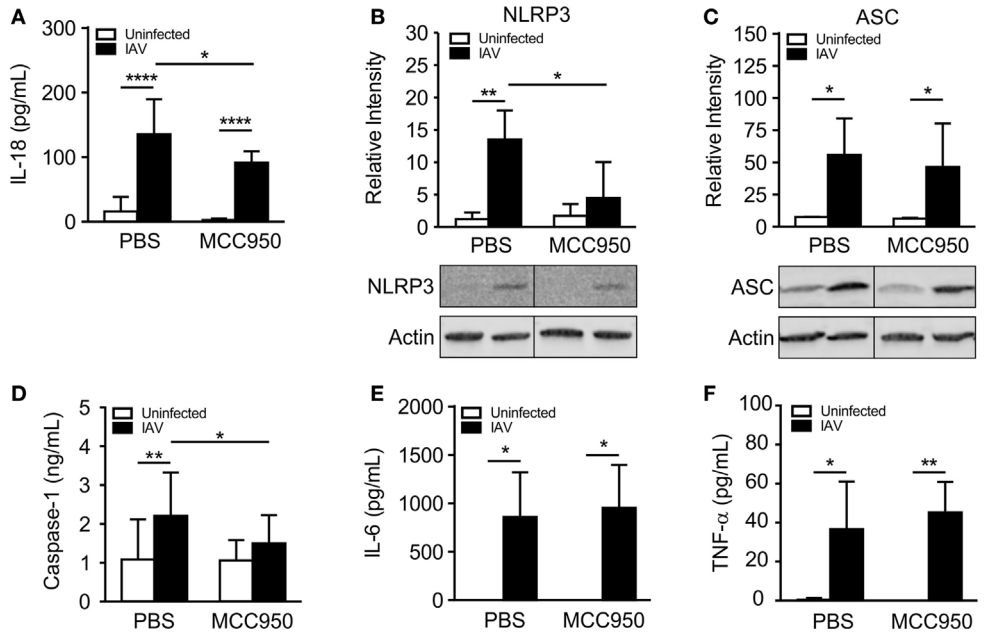

FIGURE 3 | MCC950 treatment decreases NOD-like receptor protein 3 (NLRP3) inflammasome activation in juvenile influenza A virus (IAV) infection. Juvenile mice were infected with IAV (WSN 12.5 plaque forming unit intratracheal) and treated with MCC950 (10 mg/kg intraperitoneal daily) or phosphate-buffered saline (PBS) control beginning on day 3 postinfection (p.i.). Bronchoalveolar lavage fluid (BALF) or whole lungs were collected from IAV-infected, MCC950-treated mice and IAV-infected, PBS-treated mice 7 days p.i. **(A)** Interleukin-18 (IL-18) in BALF as measured by ELISA. **(B,C)** NLRP3 and ASC in lung homogenates as measured by Western blot. **(D)** Caspase-1 in BALF as measured by ELISA. **(E)** Interleukin-6 (IL-6) and **(F)** tumor necrosis-α (TNF-α) in BALF as measured by ELISA. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$.

IAV infection caused an influx of monocyte-derived cells into the lungs. When analyzing all CD45+ cells, a similar number of alveolar macrophages (**Figure 4A**) and monocyte-derived cells (data not shown) were found in both treatment groups on day 7 p.i. To determine if MCC950 inhibited the expression of NLRP3 in these cells, we examined the median fluorescence intensity (MFI) of the inflammasome components, NLRP3 and caspase-1, and the product of its activation, IL-1β. All components of the NLRP3 inflammasome measured were significantly elevated in the alveolar macrophages of IAV-infected mice compared to uninfected controls (data not shown and **Figures 4B–D**). IAV-infected, MCC950-treated mice had significantly decreased levels of NLRP3 and IL-1β in alveolar macrophages (**Figures 4C,D**). These results are consistent with our finding that IAV-infected, MCC950-treated mice had decreased levels of NLRP3 in homogenized lungs as measured by Western blot, and IL-18 in BALF as measured by ELISA (see **Figure 3**).

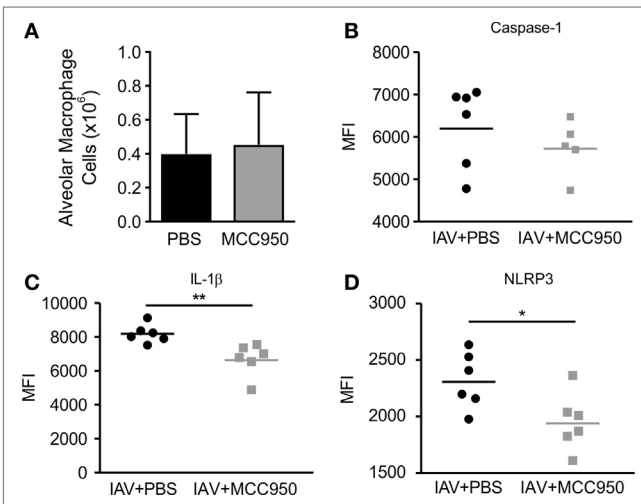

FIGURE 4 | MCC950 treatment decreases NOD-like receptor protein 3 (NLRP3) inflammasome activation in alveolar macrophages in juvenile mice infected with influenza A virus (IAV). Juvenile mice were infected with IAV (WSN 12.5 plaque forming unit intratracheal) and treated with MCC950 (10 mg/kg intraperitoneal daily) or phosphate-buffered saline (PBS) control beginning on day 3 postinfection (p.i.). Lungs were harvested 7 days p.i. and evaluated by flow cytometry for NLRP3 inflammasome activation using intracellular staining. **(A)** Number of alveolar macrophages in lung homogenates. **(B–D)** Median fluorescence intensity (MFI) of caspase-1, interleukin-1β (IL-1β), and NLRP3 in alveolar macrophages. $^{*}p < 0.05$, $^{**}p < 0.01$.

MCC950 Does Not Impact Type I Interferon Production or Viral Clearance in Juvenile IAV Infection

Interferon-α (IFN-α) is a type I interferon secreted in response to viral infection to control viral replication and prevent propagation of the infection to neighboring cells. Mice infected with IAV, as well as mice treated with MCC950, had an increase in IFN-α levels on day 7 p.i. (**Figure 5A**). Consistent with this, viral

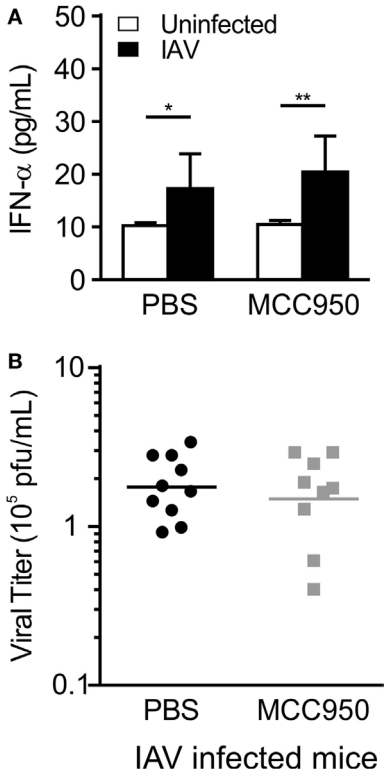

FIGURE 5 | MCC950 treatment does not impair viral clearance. Juvenile mice were infected with influenza A virus (IAV) (WSN 12.5 plaque forming unit intratracheal) and treated with MCC950 (10 mg/kg intraperitoneal daily) or phosphate-buffered saline (PBS) control beginning on day 3 postinfection (p.i.). Bronchoalveolar lavage fluid (BALF) or whole lungs were collected from IAV-infected, MCC950-treated mice and IAV-infected, PBS-treated mice 7 days p.i. **(A)** Interferon-α (IFN-α) in BALF as measured by ELISA. **(B)** Viral titer in lung homogenates was measured by plaque assay. $^{*}p < 0.05$, $^{**}p < 0.01$.

titers in the lung homogenates of IAV-infected, PBS-treated and IAV-infected, MCC950-treated mice as measured by plaque assay were equal (**Figure 5B**).

Anakinra Does Not Protect Juvenile Mice from IAV Infection

NOD-like receptor protein 3-dependent production of IL-1β and IL-18 may have downstream consequences with regard to IAV-induced inflammation and disease. IL-1β and IL-18 bind their cell-surface receptors (IL-1R and IL-18R, respectively) expressed on a range of cell types to induce potent NF-κB-dependent secondary cytokine production (20, 21). Importantly, lack of IL-1R resulted in reduced lung immunopathology following H1N1 infection, suggesting that IL-1R signaling may increase damage to the lung (22). Anakinra competes for the IL-1 receptor and blocks the actions of IL-1β. We investigated the impact of anakinra treatment on survival in IAV-infected juvenile mice. Juvenile mice were infected with IAV (A/WSN/2009 12.5 PFU i.t.) and anakinra (100 mg/kg i.p.) or an equal volume of vehicle control was administered i.p., q.d. beginning on day 3 p.i. and continuing until recovery or death. There was no statistically significant difference in the survival of IAV-infected mice treated with anakinra compared to those given vehicle control (**Figure 6A**). Initiating anakinra therapy on day 2 p.i. or day 4 p.i. also did not improve survival (data not shown). Measurement of protein leakage, IL-18, and IL-6 in BALF failed to show a difference between anakinra and control-treated mice on day 7 p.i. (**Figures 6B–D**). IFN-α secretion was not impacted by anakinra therapy, either, and viral titers from lung homogenates were equal in IAV-infected anakinra-treated mice, and IAV-infected PBS control-treated mice (**Figures 6E,F**). Finally, a similar degree of lung injury was seen on histological examination of IAV-infected, PBS-treated and IAV-infected, anakinra-treated mice on day 7 p.i. (**Figure 6G**).

DISCUSSION

The host response to IAV can exacerbate the morbidity associated with IAV infection (4, 23, 24). It is well established that the NLRP3 inflammasome is a major component of the host response to IAV (6). It is activated by the influenza M2 proton channel and results in the production of the potent inflammatory cytokines, IL-1β and IL-18 (7). While pathogen clearance and host survival depend on adequate activation of the innate immune system, an excessive inflammatory response to infection can be harmful to the young host. The NLRP3 inflammasome is protective in lethal mouse models of IAV infection (9–11). Loss of NLRP3, ASC, or caspase-1 in mice leads to decreased IL-1β and IL-18 secretion and increases mortality from IAV. Alternatively, excessive inflammasome activation may decrease survival by exacerbating the lung injury seen in lethal IAV infection (6). Therefore, inflammasome signaling must be tightly controlled to promote eradication of the virus while limiting collateral damage to the host. In severe IAV infection, this balance is not achieved, making the NLRP3 inflammasome an attractive therapeutic target. Early modulation of its activity may not only limit the production of injurious inflammatory cytokines, but also prevent the pyroptotic cell death and the ensuing tissue destruction caused by activated caspase-1. Thus, the identification of small molecule inhibitors of the NLRP3 inflammasome offers considerable therapeutic promise. To establish the optimal degree of NLRP3 inflammasome activation during IAV infection, we sought to modulate NLRP3 inflammasome signaling, with the goal of protecting juvenile mice from IAV-induced lung injury. We investigated the efficacy of MCC950, a potent inhibitor of NLRP3, as well as anakinra, a known inhibitor of the IL-1β pathway *via* IL-1 receptor.

MCC950 has been shown to be a specific NLRP3 inhibitor and to be protective in multiple models of injurious NLRP3 inflammasome activation (14–16). It can be given by oral, intravenous, and i.p. routes and is effective at doses ranging from 4 to 20 mg/kg in mouse models of autoimmune disease (experimental autoimmune encephalitis) (14), diseases of constitutive NLRP3 activation (cryopyrin-associated periodic syndrome) (14), and disorders in which NLRP3 has been shown to play an important role [including cardiac infarction (25) and non-alcoholic steatohepatitis (26)]. *In vitro* treatment of IAV-infected THP-1 macrophages with MCC950 confirmed that IAV-induced NLRP3 inflammasome

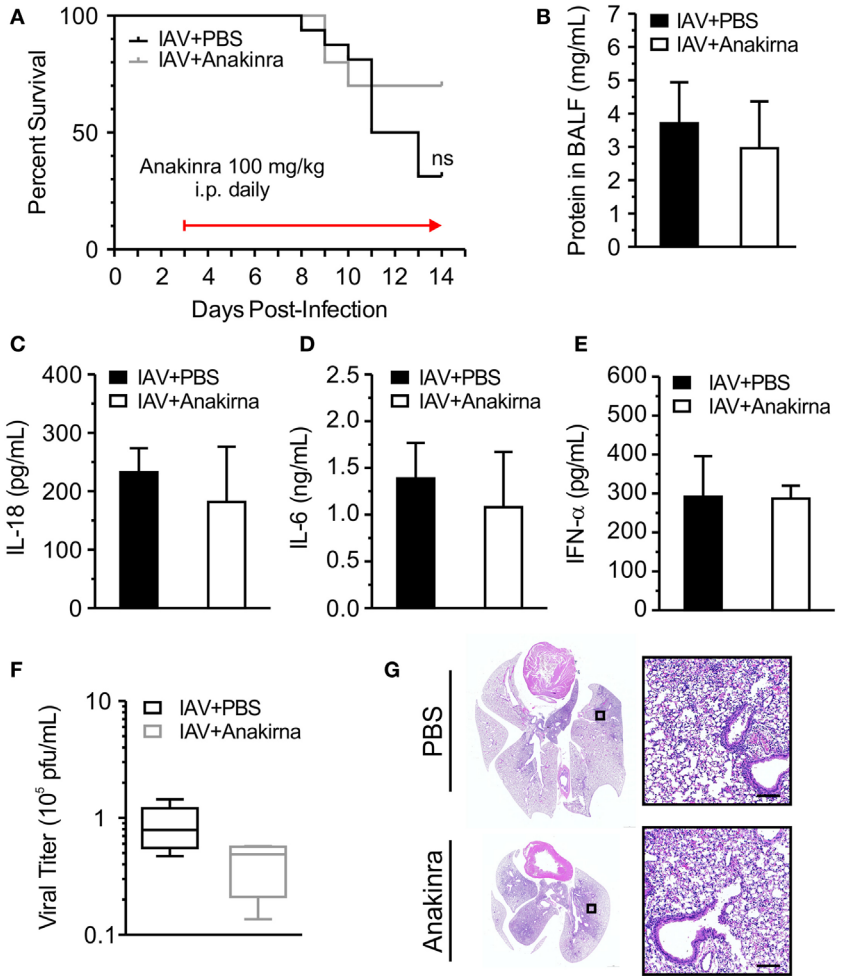

FIGURE 6 | Anakinra treatment does not protect juvenile mice from influenza A virus (IAV) infection. Juvenile mice were infected with IAV (WSN 12.5 plaque forming unit intratracheal) and treated with anakinra [100 mg/kg intraperitoneal (i.p.) daily] or vehicle control beginning on day 3 postinfection (p.i.). **(A)** Mortality. **(B)** Protein in bronchoalveolar lavage fluid (BALF) on day 7 p.i. **(C–E)** Interleukin-18 (IL-18), interleukin-6 (IL-6), and interferon-α (IFN-α) in BALF on day 7 p.i. as measured by ELISA. **(F)** Viral titer on day 7 p.i. was measured by plaque assay. **(G)** Hematoxylin and eosin stained lung sections from juvenile mice 7 days p.i. with IAV and treatment with anakinra or phosphate-buffered saline (PBS) control. Images shown are representative of three mice for each condition. Scale bars, 100 μm.

activation is effectively inhibited with this molecule. Interestingly, treatment of IAV-infected cells with the IL-1β receptor antagonist, anakinra, did not just block downstream signaling from the IL-1β receptor. It also appeared to inhibit NLRP3 inflammasome activation, as demonstrated by decreased caspase-1 and IL-1β in the supernatant from IAV-infected, anakinra-treated cells. Although anakinra classically targets IL-1β signaling by blocking the interaction of IL-1β with its receptor, it has also been shown to bind to and inhibit caspase-1, which likely explains our finding of NLRP3 inflammasome inhibition in IAV-infected cells treated with anakinra (27, 28). Consequently, we hypothesized that both MCC950 and anakinra had the potential to protect juvenile mice from IAV-induced NLRP3 inflammasome activation and lung injury.

Juvenile mice treated with MCC950 beginning 3 days p.i. were protected from IAV-induced mortality. We chose this timing for the initiation of therapy because this likely corresponds to when children infected with IAV develop symptoms and seek medical attention. The protection from IAV-induced mortality was associated with a decreased amount of NLRP3 in the lung homogenates, and decreased IL-18 levels in the BALF, from IAV-infected, MCC950-treated mice compared to IAV-infected, vehicle-treated mice, indicating that inhibition of the NLRP3 inflammasome was achieved. However, this protection from IAV-induced mortality was not associated with a decrease in traditional markers of lung injury, including cellular infiltration and protein leakage into the alveolar space. Since MCC950 therapy did not prevent IAV-induced lung injury on day 7 p.i., this suggests that MCC950 treatment improved survival by either halting disease progression or enhancing recovery. When CD45+ cells from IAV-infected mice were examined with intracellular staining, the greatest impact of NLRP3 inflammasome inhibition was found in alveolar macrophages. In these cells, MCC950 treatment decreased NLRP3 and IL-1β levels. As alveolar macrophages can

promote alveolar epithelial cell repair (24), the switch from an inflammatory to an anti-inflammatory phenotype in alveolar macrophages may play an important role in recovery from IAV. We reason that inhibiting the NLRP3 inflammasome in this cell population may have played a key role in the beneficial effects of MCC950 therapy.

Other groups have shown varying degrees of protection from IAV (12) or the IAV virulence factor PB1-F2 (29) using MCC950 in murine models of adult IAV. Tate et al. were able to delay death from two different strains of IAV (A/PR/8/34 and HKx31) by a few days with MCC950 (5 mg/kg intranasal) treatment. Notably, timing of initiation of MCC950 therapy was important in their model, with early administration of the drug on day 1 p.i. harmful, and late administration after day 3 beneficial. This could be consistent with our proposal that NLRP3 inflammasome inhibition is important for recovery from IAV infection, rather than prevention of IAV-induced lung injury. However, evaluation of lung injury was not performed in their study. They were able to demonstrate prevention of immune cell infiltration into the lungs and decreased cytokine production, including IL-1β, IL-18, TNF-α, and IL-6, in BALF and serum. In our model of juvenile IAV infection, we did not see the same inhibition of immune cell recruitment to the lungs with MCC950 therapy (10 mg/kg i.p.). We also did not see the same suppression of IL-6 or TNF-α production. However, we were not surprised by this finding because these cytokines are not dependent on NLRP3 inflammasome activation. Importantly, we did see evidence of NLRP3 inflammasome inhibition in the resident alveolar macrophages, and decreased IL-18 in BALF, which may have contributed to the increased survival we found in mice treated with MCC950. Differences in our models may explain the disparate findings regarding cellular recruitment and cytokine suppression, highlighting the importance of the microenvironment when modulating the immune response to a pathogen. We administered the drug i.p. instead of intranasal to avoid repeated exposure to anesthesia, but these two delivery methods could result in different concentrations of the drug in the alveolar space. In addition, the half-life of the drug may be altered by delivery method, potentially limiting its efficacy. Perhaps more importantly, we used juvenile mice, which may have a propensity for worse disease (4, 30, 31). Innate immune signaling has been shown to be vary widely depending on age, making age-relevant models critical when studying inflammatory diseases (32–38).

Genetic deletion of NLRP3 inflammasome components leads to worse outcomes in IAV infection (9–11). This argues that abolishment of NLRP3 inflammasome signaling during IAV infection is harmful and that early NLRP3 inflammasome activation is necessary for controlling the infection and viral clearance. The finding from Tate et al. that early inhibition of NLRP3 with MCC950 increases mortality from IAV infection is consistent with this (12). In contrast, late inhibition was protective, which supports our finding that MCC950 treatment beginning 3 days p.i. improved survival in juvenile mice infected with IAV. The sensitivity of the outcome of NLRP3 modulation to timing and degree of inhibition is not unique to IAV infection, but a common theme in inflammatory responses to pathogens, where inadequate inflammation impairs pathogen clearance, but excessive inflammation causes collateral tissue damage and enhanced injury. This emphasizes the need for careful characterization of optimal treatment strategies in clinically relevant, age appropriate, models.

In contrast to our results from MCC950 treatment, anakinra treatment of IAV-infected juvenile mice did not show protection from IAV-induced mortality or lung injury. Despite achieving NLRP3 inflammasome suppression *in vitro*, anakinra therapy did not effectively decrease NLRP3 inflammasome activation or IL-18 secretion into the alveolar space in our *in vivo* model of juvenile IAV infection. Therefore, it was not surprising that we could not demonstrate protection from IAV with anakinra treatment. Instead, it suggests that once daily dosing with i.p. delivery was not sufficient to achieve caspase-1 inhibition. Alternatively, it argues that isolated IL-1 receptor antagonism is insufficient to protect juvenile mice from IAV infection because it leaves IL-18 signaling intact. There is one report of anakinra therapy (100 μg/mouse, intravenous, daily from day 2 to 6 p.i.) improving survival in IAV infection in adult mice (A/PR/8/34) (39), but only mortality was evaluated. Differences in our model, especially the age of the mice, may explain the discrepancy in our findings.

Influenza A virus is a source of significant morbidity and mortality in children, but current therapies are limited to early antiviral treatment and supportive care. There is considerable need for new strategies to improve outcomes in pediatric IAV infection, and the use of juvenile models to test these strategies is critical. Targeting the NLRP3 inflammasome may be beneficial in juvenile IAV infection and does not appear to impact viral clearance. Better understanding of how NLRP3 inflammasome inhibition improves mortality in juvenile IAV infection, and identification of the optimal timing and method of NLRP3 inflammasome inhibition in juvenile IAV infection, deserve further study.

ETHICS STATEMENT

This study was carried out in accordance with United States federal guidelines and was approved by The Institutional Animal Care and Use Committee at Northwestern University.

AUTHOR CONTRIBUTIONS

BC contributed to all aspects of this manuscript including experimental design, conduction of experiments, interpretation of data, and manuscript preparation. NR, YC, JD, and DS contributed to the conduction of the experiments, interpretation of data, and manuscript preparation. KS and CK contributed to experimental design, conduction of experiments, interpretation of data, and manuscript preparation. KR contributed to experimental design, interpretation of data, and manuscript preparation.

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MINI REVIEW
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Inflammatory Responses Regulating Alveolar Ion Transport during Pulmonary Infections

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The respiratory epithelium is lined by a tightly balanced fluid layer that allows normal O_2 and CO_2 exchange and maintains surface tension and host defense. To maintain alveolar fluid homeostasis, both the integrity of the alveolar–capillary barrier and the expression of epithelial ion channels and pumps are necessary to establish a vectorial ion gradient. However, during pulmonary infection, auto- and/or paracrine-acting mediators induce pathophysiological changes of the alveolar–capillary barrier, altered expression of epithelial Na,K-ATPase and of epithelial ion channels including epithelial sodium channel and cystic fibrosis membrane conductance regulator, leading to the accumulation of edema and impaired alveolar fluid clearance. These mediators include classical pro-inflammatory cytokines such as TGF-β, TNF-α, interferons, or IL-1β that are released upon bacterial challenge with *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, or *Mycoplasma pneumoniae* as well as in viral infection with influenza A virus, pathogenic coronaviruses, or respiratory syncytial virus. Moreover, the pro-apoptotic mediator TNF-related apoptosis-inducing ligand, extracellular nucleotides, or reactive oxygen species impair epithelial ion channel expression and function. Interestingly, during bacterial infection, alterations of ion transport function may serve as an additional feedback loop on the respiratory inflammatory profile, further aggravating disease progression. These changes lead to edema formation and impair edema clearance which results in suboptimal gas exchange causing hypoxemia and hypercapnia. Recent preclinical studies suggest that modulation of the alveolar–capillary fluid homeostasis could represent novel therapeutic approaches to improve outcomes in infection-induced lung injury.

Keywords: ion channel, ion pumps, edema, cytokines, Na-K-ATPase, cystic fibrosis membrane conductance regulator, epithelial sodium channel, lung injury

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Abbreviations: AFC, alveolar fluid clearance; ALF, alveolar lining fluid; ALI, acute lung injury; AMPK, AMP-kinase; AQP, aquaporin; ARDS, acute respiratory distress syndrome; ASL, airway surface liquid; CaCC, Ca^{2+}-activated ion channels; cAMP, cyclic AMP; CFTR, cystic fibrosis membrane conductance regulator; CNG, cyclic nucleotide-gated cation channel; ENaC, epithelial sodium channel; IAV, influenza A virus; ICAM, intercellular adhesion molecule-1; IFN, interferons; IL, interleukin; L-NMMA, N(omega)-monomethyl-L-arginine; LPS, lipopolysaccharide; MERS-CoV, middle east respiratory syndrome coronavirus; mRNA, messenger RNA; NETs, neutrophil extracellular traps; NKCC, $Na^+/K^+/2Cl^-$ cotransporters; NO, nitric oxide; PECAM, platelet endothelial cell adhesion molecule-1; RONS, reactive oxygen and nitrogen species; ROS, reactive oxygen species; RSV, respiratory syncytial virus; SARS-CoV, severe acute respiratory syndrome coronavirus; TGF-β, transforming growth factor beta; TNFR1, TNF receptor 1; TNF-α, tumor necrosis factor alpha; TRAIL, TNF-related apoptosis-inducing ligand; VCAM, vascular adhesion molecule-1; β2AR, beta-2 adrenergic receptor.

INTRODUCTION

The major task of the respiratory tract is the exchange between inhaled atmospheric oxygen and carbon dioxide carried by the bloodstream, which is ensured by a thin but large surface area formed by type I and type II alveolar epithelial cells. Both the upper and the lower respiratory epithelia are lined by a thin (0.2 μM) aqueous layer (1), referred to as airway surface liquid (ASL) and alveolar lining fluid (AFL), respectively. This fluidic component serves—in concerted action with surfactant, mucus, and ciliary beat—to reduce alveolar surface tension and prevent atelectasis as well as to defend against invading pathogens. To maintain the composition of the ASL and AFL and to prevent alveolar flooding, lung fluid homeostasis is tightly controlled by the expression and activity of ion channels and pumps. These channels and pumps establish an osmotic gradient between airspace and interstitium, driving paracellular or aquaporin- (AQP3, 4, and 5) (2) mediated fluid movement across the respiratory epithelium. Among these, the apical amiloride-sensitive epithelial sodium channel (ENaC) and the amiloride-insensitive cyclic nucleotide-gated cation channel (CNG) acting together with the basolaterally located Na,K-ATPase (NKA) promote transcellular sodium transport (3), which is accompanied in the alveolar epithelium by chloride uptake from the apical cystic fibrosis membrane conductance regulator (CFTR) (4). However, in the airway, CFTR promotes chloride secretion to regulate mucus density (5). In addition, Ca^{2+}-activated ion channels (CaCC) promote apical chloride secretion, further supported by basolateral chloride uptake *via* $Na^+/K^+/2Cl^-$ cotransporters (NKCC) (6) as well as potassium ion channels such as Kv7.1, contributing to cellular membrane potential and buildup of an electrochemical gradient necessary for apical chloride secretion (7). Additional factors influencing fluid homeostasis are epithelial (im)permeability established by tight junction proteins as well as endothelial integrity limiting the extravasation of fluid from the blood vessels driven by changes in the capillary hydrostatic pressure (8, 9).

Pulmonary infections commonly disturb ion and thus fluid homeostasis, resulting in abnormal changes of ASL, AFL, and alveolar edema formation. Both viral and bacterial pathogens are common causative agents for acute lung injury (ALI) and the acute respiratory distress syndrome (ARDS), which are characterized by a widespread inflammation within the lungs, extensive flooding of the alveolar airspace with protein-rich exudate fluid and impaired gas exchange leading to respiratory failure and resulting in mortality rates of 40–58% (10, 11). Additionally, sepsis resulting from primary infections at other sites is often complicated by the development of severe lung injury during the onset of bacteremia, resulting in lung failure and accounting for as many as half of all cases of ARDS (12). Although some of the pathogen-derived effects on ion transport during lung injury have been reported to be caused directly by the pathogen–host cell interaction (13), accumulating evidence suggests that auto- and paracrine mediators of local and/or systemic inflammatory responses mounted upon pathogen recognition and replication induce—among other pathophysiological changes—impaired ion transport and alveolar fluid clearance (AFC), resulting in edema formation and persistence. Importantly, mortality in ARDS patients has repeatedly been found to correlate with persistence of alveolar edema (11, 14).

In this review, we will highlight advances in the understanding of how inflammatory responses in pulmonary infection affect ion transport, including common patterns and unique pathways activated by different respiratory pathogens, and how these mechanisms might be modulated to improve the outcomes of ARDS patients.

MEDIATORS MODULATING ION AND FLUID HOMEOSTASIS

There are numerous reports showing that pulmonary infection leads to loss of barrier integrity and edema accumulation as well as the role of distinct mediators on impairing ion channel or transporter function on the alveolar, bronchial, and gut epithelia. However, there have been few studies showing how infectious agents modulate soluble signaling molecules that affect ion and fluid homeostasis. Several reports from the last decade have reestablished an important role for soluble, inflammatory mediators in the progression of ARDS. For example, Lee et al. demonstrated that exposure of human ATII cells to pulmonary edema fluid derived from ARDS patients alone was sufficient to downregulate the ion channels and pumps involved in AFC, including ENaC, the NKA, and CFTR (15). Concomitantly, it was established that viral or bacterial lung infections lead to edema accumulation and impair clearance *via* the induction of paracrine factors. For example, influenza A virus (IAV) has been shown to increase apical potassium secretion by upregulation of the apical potassium channel KCNN4 by a paracrine signaling event, thus disturbing the osmotic gradient necessary for edema clearance (16). Similarly, *Pseudomonas aeruginosa* evokes a strong inflammatory response and lung edema accumulation related with the modulation of ENaC subunit expression (17, 18). In the next paragraphs, we will provide an overview on interconnections of mediators released in pulmonary infection and their effects on ion and fluid homeostasis (**Figure 1**).

Interferon

Once cells detect pathogens by their specific and specialized pattern recognition receptors, they produce interferons (IFN), which can be detected—if not actively suppressed by a given pathogen—in most pulmonary infection scenarios. Effects of IFN on fluid homeostasis seem to be mostly limited to gamma IFN (IFN-γ), which have been attributed a modulatory role in both innate and adaptive immunity (19, 20). IFN-γ has been reported to decrease sodium transport at levels as low as 10 U/ml (21). Moreover, IFN-γ can also directly decrease chloride currents along the bronchial epithelium by downregulating CFTR due to a posttranscriptional modulation of CFTR messenger RNA (mRNA) stability and thus half-life (21–23). In contrast, both class I IFN, IFN-α, and IFN-β that are usually implicated in mounting a direct cellular pathogen-restrictive response do not modulate CFTR mRNA or protein abundance (22). IFN-α appears to negatively impact NKA cell membrane protein abundance during IAV infection *via* activating the metabolic sensor AMP-kinase (AMPK) (24). However, to date,

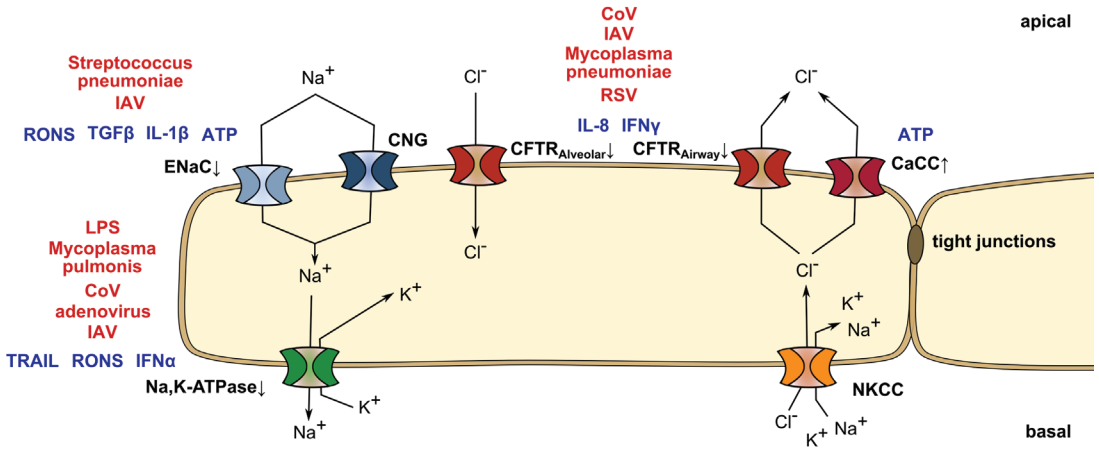

FIGURE 1 | Mediators released in pulmonary infection and their effects on ion homeostasis. Ion transport of the lung epithelial cell is mediated by various ion channels and pumps. Sodium enters the epithelial cell *via* the apical cyclic nucleotide-gated cation channel (CNG) or the epithelial sodium channel (ENaC), that can be downregulated by reactive oxygen and nitrogen species (RONS) and ATP, transforming growth factor beta (TGF-β) or interleukin-1 beta (IL-1β) upon *Streptococcus pneumoniae* and influenza A virus (IAV) infection. Sodium is secreted at the basolateral side by the Na,K-ATPase (NKA), which is modulated in lipopolysaccharide (LPS)-induced lung injury as well as upon *Mycoplasma pulmonis*, IAV, coronavirus (CoV), or adenovirus challenge. RONS, interferon-alpha (IFN-α), and TNF-related apoptosis-inducing ligand (TRAIL) lead to a decrease in NKA abundance or activity. In parallel, chloride is taken up (alveolar epithelium) or secreted (airway) by the cystic fibrosis membrane conductance regulator (CFTR) and secreted by apical Ca^{2+}-activated ion channels (CaCC), supported by basolateral potassium channels (not shown) and $Na^+/K^+/2Cl^-$ cotransporters (NKCC). While extracellular ATP enhances chloride secretion by CaCC, CFTR action is reduced by IFN-γ and interleukin-8 (IL-8) in CoV, IAV, respiratory syncytial virus (RSV), or *Mycoplasma pneumoniae* infection.

there is no data supporting whether this effect of IFN-α on ion transport is a generalized response during pulmonary infections.

Tumor Necrosis Factor Alpha (TNF-α)

Tumor necrosis factor alpha is a classical cytokine produced upon local or systemic inflammation, regulating differential processes such as proliferation and differentiation of immune cells as well as cell death (25–27). After initial conflicting studies, it has by now become clear that it plays a dichotomic role in lung fluid reabsorption (28). On one hand, TNF-α ligation to its receptor TNF receptor 1 (TNFR1, also named CD120a or p55) inhibits ENaC activity both *in vitro* and *in vivo via* a PKC-dependent mechanism (29). On the other hand, a distinct lectin-like domain of TNF different from the receptor-binding domain, which can be mimicked by the 17-amino acid circular TIP peptide (30), has been reported to increase edema reabsorption in rat bacterial pneumonia (31). Application of the TIP peptide has been demonstrated to elevate ENaC expression and open probability (32) resulting in enhanced AFC in *P. aeruginosa*-treated rats *in vivo* (31) and has furthermore been reported to increase NKA activity (33). In addition to its direct effects on ion channels and pumps of the alveolar epithelium, the TNF-α/TNFR1 interaction also modulates the integrity of the alveolar barrier, as it increases endothelial expression of chemoattractants and adhesion molecules including the interleukin-8 (IL-8; formerly called neutrophil chemotactic factor)/IL-8-receptor 2 axis, the intercellular adhesion molecule-1, platelet endothelial cell adhesion molecule-1, and vascular adhesion molecule-1, and thus promotes excessive recruitment of mononuclear phagocytes and neutrophils during lung inflammation (30, 34, 35). Importantly, besides cellular transmigration itself, neutrophil-derived proteases and neutrophil extracellular traps are central drivers of both endothelial and epithelial injury (36).

Interleukin-1 Beta (IL-1β)

Interleukin-1 beta is one of the most commonly found cytokines in pulmonary edema and bronchoalveolar lavage fluids in experimental and human ARDS (37, 38) and is, for example, induced during *Klebsiella pneumoniae* bacterial pneumonia (39–41). It is mainly produced by macrophages and, similarly to TNF-α, has a major impact on cell proliferation, differentiation, and cell death. In pulmonary inflammation, IL-1β increases lung barrier permeability in *in vitro* and *in vivo* models of ARDS (41, 42) and may contribute to alveolar edema in lung injury models by impairing fluid reabsorption from the lungs. This can in part be attributed to decreased sodium absorption due to a decrease in αENaC expression and trafficking to the apical membrane of ATII cells (43). In addition, IL-1β in *Streptococcus pneumonia* infection (44)—and also TNF-α and IFN-γ (45)—can influence ion transport processes *via* activation of the pro-coagulant factors (46). Thrombin in particular has been demonstrated to impair AFC by increasing the PKC-ζ-dependent endocytosis of the alveolar NKA (47).

Interleukin-8

Interleukin-8 is a chemotactic factor that correlates with neutrophil accumulation in distal airspaces of patients with ARDS and is a predictor of mortality (48–50). IL-8 is secreted by bronchial epithelial cells and can be induced by *Mycoplasma pneumoniae* antigen or live *M. pneumoniae* (51) as well as by severe acute respiratory syndrome coronavirus spike protein or respiratory syncytial virus infection (52, 53). The rate of AFC is impaired by high levels of IL-8 and is significantly lower in patients who have

a pulmonary edema fluid concentration of IL-8 above 4,000 pg/ml (54). Mechanistically, IL-8 inhibits beta-2 adrenergic receptor (β2AR) agonist-stimulated fluid transport across rat and human alveolar epithelia. This inhibition is mediated by a PI3K-dependent desensitization and downregulation of the β2AR from the cell membrane associated with an inhibition of cyclic AMP generation normally observed in response to β2AR agonist stimulation (54).

Transforming Growth Factor Beta (TGF-β)

The cytokine TGF-β is a critical factor for the development of ARDS. Besides its established role in dampening inflammatory responses (55), e.g., by driving macrophages toward an anti-inflammatory phenotype (56), it increases alveolar epithelial permeability to promote edema formation upon lipopolysaccharide (LPS) stimulation (57). Furthermore, TGF-β has been shown to inhibit amiloride-sensitive sodium transport by an ERK1/2-dependent inhibition of the αENaC subunit promoter activity, decreasing αENaC mRNA and protein expression (58). In addition, Peters et al. (59) demonstrated that TGF-β leads to the subsequent activation of phospholipase D1, phosphatidylinositol-4-phosphate 5-kinase 1α, and NADPH oxidase 4 (Nox4). Nox4 activation results in the production of reactive oxygen species (ROS) that in turn reduce cell surface stability of the αβγENaC complex and thus promote edema fluid accumulation. Moreover, TGF-β decreases NKA β1 subunit expression, resulting in decreased NKA activity in lung epithelial cells (60, 61). In further support of a role for TGF-β in lung injury, TGF-β levels are increased in lung fluids from patients with ALI/ARDS (62) and in murine models of *Streptococcus pneumoniae* and IAV infection (63, 64). Of note, TGF-β has been proposed to further aggravate edema formation in IAV infection by increasing epithelial cell death, causing a disruption of epithelial barrier integrity (64). Moreover, it has been implicated in the upregulation of cellular adhesins which increase host susceptibility to bacterial co-infections (65) posing a major risk for increased viral pneumonia-associated morbidity and mortality during influenza epidemics (66).

TNF-Related Apoptosis-Inducing Ligand (TRAIL)

The principal role of TRAIL, highly released by lung macrophages upon viral infection, is to drive infected cells into apoptosis to limit pathogen spread. TRAIL has been reported to be produced especially during viral respiratory infections, including IAV-, adenovirus-, and paramyxovirus infection, and cell sensitivity to TRAIL-induced apoptosis is enhanced in infected cells by increased TRAIL-receptor expression (67, 68). However, this process also affects alveolar epithelial barrier integrity leading to edema accumulation (67, 69). Moreover, TRAIL signaling leads to NKA downregulation in IAV infection in non-infected neighboring alveolar epithelial cells mediated by AMPK (24). Accordingly, TRAIL signaling reduces AFC and promotes edema formation. In addition, TRAIL release upon IAV infection further favors bacterial superinfection with *S. pneumoniae*, aggravating lung injury (70).

Nucleotides

During acute infection, extracellular nucleotides often serve as danger signals involved in recognition and control of pathogens by promoting the recruitment of inflammatory cells, stimulating pro-inflammatory cytokines, and increasing the production of ROS or nitric oxide (NO) (71, 72). Extracellular ATP, which can be released from the airway epithelia and is produced by endothelial cells upon acute inflammation, binds to P2 purinergic receptors to promote a calcium signaling-dependent stimulation of CaCC and a decreased open probability of ENaC (73, 74). Moreover, extracellular adenosine, produced from ATP by hydrolysis *via* the ecto-5′-nucleotidase CD73, is increased in bronchoalveolar lavage fluid of IAV-infected mice, and genetic deletion of the A1-adenosine-receptor is protective (75). However, CD73 is only to a limited extent involved in the progression of lung injury and has no effect on pulmonary edema formation (76).

Reactive Oxygen and Nitrogen Species (RONS)

Reactive oxygen and nitrogen species have been shown to be involved in the development of epithelial injury in pathologic situations, including LPS-/sepsis-induced lung injury as well as viral pneumonia, in which RONS are produced in large quantities by alveolar phagocytes (77). Studies in rabbit and piglet lungs further elucidated that RONS affect AFC and edema persistence by inhibiting both the activity of ENaC and alveolar epithelial NKA (78, 79).

EFFECTS OF ION CHANGES ON CYTOKINE PRODUCTION

To add to the complexity of airway and alveolar fluid regulation, it has been suggested that not only ion channels, pumps, and transporters are modulated by signaling factors released upon pulmonary infection but also changes in ion transport influence the respiratory inflammatory response. For example, the transporter NKCC1—which plays a critical role in basolateral ion transport—can affect the severity of pneumonia and sepsis and consequently severity of lung injury, by regulating the ability of the alveolar–capillary barrier to modulate neutrophil infiltration into the air spaces of the lung (80). Lack of NKCC1 in a mouse model of pneumonia infection with *K. pneumonia* or LPS resulted in increased numbers of neutrophils in the lavage fluid, decreased bacteremia, and importantly mortality. It has, therefore, been suggested that the activity of NKCC1 contributes to edema formation and decreased neutrophil migration into the lung air spaces, probably contributing to reduce bacterial killing and the subsequent development of severe sepsis (81–83). Similarly, mutations of CFTR can amplify lung inflammation by upregulating pro-inflammatory responses caused by an increase in cytokine production upon NFκB activation in lung epithelial cells (84). Lack of functional neutrophilic CFTR in a model of LPS-induced lung inflammation contributes to inflammatory imbalance with NFκB translocation and a reduction of anti-inflammatory cytokines such as IL-10, favoring the increase in lung vascular permeability (85). Also ion imbalances in

response to expression of viral ion channels or viroporins, has been recognized as potential pathogen recognition pathway that favors inflammasome activation and the release of IL-1β, TNF, and IL-6, which might contribute to the limitation of virus spreading (86, 87).

THERAPEUTIC MODULATION OF THE ALVEOLAR–CAPILLARY FLUID BALANCE DURING PULMONARY INFECTION

As stated above, pulmonary infections—especially in severe cases—can lead to lung edema accumulation and impaired edema clearance. Lung edema results in impaired oxygenation and organ dysfunction which if not resolved leads to high mortality of patients with ARDS (11, 14). Current treatment options for infection-induced ARDS include antivirals and antibiotics. However, there is increased antibiotic resistance—reported for pathogens such as *K. pneumoniae*, *Escherichia coli*, *Staphylococcus aureus* and *P. aeruginosa* (82, 83, 88)—or lack of readily available treatment options for some acute emerging agents such as zoonotic influenza viruses or middle east respiratory syndrome coronavirus (89–91). Current approaches to treat ARDS patients include low tidal volume mechanical ventilation, positive end expiratory pressure, fluid management, and extracorporeal membrane oxygenation as measures to primarily improve oxygenation (92). Interestingly, lung-protective ventilation strategies have not only been reported to reduce mortality by 22% in patients with ARDS but also to diminish the number of neutrophils and the concentration of pro-inflammatory cytokines released in patient lavage fluids.

Novel approaches targeting host mediators known to promote lung edema formation and impair clearance such as studies on TIP peptide [see Tumor Necrosis Factor Alpha (TNF-α) above] administration in ARDS are being studied. Initial reports showed that AP301, a synthetic peptide mimicking TIP, induces ENaC activity in type II alveolar epithelial cells from dogs, pigs, and rats (93) and improves lung function in a porcine lung injury model (94). A subsequent phase II clinical trial with AP301 in ventilated ARDS patients resulted in improved AFC and oxygenation of these patients (95). Also, mesenchymal stem cells, which have been reported to improve epithelial barrier integrity in human AEC II treated with a cytokine mix composed of a combination of IL-1β, TNFα, and IFNγ (96), are currently tested for safety and efficacy in phase II trials (clinical trial identifiers NCT02097641, NCT01775774, NCT02112500). Studies on β2-agonists, which had been previously shown to improve vectorial sodium transport and edema clearance (97, 98), did not improve ARDS outcomes (99, 100), possibly due to an enhanced inflammatory response driven by lung macrophages (101). Further treatment options targeting para- or autocrine signaling events affecting AFC in preclinical models include glucocorticoids that suppress inflammation and upregulate both NKA (102) and ENaC (103, 104), neutralizing antibodies directed against virus-specific release of macrophage TRAIL that improve NKA expression as well as AFC in IAV-infected mice (24) and nitric oxide synthase inhibitors aminoguanidine or N(omega)-monomethyl-L-arginine (L-NMMA) that protect against pulmonary edema in LPS-induced lung injury as well as in IAV infection (77, 105).

CONCLUSION

Pathogen-induced lung injury but also sepsis can lead to widespread respiratory inflammation that favors accumulation of lung edema leading to multiorgan dysfunction and poor outcomes. Recent advances in the development of novel treatment strategies targeting respiratory ion homeostasis show encouraging results, identifying them as promising candidates to improve AFC in ALI which could potentially improve the survival of patients with ARDS.

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CP, SH, JS, and EL have performed bibliographic research and drafted the manuscript.

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The Role of Transient Receptor Potential Vanilloid 4 in Pulmonary Inflammatory Diseases

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Ion channels/pumps are essential regulators of organ homeostasis and disease. In the present review, we discuss the role of the mechanosensitive cation channel, transient receptor potential vanilloid 4 (TRPV4), in cytokine secretion and pulmonary inflammatory diseases such as asthma, cystic fibrosis (CF), and acute lung injury/acute respiratory distress syndrome (ARDS). TRPV4 has been shown to play a role in lung diseases associated with lung parenchymal stretch or stiffness. TRPV4 indirectly mediates hypotonicity-induced smooth muscle contraction and airway remodeling in asthma. Further, the literature suggests that in CF TRPV4 may improve ciliary beat frequency enhancing mucociliary clearance, while at the same time increasing pro-inflammatory cytokine secretion/lung tissue injury. Currently it is understood that the role of TRPV4 in immune cell function and associated lung tissue injury/ARDS may depend on the injury stimulus. Uncovering the downstream mechanisms of TRPV4 action in pulmonary inflammatory diseases is likely important to understanding disease pathogenesis and may lead to novel therapeutics.

Keywords: transient receptor potential vanilloid 4, ion channels, asthma, pulmonary vascular disease, acute respiratory distress syndrome

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INTRODUCTION

Ion channels and pumps play multiple important roles in cell homeostasis (1). They function to allow passive, agonist-induced, or voltage-dependent flux of specific ions in and out of the cell (1, 2). Dysregulation of channel function and/or expression can lead to organ dysfunction and disease (1–3). Recent studies have shown that a transient receptor potential (TRP) channel family member, transient receptor potential vanilloid 4 (TRPV4), is implicated in inflammatory lung diseases such as asthma, cystic fibrosis (CF), acute lung injury/acute respiratory distress syndrome (ARDS), and pulmonary fibrosis (4–10). In fact, these studies show that TRPV4 can regulate inflammatory cytokines that play key roles in orchestrating lung tissue homeostasis and inflammatory lung disease (4, 7, 10–14). Dysregulation of cytokines leads to alterations in cell–cell interactions, lung tissue remodeling, and repair (15). Regulating cytokine secretion through the modulation of ion channels such as TRPV4 may mediate inflammatory lung diseases. Therefore, TRPV4 may be a potential target for lung disease pathogenesis (16). This review summarizes and integrates the data from our laboratory and others to further the understanding of the TRPV4–cytokine interaction in pulmonary inflammation.

THE TRPV4 CHANNEL

Intracellular calcium is tightly regulated in a spatiotemporal manner through a system of ion channels and membrane pumps (17). One such channel is TRPV4, a transmembrane (TM) cation channel of the TRP superfamily (18). TRPV4 is an 871 amino acid protein that has 6 TM domains, an ion pore located between TM5 and 6, an NH_2 terminal intracellular sequence with several ankyrin-type repeats, and a COOH-terminal intracellular tail (19, 20). Both the NH_2 and COOH termini interact with signal kinases, other molecules [e.g., nitric oxide (NO)], and scaffolding proteins (21). The intracellular tails contain several activity-modifying phosphorylation sites. TRPV4 is sensitized and activated by both chemical [5,6-epoxyeicosatrienoic acid (EET) and 4 alpha-phorbol 12,13-didecanoate (4-αPDD)] and physical stimuli (temperature 27–35°C, membrane stretch, and hypotonicity) (22–25). TRPV4 is ubiquitously expressed in many cell types in the respiratory system. In the setting of pulmonary inflammation, TRPV4 has been found to be highly expressed and upregulated in airway smooth muscle, vascular endothelial cells, alveolar epithelial cells, and immune cells such as macrophages and neutrophils (12, 16, 21, 26–28). TRPV4 has been implicated in the pathogenesis of asthma, CF, and sterile and infection-associated ARDS (4–10, 29).

THE ROLE OF TRPV4 IN INFLAMMATORY LUNG DISEASES

Asthma

Asthma is a chronic lung disease characterized by airway inflammation and remodeling, excess bronchial secretions, and smooth muscle hypertrophy and contraction leading to airway narrowing (bronchoconstriction). Recent work shows that TRPV4 mediates airway wall thickness, goblet cell recruitment, collagen expression, fibrotic airway remodeling, and increased expression of transforming growth factor-β (TGF-β) in a house dust mite (*Dermatophagoides farinae*) mouse model of asthma (30). The authors also show that TRPV4 mediates TGF-β-dependent myofibroblast differentiation *in vitro* through the ras homolog gene family member A (RhoA), p38, and PI3Kα (30). *In vitro* exposure of airway smooth muscle or tracheal rings to hypotonic solutions causes smooth muscle cell contraction, and some asthmatic patients are hypersensitive to this stimulus. To that end, it has been found that small nucleotide polymorphisms in the G allele in the coding region and 3′ flanking region of the TRPV4 gene, as first identified in COPD, are associated with a greater reduction in pulmonary function after hypotonic saline administration (8, 31). Interestingly, the calcium and contractile response of smooth muscle cells to hypotonic saline involves interactions between the cysteinyl leukotriene pathway and TRPV4 (12, 32). These findings suggest that downregulation of TRPV4 may be a therapeutic target in some etiologies and genetic variants of asthma. Of note, different TRPV4 activation stimuli beyond hypotonicity utilize different pathways for TRPV4 activation. For example, hypotonicity induces TRPV4 activation through phospholipase A2 (PLA2)/P450 epoxygenase-dependent generation of EETs, while heat and 4αPDD are PLA2/P450-independent (25). Further study of the mode of TRPV4 activation in individual diseases would support disease-specific, pathway-targeted therapy. While asthma is an inflammatory disease, there is no current evidence linking Th2-type cytokines and TRPV4 in the pathogenesis of asthma. Hence, this is an avenue for future studies.

Cystic Fibrosis

Cystic fibrosis is characterized by a mutation in the cystic fibrosis transmembrane conductance regulator (CFTR), a membrane-based chloride channel, which initially causes dehydration of the airway surface liquid thereby increasing susceptibility to bacterial and fungal infections (e.g., *Pseudomonas*, *Staphylococcus*, *Burkholderia*, atypical mycobacterium) (33). TRPV4 interacts with CFTR on several levels. TRPV4-dependent calcium influx in response to hypotonicity is reduced in human CF epithelial cells (34). Furthermore, other hypotonicity-induced TRPV4 chemical activators (5,6-, 8,9-, 11,12-, and 14,15-EET) and their metabolites (5,6 DHET) have been measured in the sputum of CF patients (10). Although the current consensus suggests that dehydration of airway mucous is the predominant cause of impaired mucociliary clearance in CF, recent considerations have been put forth to increase ciliary function or ciliary beat frequency (CBF) as a means to improve mucociliary clearance (35, 36). Concordantly, TRPV4-deleted tracheal epithelial cells have decreased CBF in response to ATP, 4αPDD, and temperature, whereas CBF in response to hyperviscosity was similar in wild-type (WT) and TRPV4 deleted cells. These data suggest that TRPV4 agonism might increase CBF; however, the effects on CF prognosis remain to be determined (37).

The pathogenesis of CF is also characterized by cytokine-mediated airway inflammation. Recently, both cytokines/chemokines and lipid mediators secreted from epithelial cells have been identified as key components in the inflammatory process. In this regard, TRPV4 activation induces epithelial cell secretion of pro-inflammatory cytokines/chemokines and active lipid mediators (e.g., IL-8, cytosolic PLA2, prostaglandin E2, NF-κB, AA, etc.) in response to lipopolysaccharide (LPS) (10). Secretion of IL-8/KC, in both bronchial epithelial cells and in intact mice lungs in response to TPRV4 activation, was increased upon inhibition of CFTR (10). These data demonstrate that TRPV4 has pleotropic effects on CF pathogenesis. Further study of the individual molecular pathways downstream of TRPV4 in CF may identify selectivity in the TRPV4 responses that can then be marshaled for therapeutic intent.

ACUTE LUNG INJURY/ARDS

Acute respiratory distress syndrome is a syndrome characterized by patchy lung inflammation along with cytokine release leading to alveolar space edema, exudate, and collapse. The pathogenesis of ARDS is complex; it is characterized by endothelial and alveolar epithelial injury followed by recruitment and accumulation of inflammatory cells in the injured alveolus (38). ARDS is a consequence of non-infectious (trauma, hemorrhage, lung ventilator stretch) or infectious (sepsis, pneumonia) causes (39).

As the biological processes that underlie the lung injury and their molecular drivers are not fully understood, medical therapy directed at the lung inflammatory response has yet to successfully modify the course of ARDS. Experimental animal and patient studies demonstrate the lung injury and resolution phases of ARDS are mediated through a complex orchestration of cytokines/chemokines (e.g., IL-1β, TNFα, IL-8, IL-6, and IL-10) (40–44). Studies show that both sterile (e.g., ventilator-induced stretch) and infectious [e.g., intra-tracheal (IT) LPS] triggers of ARDS result in stiffening (reduced compliance) of the lung tissue (45, 46).

The role of TRPV4 in ARDS is context/etiology-dependent. It has been shown that TRPV4 mediates the lung injury response to a sterile stimulus *in vivo* [i.e., hydrochloric acid (HCl)], as assessed by inflammatory cell influx, lung vascular permeability (wet/dry ratio, Evans blue dye extravasation, and total protein), lung histopathology and physiology, and pro-inflammatory cytokine levels (IL-1β, VEGF, KC, G-CSF, MCP-1, RANTES, MIP-2, and IL-6) (7, 14). Protection from the acute lung injury response to IT HCl was noted in mice that lack TRPV4 (TRPV4 KO), or in mice that were treated with three different small molecule inhibitors of TRPV4 (7, 14). Importantly, two of these inhibitors (GSK2220691 and GSK2337429A) show efficacy when administered 30 min after IT HCl (7). Thus, these inhibitors show promise as a novel and exciting therapeutic/preventative approach for acute lung injury (7). *In vitro* stimulation of human and murine neutrophils (with platelet-activating factor or LPS) induced TRPV4-dependent calcium influx, reactive oxygen species (ROS) production, adhesion chemotaxis, and Rac activation (14). Taken together, these data suggest that neutrophils possess the capacity to mediate acute lung injury in a TRPV4-dependent manner. Whether the *in vivo* lung injury response to HCl is solely dependent on neutrophil TRPV4, as opposed to TRPV4 in other cell types, remains to be determined. In addition to TRPV4's effect on the cytokine/inflammatory changes in ARDS, TRPV4 actions can induce lung endothelial barrier dysfunction *in vitro* and *in vivo*, as well as cause disruption of alveolar type I epithelial cells leading to lung vascular leak and alveolar edema (9, 29). These findings are the rationale for a clinical trial of TRPV4 antagonists in high venous pressure-induced pulmonary edema (https://clinicaltrials.gov).

TRPV4 AND MACROPHAGE FUNCTION IN LUNG INJURY

A similar TRPV4-dependent lung injury response has been demonstrated in macrophages in high volume ventilator-induced lung injury (6, 47). Mice lacking TRPV4 (TRPV4 KO) had less vascular leak, pulmonary edema (wet/dry ratio, filtration coefficient), and NO production in response to high volumes (peak inflation pressure 35 cm H_2O) when compared to WT controls. TRPV4 also seemed to partially mediate the increase in injury due to the combined effects of high volume ventilation and induced hyperthermia (40°C). Analysis of alveolar macrophages after high volume ventilation revealed that TRPV4 KO macrophages had less production of NO and ROS than those from WT mice. As in the HCl model, pretreatment with a non-selective TRP inhibitor (ruthenium red) prevented the increase in vascular permeability from combined high volume ventilation/hyperthermia in WT mice (48). Adoptive transfer of WT macrophages to TRPV4 KO mice reestablished the lung injury seen in WT mice. These data suggest that macrophage-specific TRPV4 acts as a mechanical and temperature sensor to initiate/mediate the acute lung injury induced by high volume ventilation (47).

Our laboratory is studying the role of TRPV4 in macrophage function during infection-associated lung injury. Alveolar macrophages are known to be effector cells in bacterial and particle clearance, and in the injury/repair process (49). We chose to explore the role of the calcium ion channel, TRPV4, in macrophage phagocytosis, as intracellular calcium is known to be required for the phagocytic process, and because TRPV4 plays a role in force-dependent cytoskeletal changes in other systems/cell types (7–9, 29, 47, 50, 51). Studies show that the process of phagocytosis in macrophages requires integration of the signals from macrophage surface receptors, pathogens, and the extracellular matrix (52–54). However, the effects of matrix stiffness on the macrophage phenotypic response or its signal transduction pathways have yet to be fully elucidated.

We recently published the novel observation that TRPV4 integrates the LPS and matrix stiffness signals to control macrophage function, which promotes host defense and resolution from lung injury (4). After demonstrating that TRPV4 is expressed and functionally active in murine bone marrow-derived macrophages, we studied the macrophage response to LPS on matrices of varying physiological-range stiffnesses. We demonstrated that TRPV4 mediates LPS-stimulated macrophage phagocytosis of both opsonized particles (IgG-coated latex beads) and non-opsonized particles (*Escherichia coli*) *in vitro*. Matrix stiffness in the range seen in inflamed or fibrotic lung (>25 kPa) augmented the LPS phagocytic response by 151 ± 3% (4). Inhibition of TRPV4 by siRNA or pharmacologic inhibitors completely abrogated both the LPS effect, as well as the matrix stiffness effect, on phagocytosis. These data indicate that both the LPS and stiffness effect on macrophage phagocytosis are TRPV4 dependent (4).

As TRPV4 is required for macrophage phagocytosis *in vitro* in a stiffness-dependent manner, we next sought to examine the role of TRPV4 on macrophage phagocytosis after intratracheally (IT) administered LPS *in vivo*. Despite the influx of neutrophils, alveolar macrophages were the predominant cell type that phagocytosed IT administered IgG-coated beads following IT LPS (24 h) in WT mice (4). As seen *in vitro*, the *in vivo* enhancement effect of IT LPS on alveolar macrophage phagocytosis was lost upon deletion of TRPV4 (TRPV4 KO mice) (**Figure 1**) (4). This effect is not explained by a difference in macrophage recruitment. Concordant with the *in vitro* data, our *in vivo* data demonstrate that LPS-induced alveolar macrophage phagocytosis is TRPV4 dependent.

Studies suggest that macrophage-released cytokines modulate bacterial clearance and the lung injury/repair process, in the context of injury-related stiffened matrix (52–55). Recognizing the complexity of tissue responses to individual cytokines/chemokines, we chose to focus initially on IL-1β and IL-10, as they are well-known key mediators of lung injury/resolution (56–58). TRPV4 also modulates the LPS signal for cytokine production. Specifically, IL-1β secretion was decreased by half,

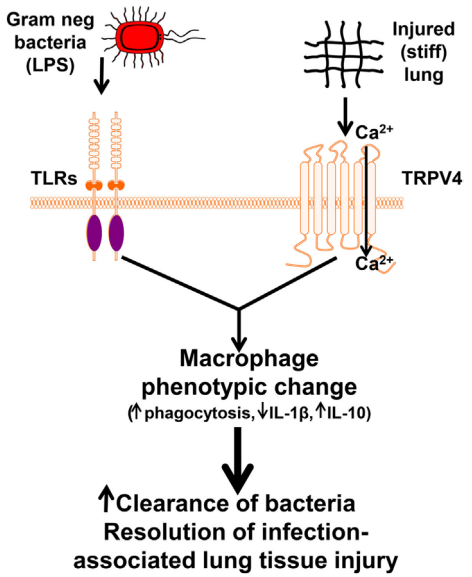

FIGURE 1 | Working model illustrating that lipopolysaccharide (LPS) and transient receptor potential vanilloid 4 (TRPV4) signal cooperate to alter macrophage phenotypic change leading to enhanced clearance of bacteria and resolution of lung injury. Our data suggest that TRPV4 is sensitized by extracellular matrix stiffness in the range of inflamed/fibrotic lung. Interaction between the LPS signal and the matrix stiffness signal through TRPV4 promote increased TRPV4 channel activity and macrophage phenotypic change leading to increased clearance of bacteria and resolution of infection-associated lung injury (4). Copyright 2016, The American Association of Immunologists, Inc.

and IL-10 secretion increased approximately twofold in WT alveolar macrophages compared with TRPV4 KO macrophages in response to LPS. Such a profile would predict that TRPV4 mediates a net inflammation-suppressive response to LPS. Interestingly, this TRPV4 modulation of the LPS signal required a matrix stiffness in the range of injured or fibrotic lung (≥25 kPa). As illustrated in the schematic model, macrophage TRPV4 is sensitized by a stiff matrix (as seen in ARDS) to modulate the infectious (LPS—experimental surrogate for Gram-negative bacterial lung infection) signal toward an anti-inflammatory macrophage phenotype (**Figure 1**).

Collectively, our data demonstrate that TRPV4 responds to extracellular matrix stiffness, thereby altering the LPS signal to mediate macrophage phagocytosis and cytokine production (4). Despite the limitations in extrapolating our simplified experimental system to *in vivo* lung injury, the data point to TRPV4 as an important mechanosensor that mediates macrophage function differently in lung homeostasis, and in the context of pulmonary infection-induced inflammation. We speculate that under basal conditions, the resident lung macrophage response to LPS is modified (less phagocytic, more IL-1β) as a consequence of low lung tissue stiffness (i.e., 1–3 kPa) thereby enhancing recruitment of professional bactericidal cells (neutrophils) (55). After an acute inflammatory or infectious insult, a separate population of monocytes is recruited from the bone marrow to populate both interstitial and injured alveolar compartment in the context of denuded, exposed interstitial matrix (40). There are two overlapping phases of ARDS. During the initial injury phase (days 1–10), lung tissue is predominantly edematous and exudative, while during the fibroproliferative phase (days 7–28), there is increased deposition of interstitial and alveolar type I and III collagen (40). Both phases of ARDS (fibroproliferative > acute) exhibit clear evidence of increased stiffness at the whole organ level (40, 46, 59), but, limited mechanical data are available at the cellular level of resolution. A recent study shows that lung alveolar vessel wall stiffness is increased >10-fold (3 versus 43 kPa) after IT LPS (48 h) in mice compared to controls, as measured by atomic force microscopy, well within the range examined in our study (>8–25 kPa) (45, 46). We further speculate that, after injury, the macrophage phagocytic response to LPS is upregulated along with a cytokine profile that promotes resolution in a TRPV4-dependent manner as a consequence of tissue stiffening. Such a scenario would support tissue stiffness, TRPV4-dependent shift in the macrophage phenotype that is commensurate with the appropriate phase of the injury/repair process.

Thus, our findings suggest that TRPV4 regulates a feed-forward mechanism of phagocytosis in activated lung tissue macrophages when they interact with stiffened infection/injury-associated lung matrix. This concept is further supported by the observation that surfactant protein B-deficient mice have altered alveolar macrophage shape and function in association with increased alveolar surface tension (60). The macrophage activation phenotypes (M1/M2 classification) are well established *in vitro*. The classically activated M1 macrophage phenotype, induced by INFγ, TNFα, and LPS, exhibits inflammatory/bactericidal properties. In contrast, the alternatively activated M2 macrophage phenotype, induced by IL-4 and IL-13, exhibits tissue repair/fibrotic properties (49, 55). Data are emerging that the *in vivo* macrophage phenotypes are more heterogeneous and plastic than the *in vitro* derived M1/M2 classification. Our published cytokine data (↑IL-1β, ↓IL-10) with inhibition of TRPV4 indeed suggests that TRPV4 mediates polarization toward M1-like phenotype (4, 61, 62). However, a complex array of cytokines contributes to the pathogenesis of ARDS, and targeting individual cytokines has not been shown to alter the disease process, indicating the net inflammatory balance is important (41–44, 63).

Our findings regarding the role of TRPV4 in downregulating the pro-inflammatory, bacterial clearance-inducing LPS signal are opposite to those in neutrophils in response to sterile inflammation, or in macrophages upon stretch-induced tissue injury. Lung injury is dependent on cytokine production and inflammatory cell influx in response to activation of pattern recognition receptors by damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs). There are multiple known ligand–receptor interactions and intracellular signaling pathways that are both DAMP/PAMP-receptor specific and overlapping. We speculate that differences in the interaction of TRPV4 signals with infectious PAMP signals versus sterile tissue injury DAMP signals might explain the differences between our infectious model and the sterile lung injury model. Defining the specific molecular pathways and interactions in individual injury models is a fruitful avenue of research that may lead to novel therapeutic targets.

TABLE 1 | *In vitro* and *in vivo* studies of the role of transient receptor potential vanilloid 4 (TRPV4) in inflammatory pulmonary diseases.

Disease	Cell type	Key findings	Reference
Asthma	Fibroblasts	Transforming growth factor-β-dependent airway remodeling	(30)
	Smooth muscle cells	Hypotonicity-induced calcium and contractile response	(12, 32)
Cystic fibrosis (CF)	Epithelial cells (tracheal and airway)	Regulates ciliary beat frequency Decreased ATP-induced calcium influx Pro-inflammatory cytokine production (e.g., IL-8, cytosolic PLA2, prostaglandin E2, NF-κB, arachidonic acid, etc.)	(10, 33–37)
Acute lung injury/acute respiratory distress syndrome (ARDS)	Epithelial cells	Maintains epithelial barrier function	(9, 29)
	Endothelial cells	Maintains endothelial septal barrier	(11)
	Neutrophils	Calcium influx Reactive oxygen species production Adhesion chemotaxis Rac activation	(7, 14)
	Macrophages	Lipopolysaccharide-induced macrophage phagocytosis *in vitro* and *in vivo* Anti-inflammatory cytokine production (IL-1β, IL-10)	(4, 6, 47, 48)
Pulmonary fibrosis	Fibroblasts	Myofibroblast differentiation Experimental pulmonary fibrosis in mice	(5)

This table is only a partial representation of the literature, given the focused nature of the mini review. We apologize for any work omitted from this review. We summarize the cited literature on the role of TRPV4 in asthma, CF, acute lung injury/ARDS, and pulmonary fibrosis.

SUMMARY

In summary, ion channels are important in the pathogenesis of inflammatory lung diseases, and the ion channel TRPV4 plays a specific role in mediating lung diseases associated with parenchymal stretch and inflammation or infection. The data reviewed in this work on the role of TRPV4 in pulmonary inflammatory diseases are summarized in **Table 1**. TRPV4 activation and its downstream signaling pathways differ in response to varying stimuli, cell types, and contexts. In asthma, TRPV4 mediates hypotonicity-induced airway hyperresponsiveness, but not release of Th2 cytokines (12, 32). In CF, TRPV4 appears to play important, yet paradoxical, roles in CBF/mucociliary clearance and epithelial cell pro-inflammatory cytokine (IL-8/KC) secretion (35, 36). TRPV4 may also play different roles in ARDS depending on the underlying etiology (4, 7, 14, 48). We, and others, have shown that macrophage and neutrophil TRPV4 regulate pro-inflammatory cytokine secretion. Lastly, in pulmonary fibrosis, TRPV4 has been shown to mediate the mechanosensing that drives myofibroblast differentiation and experimental lung fibrosis in mice (5). Collectively, TRPV4 is shown to play a novel role in modulating cytokine secretion and pulmonary inflammation and therefore may be involved in the pathogenesis of many respiratory diseases.

AUTHOR CONTRIBUTIONS

RS, BS, LG, and MO reviewed the literature and wrote the paper.

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Hypercapnia Impairs ENaC Cell Surface Stability by Promoting Phosphorylation, Polyubiquitination and Endocytosis of β-ENaC in a Human Alveolar Epithelial Cell Line

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Acute lung injury is associated with formation of pulmonary edema leading to impaired gas exchange. Patients with acute respiratory distress syndrome (ARDS) require mechanical ventilation to improve oxygenation; however, the use of relatively low tidal volumes (to minimize further injury of the lung) often leads to further accumulation of carbon dioxide (hypercapnia). Hypercapnia has been shown to impair alveolar fluid clearance (AFC), thereby causing retention of pulmonary edema, and may lead to worse outcomes; however, the underlying molecular mechanisms remain incompletely understood. AFC is critically dependent on the epithelial sodium channel (ENaC), which drives the vectorial transport of Na^+ across the alveolar epithelium. Thus, in the current study, we investigated the mechanisms by which hypercapnia effects ENaC cell surface stability in alveolar epithelial cells (AECs). Elevated CO_2 levels led to polyubiquitination of β-ENaC and subsequent endocytosis of the α/β-ENaC complex in AECs, which were prevented by silencing the E3 ubiquitin ligase, Nedd4-2. Hypercapnia-induced ubiquitination and cell surface retrieval of ENaC were critically dependent on phosphorylation of the Thr615 residue of β-ENaC, which was mediated by the extracellular signal-regulated kinase (ERK)1/2. Furthermore, activation of ERK1/2 led to subsequent activation of AMP-activated protein kinase (AMPK) and c-Jun N-terminal kinase (JNK)1/2 that in turn phosphorylated Nedd4-2 at the Thr899 residue. Importantly, mutation of Thr899 to Ala markedly inhibited the CO_2-induced polyubiquitination of β-ENaC and restored cell surface stability of the ENaC complex, highlighting the critical role of Nedd4-2 phosphorylation status in targeting ENaC. Collectively, our data suggest that elevated CO_2 levels promote activation of the ERK/AMPK/JNK axis in a human AEC line, in which ERK1/2 phosphorylates β-ENaC whereas JNK mediates phosphorylation of Nedd4-2, thereby facilitating the channel–ligase interaction. The hypercapnia-induced ENaC dysfunction may contribute to impaired alveolar edema clearance and thus, interfering with these molecular mechanisms may improve alveolar fluid balance and lead to better outcomes in patients with ARDS.

Keywords: carbon dioxide, epithelial sodium channel, sodium transport, ubiquitination, alveolar fluid clearance, alveolar epithelium, mitogen-activated protein kinase signaling

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INTRODUCTION

Carbon dioxide (CO_2) is formed as a by-product of cellular respiration and is eliminated from the body during breathing (1). In respiratory disorders that are associated with alveolar hypoventilation, retention of CO_2 is often detected, which leads to elevated CO_2 concentrations in the blood, also known as hypercapnia (2). For example, patients with severe acute respiratory distress syndrome (ARDS) frequently present with hypercapnia, which may be enhanced due to mechanical ventilation with low tidal volumes to minimize further ventilator-induced injuries to the lung (3). It is increasingly evident that the alveolar epithelium is capable of sensing of elevated CO_2 levels, which initiate specific signaling signatures and alter the function of alveolar epithelial and other cells (2, 4). While some of these effects are anti-inflammatory, which may be beneficial in the context of excessive inflammation, others impair innate immunity, mitochondrial function, cellular repair, and alveolar epithelial barrier function, which are clearly detrimental in the setting of ARDS (5–10).

A fully functional alveolar epithelial barrier is crucial for maintaining optimal fluid balance and gas exchange in the lung (11, 12). In order to keep the alveolar space "dry," excess alveolar liquid is reabsorbed from the air space into the interstitium by a well-characterized active sodium transport process in which Na^+ enters the alveolar epithelial type I and type II cells through the apically located epithelial sodium channel (ENaC) and is subsequently pumped out basolaterally by the Na,K-ATPase. This creates a Na^+ gradient, which drives paracellular movement of water leading to its clearance from the alveolar space (11, 12). Importantly, it has been clearly demonstrated that in most patients with ARDS alveolar fluid clearance (AFC) is impaired and that those patients with ARDS and impaired AFC the mortality is significantly higher than in ARDS patients with normal AFC (13).

Other than in the alveolar epithelium, ENaC molecules are expressed in the apical surface of various tight epithelia including kidney, colon, and respiratory airways where ENaC is located along the entire length of motile cilia and regulate osmolarity of the periciliary fluid (14). ENaC usually consists of three subunits (α [or δ, depending on the species, tissue, and cell type], β, and γ) (15–17). A functional ENaC complex requires at least one α- or δ-subunit, whereas the β- and γ-subunits are necessary for proper trafficking and activity of the channel (15, 16, 18). In line with this notion, mice lacking α-ENaC are unable to clear lung fluid from the alveoli and die nearly immediately after birth (19). The significance of β-ENaC in the regulation of channel activity has been highlighted in transgenic mice overexpressing this subunit in the lung (20). In these animals, an increase in alveolar epithelial Na^+ uptake due to β-ENaC overexpression, probably by promoting trafficking of ENaC to the cell surface and enhancing channel activity, leads to lung dehydration and causes a CF-like phenotype. Cell surface abundance of ENaC is modified by the E3 ubiquitin ligase Nedd4-2, which by interaction with the PY motif, located at the C-termini of each subunit of the channel, promotes ubiquitination and subsequent clathrin-mediated endocytosis of the channel (21–23). Nedd4-2$^{-/-}$ mice have enhanced ENaC expression and function; however, this genetic manipulation has lethal consequences (24). In contrast, overexpression of the ligase causes a decrease in ENaC density at the plasma membrane (PM) and reduces Na^+ transport (25).

Previous studies have proposed the involvement of phosphorylation in the mechanisms regulating Nedd4-2 binding to ENaC (26, 27). For example, extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and recently also the cellular energy sensor, AMP-activated protein kinase (AMPK), have been described as potential modulators of the ENaC/Nedd4-2 interaction by phosphorylating the E3 ligase or the target (28–32). We have previously described that hypercapnia markedly impairs AFC and initiates a specific signaling pattern in the alveolar epithelium, including rapid activation of ERK, AMPK, and JNK and subsequent downregulation of the Na,K-ATPase (7, 8, 33). Considering the pivotal role of ENaC in AFR and that several kinases, which have previously been suggested to alter ENaC/Nedd4-2 interaction, are activated by elevated CO_2 levels, in the current study we sought to determine whether ENaC is effected by hypercapnia and provide evidence that excess CO_2 initiates ERK-mediated β-ENaC phosphorylation and AMPK/JNK-dependent activation of Nedd4-2 leading to an enhancement of β-ENaC polyubiquitination and, thus, to endocytosis of the ENaC complex form the cell surface. Since ENaC activity is essential for optimal lung fluid balance, the hypercapnia-induced alterations in ENaC cell surface stability may cause further aggravation of lung injury.

MATERIALS AND METHODS

Cell Culture

Human epithelial A549 cells (ATCC, CCL 185) were grown in DMEM supplemented with 10% fetal bovine serum and 100 U/ml penicillin, 100 μg/ml streptomycin as previously described (8). Experiments were performed on subconfluent monolayers of cells. Cells were incubated in a humidified atmosphere of 5% CO_2/95% air at 37°C.

CO_2 Exposure

A549 cells were treated with 40 or 120 mmHg CO_2 (normocapnia and hypercapnia, respectively). Before each experiment, fresh solutions were prepared with DMEM-Ham's F-12 medium and Tris base. The buffering capacity of the experimental media was modified by changing the initial pH using Tris base to obtain a pH of 7.4 at 40 and 120 mmHg CO_2 (8). The desired CO_2 concentrations and pH levels were obtained by equilibrating the experimental media overnight in a humidified chamber from BioSpherix Ltd. (NY, USA). The C-Chamber's atmosphere was controlled with a PRO-CO_2 Carbon Dioxide controller (Biospherix Ltd.). In the chamber, cells were treated with a pCO_2 of 40 or 120 mmHg while keeping 21% O_2 balanced with N_2. Before and after CO_2 exposure, pH, pCO_2, and pO_2 levels in the media were measured using a Rapidlab blood gas analyzer (Siemens, Erlangen, Germany).

Plasmids, Constructs, Site-Directed Mutagenesis, Antibodies, and Inhibitors

pEYFP-C1-expressing α-ENaC was constructed by PCR amplifying α-ENaC gene using as a template pTNT-α-ENaC and

oligonucleotide primers α-ENaC forward 5′-GAATTCAATGG-AGGGGAACAAGCTGGAGG-3′ and α-ENaC reverse 5′-GGATCCCTTGTCATCGTCATCCTTGTAATCGGGCCCC CCCAGAGGAC-3′. The resulting amplicon was digested with *Eco*RI/BamH1 and ligated to the multiple cloning site (MCS) of pEYFP-C1 plasmid. The pEYFP-C1 vector contained the epitope-tag eYFP at the N-terminus and a FLAG-tag at the C-terminus. Thus, anti-GFP or anti-FLAG antibodies recognize the α-ENaC construct at a predicted size of 118 kDa, 1,073 amino acids [α-ENaC (90 kDa) plus YFP (27 kDa) and FLAG (1 kDa)]. pcDNA3.1V5/His expressing β-ENaC was constructed by PCR amplifying β-ENaC gene using as a template cDNA transcribed from total mRNA isolated from A549 cells and oligonucleotide primers β-ENaC forward 5′-CTCGGATCCACATGCACGTGAAGAAGTACCT-3′ and β-ENaC reverse 5′-GCACTCGAGGATGGCATCACCCT-CACTGT-3′. The resulting amplicon was digested with Xho1/BamH1 and ligated to MCS of pcDNA3.1V5/His plasmid. Finally, *E. coli* DH5α were transformed using the constructed plasmid. Anti-V5 antibodies recognize the β-ENaC construct at a predicted size of 96 kDa, 872 amino acids [β-ENaC plus V5 at the C-terminus (1 kDa)]. pCMV-HA-C-expressing γ-ENaC was constructed by PCR amplifying γ-ENaC gene using as a template pTNT-γENaC and oligonucleotide primers γ-ENaC forward 5′-AGGCCCGAATTCATGGCACCCGGAGAGAAGAT-3′ and γ-ENaC reverse 5′-GTAGCCGGTACCGAGCTCATC-CAGCATCTGGG-3′. The resulting amplicon was digested and ligated to MCS of pCMV-HA-C plasmid. The pCMV-HA-C vector contained the epitope-tag myc at the N-terminus and an HA-tag at the C-terminus. Anti-HA antibodies recognize γ-ENaC at a predicted size of 97 kDa, 881 amino acids [γ-ENaC plus myc (1 kDa) and HA (1 kDa)]. pRK5-HA-ubiquitin was a gift from Ted Dawson [Addgene 17608 (34)] and the pCI HA NEDD4L plasmid was a gift from Joan Massague [Addgene 27000 (35)]. Site-directed mutagenesis was used to perform point mutation of T899A in human Nedd4-2 using Quick Change Mutagenesis Kit from Stratagene (La Jolla, CA, USA) in accordance to the manufacturer's instructions. The primer sequences were as follows: Nedd4-2 forward: 5′-ACTGCAGTTTGTCGCAGGGACATCG-CGAG-3′, Nedd4-2 reverse: 5′ CTCGCGATGTCCCTGCGAC-AAACTGCAGT-3′. Immunoblot analysis of epitope-tagged ENaC expressed in A549 cells were performed with a mouse anti-GFP antibody from Roche (Basel, Switzerland), a mouse anti-HA antibody (Covance, Princeton, NJ, USA), a mouse anti-V5 antibody and a mouse antibody against transferrin receptor used as loading control of biotinylated proteins from Invitrogen (Waltham, MA, USA; Figure S1 in Supplementary Material). A rabbit antibody directed against β-actin was used as loading control of cytoplasmic ENaC and was purchased from Sigma Aldrich (Saint Louis, MO, USA). The inhibitor of AMPK, Compound C, was from Merck Millipore (Darmstadt, Germany). The inhibitor of MEK, U0126 was from Promega (Fitchburg, WI, USA). siRNA against AMPK-α1 and Nedd4-2 and scrambled siRNA control were purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

Transient Transfection

A549 cells were transiently transfected with eYFP-α-ENaC, β-ENaC-V5, HA-ubiquitin, HA-Nedd4-2 wild type, or mutant by using nucleofection, as previously described (36). Briefly, cells were resuspended in 100 µl of the nucleofection solution SF (Lonza, Cologne, Germany), and 4–6 µg of DNA was added. Cells were placed in a cuvette and pulsed with the specified cell-type nucleofector program. After 10 min of incubation time, cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. In some studies, 24 h before nucleofection with ENaC plasmids, cells were transfected with siRNA using Lipofectamine RNAiMAX (Invitrogen, Waltham, MA, USA) according to the instructions of the manufacturer. Experiments were performed 48 h later.

Cell Surface Biotinylation

A549 cells were labeled for 20 min using 1 mg/ml EZ-Link *N*HS-SS-biotin (Pierce Biotechnology, Waltham, MA, USA) and lysed in lysis buffer (50 mM HEPES, 150 mM NaCl, 1 mM EGTA, 10% glycerol, 1% TritonX100). Surface proteins were pulled down with streptavidin-agarose beads from Pierce Biotechnology (Waltham, MA, USA) and analyzed by SDS-PAGE and immunoblot, as described previously (8).

Ubiquitination Studies

A549 cells were transfected with ENaC plasmids (2 µg of each) and HA-ubiquitin (3 µg). In some studies, cells were co-transfected with a plasmid coding HA-Nedd4-2 (wild type or mutant T899A, 2 µg) or siRNAs (against AMPK-α1, Nedd4-2, or scrambled). Cells were exposed to 40 mmHg CO_2 (Ctrl) or to 120 mmHg CO_2 (CO_2) for 15 or 30 min and lysed on ice in lysis buffer (50 mM HEPES, 150 mM NaCl, 1 mM EGTA, 10% glycerol, 1% TritonX100), containing a protease inhibitor cocktail from Roche. After lysing the samples, proteins were resolved in 8% polyacrylamide gel, transferred to nitrocellulose membrane (Optitran; Schleicher & Schuell, Dassel, Germany) using a semidry apparatus from Bio-Rad (Hercules, Berkeley, CA, USA). Membranes were blocked in 5% fat-free dried milk powder and immunoblotted with anti-GFP or anti-V5 to detect α- or β-ENaC, respectively. Films were overexposed to detect ENaC ubiquitin conjugates.

Phosphorylation Experiments

Phosphorylation studies of ERK1/2, AMPK-α1, and c-Jun were performed using antibodies from Cell Signaling (Danvers, MA, USA). The anti-phospho-β-ENaC (T615) antibody was from Abcam (Cambridge, UK). A549 cells were treated with normal or elevated CO_2 concentrations (40 or 120 mmHg, respectively) for the desired times, and then were washed with PBS twice and were lysed on ice in lysis buffer (50 mM HEPES, 150 mM NaCl, 1 mM EGTA, 10% glycerol, 1% TritonX100). Samples having the same amount of protein were resuspended in Laemmli sample buffer and boiled for 10 min at 98°C and immunoblotted with specific antibodies.

Statistics

Data are presented as mean ± SEM and were analyzed using one-way analysis of variance (ANOVA) followed by a multiple comparison with the Dunnet test. p values of less than 0.05 were considered significant. GraphPad prism 6 (GraphPad software, San Diego, CA, USA) was used for the analysis and presentation of data.

RESULTS

Acute Exposure to Elevated CO_2 Levels Leads to ENaC Endocytosis by Promoting Polyubiquitination of β-ENaC

To test whether high CO_2 levels promote endocytosis of ENaC, A549 cells were co-transfected with plasmids encoding the human α- and β-subunit of ENaC and the PM abundance of these proteins was measured after exposure of cells to physiological (pCO_2 40 mmHg; normocapnia) or elevated (pCO_2 120 mmHg; hypercapnia) CO_2 concentrations at a pH_e of 7.4 for 30 min. Exposure of cells to elevated CO_2 levels decreased α- and β-ENaC cell surface abundance by approximately 60% (**Figure 1A**), whereas the total protein level remained unaffected (**Figure 1B**). To determine whether elevated CO_2 levels lead to ubiquitination of either ENaC subunit, A549 cells were co-transfected with α- or β-ENaC and ubiquitin containing HA-tag (HA-Ub) and exposed the cells to 40 or 120 mmHg CO_2 for 15 min. Whereas no ubiquitination of α-ENaC in response to hypercapnia was

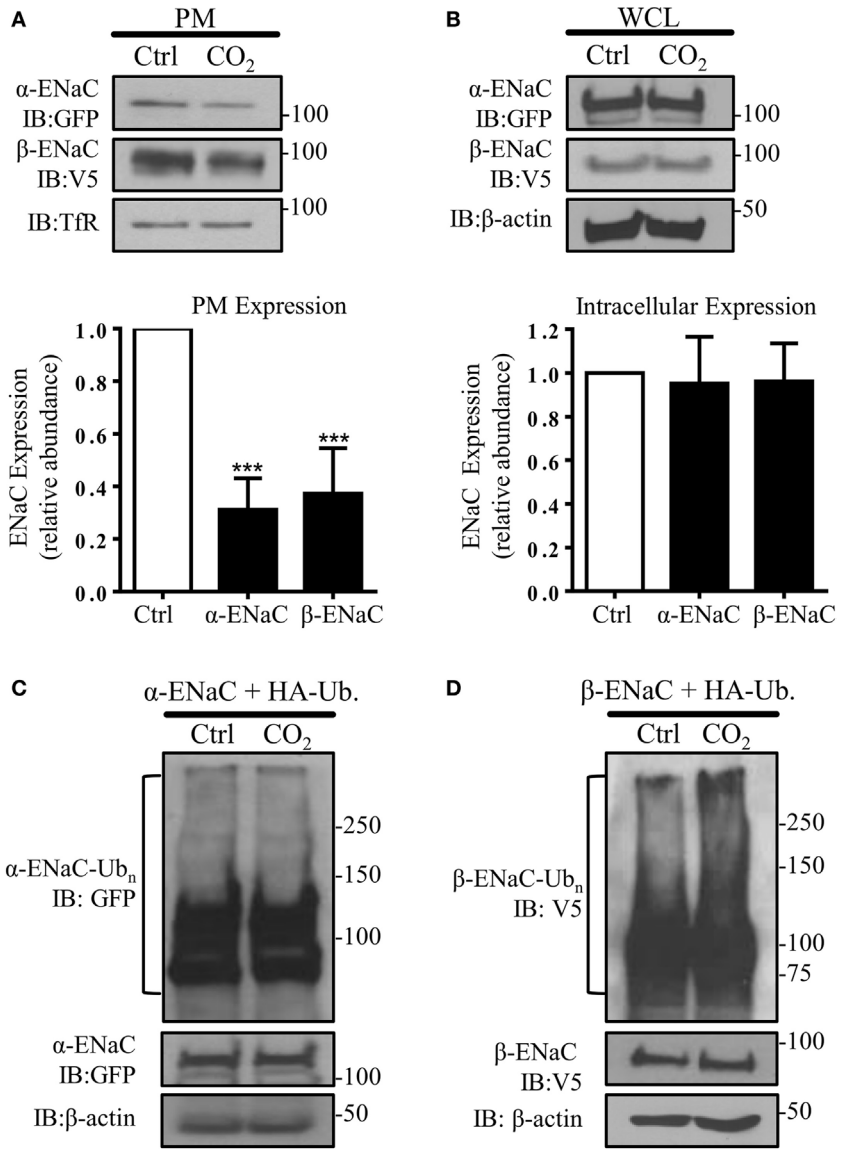

FIGURE 1 | Acute exposure to elevated CO_2 levels leads to epithelial sodium channel (ENaC) endocytosis by promoting polyubiquitination of β-ENaC. **(A)** A549 cells were co-transfected with α- and β-ENaC and were exposed to 40 mmHg CO_2 (Ctrl) or 120 mmHg CO_2 (CO_2) for 30 min at a pH_e of 7.4. Plasma membrane (PM) proteins were determined by streptavidin pull-downs and immunoblotting with anti-GFP to detect α-ENaC and anti-V5 to detect β-ENaC. Representative immunoblots of α-, β-ENaC, and transferrin receptor (TfR) at the PM are shown. **(B)** A549 cells were co-transfected with α- and β-ENaC and were treated as described above. Protein abundance in whole cell lysate (WCL) was measured by immunoblotting. Representative immunoblots of α-, β-ENaC, and β-actin are shown. Bars represent mean ± SEM [*n* (number of independent experiments) = 3; ***$p < 0.001$]. **(C,D)**, A549 cells were co-transfected with ubiquitin containing HA-tag (HA-Ub) and α-ENaC **(C)** or β-ENaC **(D)** and were exposed to 40 or 120 mmHg CO_2 for 15 min. Total ubiquitinated α-ENaC and β-ENaC was detected by immunoblots with anti-GFP or anti-V5 antibody.

evident (**Figure 1C**), a marked increase in β-ENaC ubiquitination in total cell lysates was observed when cells were treated with elevated CO_2 and immunoblotted with an antibody against V5 (**Figure 1D**). We detected a "polyubiquitin smear" above the molecular size of ENaC suggesting the presence of β-ENaC ubiquitin conjugates. This observation suggested that β-ENaC is a substrate of CO_2-induced ubiquitination.

Nedd4-2 Mediates the Hypercapnia-Induced ENaC Polyubiquitination and Endocytosis

In subsequent studies, we silenced the endogenous Nedd4-2 with a specific siRNA to study whether Nedd4-2 mediates β-ENaC polyubiquitination during hypercapnia. Of note, the elevated CO_2-induced ENaC ubiquitination was prevented by Nedd4-2 silencing (**Figure 2A**). To further test whether Nedd4-2 silencing altered ENaC PM stability, we measured cell surface ENaC abundance in A549 cells co-transfected with a scrambled siRNA (si-Scr.) or siRNA against Nedd4-2. Importantly, cells exposed to increased CO_2 concentrations treated with siRNA targeting Nedd4-2 had increased ENaC α- and β-subunit density at the PM (**Figure 2B**). Thus, upon hypercapnia, Nedd4-2 targets β-ENaC, leading to polyubiquitination of the β-subunit of the channel, which results in decreased abundance of the α/β-ENaC complex at the cell surface.

Hypercapnia Induces ERK1/2-Dependent Phosphorylation of β-ENaC at Thr615 and Downregulates Surface Abundance of the Channel by Facilitating β-ENaC Polyubiquitination and Endocytosis of the α/β-ENaC Complex

In agreement with a previous report describing increased ERK 1/2 activity in alveolar epithelial cells (AECs) exposed to hypercapnia (33), we found a rapid and transient phosphorylation of ERK1/2 in A549 cells exposed to elevated CO_2 (**Figure 3A**). Moreover, ERK1/2 activation was paralleled by phosphorylation of β-ENaC at the Thr 615 residue (**Figure 3B**). To test whether ERK-dependent β-ENaC phosphorylation was sufficient to promote polyubiquitination of β-ENaC in response to high CO_2, we co-transfected A549 cells with β-ENaC and HA-ubiquitin. Cells were pre-treated with the MEK (upstream of ERK) inhibitor U0126 and exposed to elevated CO_2 concentrations for 30 min. Inhibition of ERK prevented phosphorylation and polyubiquitination of the ENaC β-subunit (**Figure 3C**). To further prove that the hypercapnia-induced ENaC cell surface retrieval is dependent on ERK1/2, A549 cells were co-transfected with ENaC plasmids, and exposed to normo- or hypercapnia as described above and observed that inhibition of ERK1/2 markedly increased the number of the ENaC molecules at the cell surface upon hypercapnia exposure (**Figure 3D**). Together, these data indicate that the hypercapnia-induced ERK1/2 activation promotes ENaC internalization by phosphorylation-dependent ubiquitination of the β-subunit of the channel.

JNK1/2-Dependent Nedd4-2 Phosphorylation at Thr899 Facilitates β-ENaC Polyubiquitination and Endocytosis

Because JNK activation has been implicated in the CO_2-induced signaling pattern in AEC, which led to inhibition of the Na, K-ATPase (7), we next investigated the effects of JNK phosphorylation in AEC exposed to hypercapnia on ENaC. Activity of JNK1/2 was assessed by phosphorylation of c-Jun, a downstream target of JNK1/2. In line with the previously published data, we observed a rapid and time-dependent JNK activation induced by hypercapnia, which returned to baseline within 30 min of exposure to elevated CO_2 levels (**Figure 4A**). To further investigate whether increased activity of Nedd4-2 is crucial to decrease hypercapnia-induced ENaC cell surface abundance, we next mutated a single amino acid in the catalytic domain of Nedd4-2 (T899A). The Thr899 residue within the HECT domain of the E3 ligase has previously been reported to be involved in the Nedd4-2-mediated ubiquitination of α-ENaC (37). A549 cells were co-transfected with HA-Ub and HA-Nedd4-2 wild type (WT) or HA-Nedd4-2 mutant (T899) constructs and exposed to 40 or 120 mmHg CO_2 for 30 min. Of note, we found that phosphorylation of Thr899 played a central role in the hypercapnia-induced ubiquitination of β-ENaC, as in A549 cells expressing the Nedd4-2 in which the Thr899 has been mutated to an alanine (T899A), which cannot be phosphorylated, the level of β-ENaC polyubiquitination significantly decreased (**Figure 4B**). To further investigate whether this decrease in the ubiquitination of β-ENaC due to the lack of phosphorylation at the Thr899 residue of Nedd4-2 correlated with an increase in ENaC cell surface stability, cell surface biotinylation studies were performed. Importantly, overexpression of the Nedd4-2 mutant (T899A) also prevented endocytosis of the α/β-ENaC complex during hypercapnia (**Figure 4C**). Moreover and further confirming the central role of Nedd4-2 phosphorylation at Thr899 in the ubiquitination and subsequent endocytosis of ENaC, we observed increased levels of ENaC proteins at the cell surface after overexpression of the Nedd4-2 T899A mutant. Finally and in line with the above described findings, pretreatment of A549 cells with the potent and specific JNK inhibitor, SP600125, also fully prevented the hypercapnia-induced endocytosis of ENaC (**Figure 4D**).

Hypercapnia Induces ENaC Endocytosis by ERK1/2-Dependent AMPK-α1 Activation

AMP-activated protein kinase, which has been shown to activate Nedd4-2 and inhibit ENaC (29), has also been described as one of the central mediators of the hypercapnia-induced alveolar epithelial dysfunction and a downstream target of ERK upon CO_2 exposure (8, 33). Furthermore, we have previously observed that AMPK activates JNK1/2 in AEC when exposed to elevated CO_2 levels (7). In line with these previously published observations, we measured a rapid and transient phosphorylation of AMPK-α1 in A549 cells exposed to hypercapnia (**Figure 5A**), which was dependent on activation of ERK (**Figure 5B**) and

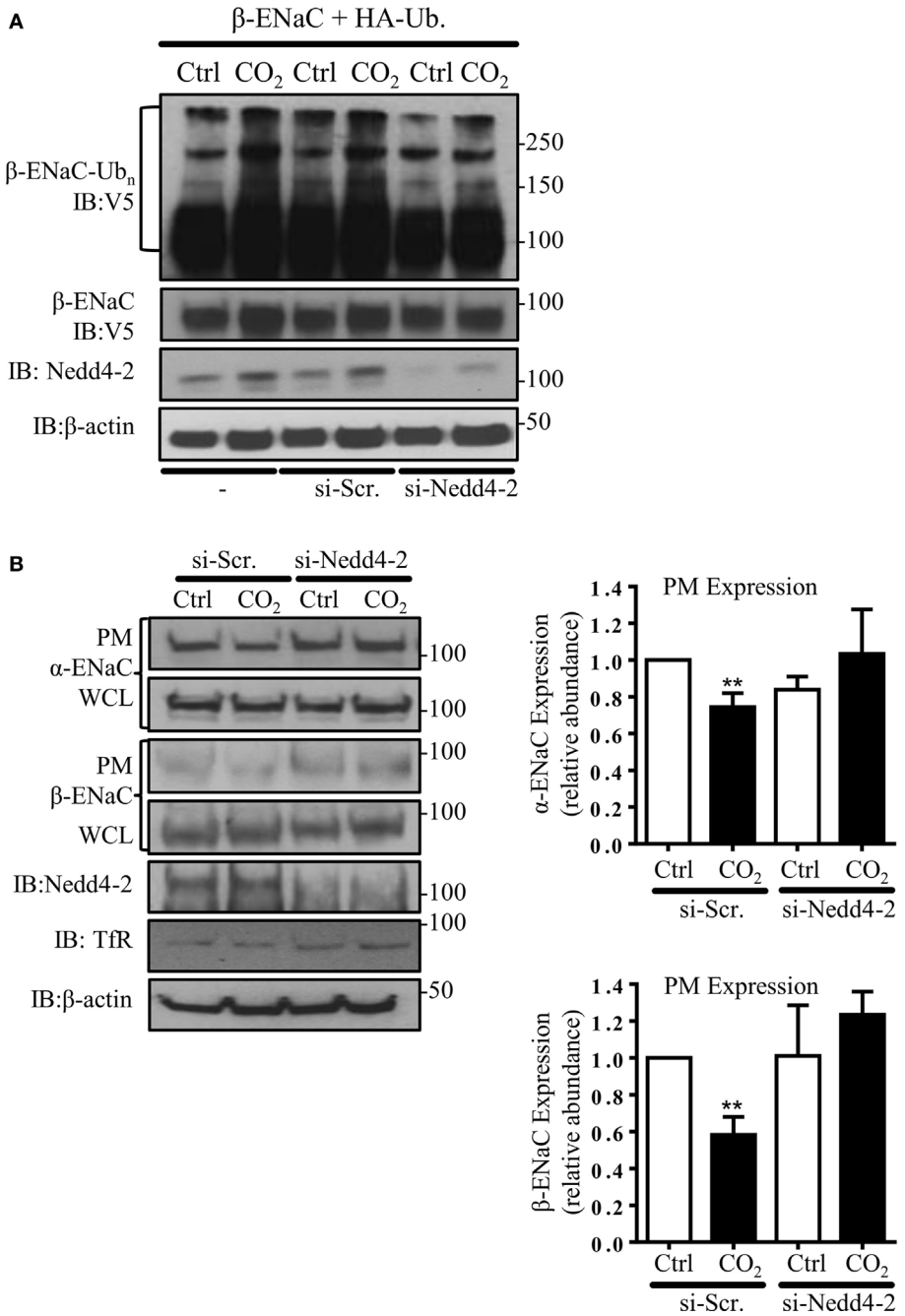

FIGURE 2 | **Nedd4-2 mediates hypercapnia-induced epithelial sodium channel (ENaC) polyubiquitination and endocytosis**. **(A)** A549 cells were co-transfected with β-ENaC, HA-ubiquitin, and siRNA against Nedd4-2 or a scrambled siRNA (si-Scr.) and were treated with 40 or 120 mmHg CO_2 for 30 min and β-ENaC polyubiquitinated isoforms were determined. **(B)** A549 cells were co-transfected with α-, β-ENaC, and siRNA targeting Nedd4-2 or scrambled siRNA. Biotinylated ENaC proteins were detected by immunoblotting. Representative immunoblots of α-, β-ENaC, and transferrin receptor (TfR) at the plasma membrane (PM) and total protein abundance [whole cell lysate (WCL)] of ENaC proteins, β-actin, and Nedd4-2 are shown. Bars represent mean ± SEM ($n = 3$; ** $p < 0.01$).

upstream of JNK (Figure S2 in Supplementary Material). To determine whether activation of AMPK was necessary for the hypercapnia-induced polyubiquitination of β-ENaC, A549 cells were co-transfected with β-ENaC, HA-ubiquitin, and a specific siRNA against AMPK-α1 (or a scrambled siRNA) and were exposed to normal or elevated CO_2 levels and observed a significant decrease in polyubiquitination of β-ENaC after CO_2 exposure (**Figure 5C**). As a second approach, endogenous AMPK was inhibited by compound C after co-transfection of A549 cells with β-ENaC and HA-ubiquitin. Similar to our data that we obtained with AMPK silencing, exposure of the cells to hypercapnia in the presence of the inhibitor markedly

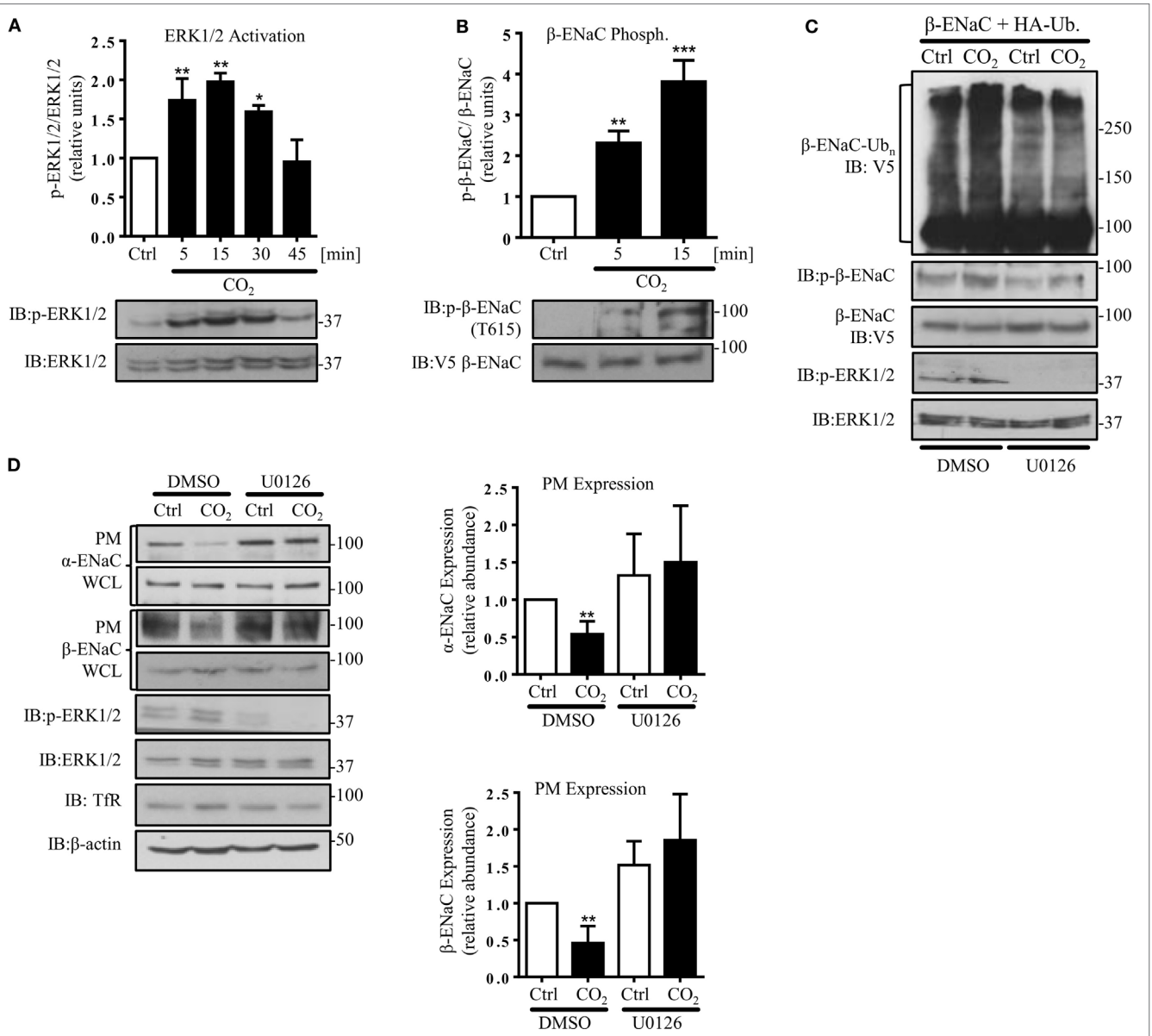

FIGURE 3 | Hypercapnia induces extracellular signal-regulated kinase (ERK)1/2-dependent phosphorylation of β-ENaC at T615 facilitating β-ENaC polyubiquitination and endocytosis of the α/β-ENaC complex. **(A)** A549 cells were exposed to 40 mmHg CO_2 (Ctrl) for 15 min or to 120 mmHg CO_2 (CO_2) for 5–45 min at a pH_e of 7.4. Phosphorylation of ERK1/2 and the total amount of ERK1/2 were measured. The graph represents the p-ERK1/2/ERK1/2 ratio. Representative immunoblots of p-ERK1/2 and total ERK1/2 are shown. **(B)** A549 cells were co-transfected with β-ENaC and were treated with 40 mmHg CO_2 for 15 min or 120 mmHg CO_2 for 5 and 15 min at a pH_e of 7.4. Phosphorylation of β-ENaC at T615 (p-β-ENaC) and total β-ENaC were determined by immunoblotting. Graphs represent p-β-ENaC/β-ENaC ratio. Representative immunoblots of p-β-ENaC and β-ENaC are shown. Values are expressed as mean ± SEM ($n = 3$; $^*p < 0.05$; $^{**}p < 0.01$). **(C)** A549 cells were transfected with β-ENaC, HA-ubiquitin, and were exposed to 40 mmHg CO_2 or 120 mmHg CO_2 for 30 min at a pH_e of 7.4 in the presence or absence of U0126 (10 μM, 30 min pretreatment). Total ubiquitinated β-ENaC was detected by immunoblotting with anti-V5 antibody. **(D)** A549 cells were co-transfected with α- and β-ENaC and were exposed to CO_2 as described above. ENaC subunits at the plasma membrane (PM) were determined by biotin-streptavidin pull-downs and immunoblotting. Representative immunoblots of α- and β-ENaC at the PM, total protein abundance of epithelial sodium channel (ENaC) and p-ERK 1/2 are shown. Bars represent mean ± SEM ($n = 3$; $^*p < 0.05$; $^{***}p < 0.001$).

decreased the hypercapnia-induced β-ENaC polyubiquitination (**Figure 5D**). To further confirm the role of AMPK-α1 in the CO_2-induced downregulation of ENaC, we transfected A549 cells with α- and β-ENaC and exposed to elevated CO_2 levels for 30 min in the presence or absence of the above mentioned siRNA against AMPK-α1 (**Figure 5E**) or compound C (Figure S3 in Supplementary Material) and observed that silencing or inhibition of AMPK stabilized ENaC proteins at the PM upon hypercapnia. Taken together, these latter studies suggest that AMPK by activation of JNK and subsequent phosphorylation of Nedd4-2 plays a central role in the hypercapnia-induced ubiquitination and endocytosis of ENaC.

DISCUSSION

In the present study, we show that elevated CO_2 levels initiate a specific signaling pattern leading to ubiquitination-mediated retrieval of ENaC from the PM, thereby reducing cell surface abundance of the channel in a human AEC line. Hypercapnia is associated with a number of acute and chronic pulmonary diseases; however, it is not evident to what extent and by which mechanisms these elevated levels of CO_2 may further impact on disease states. While hypercapnia and the associated acidosis have been shown to have anti-inflammatory effects, which might be advantageous at sites of excessive inflammation, recently, it has been clearly demonstrated that by impairing innate immunity, cellular repair, and alveolar epithelial function, elevated CO_2 may play a role in the pathogenesis of ARDS and COPD (2, 5, 9, 10, 38). Furthermore, it is increasingly evident that patients with ARDS and COPD who present with hypercapnia have worse outcomes (3, 39, 40).

A major function of the alveolar epithelium is the clearance of excess alveolar fluid, thereby promoting effective gas exchange. This clearance is mediated by the concerted action of various sodium transporters, among which the apically located ENaC

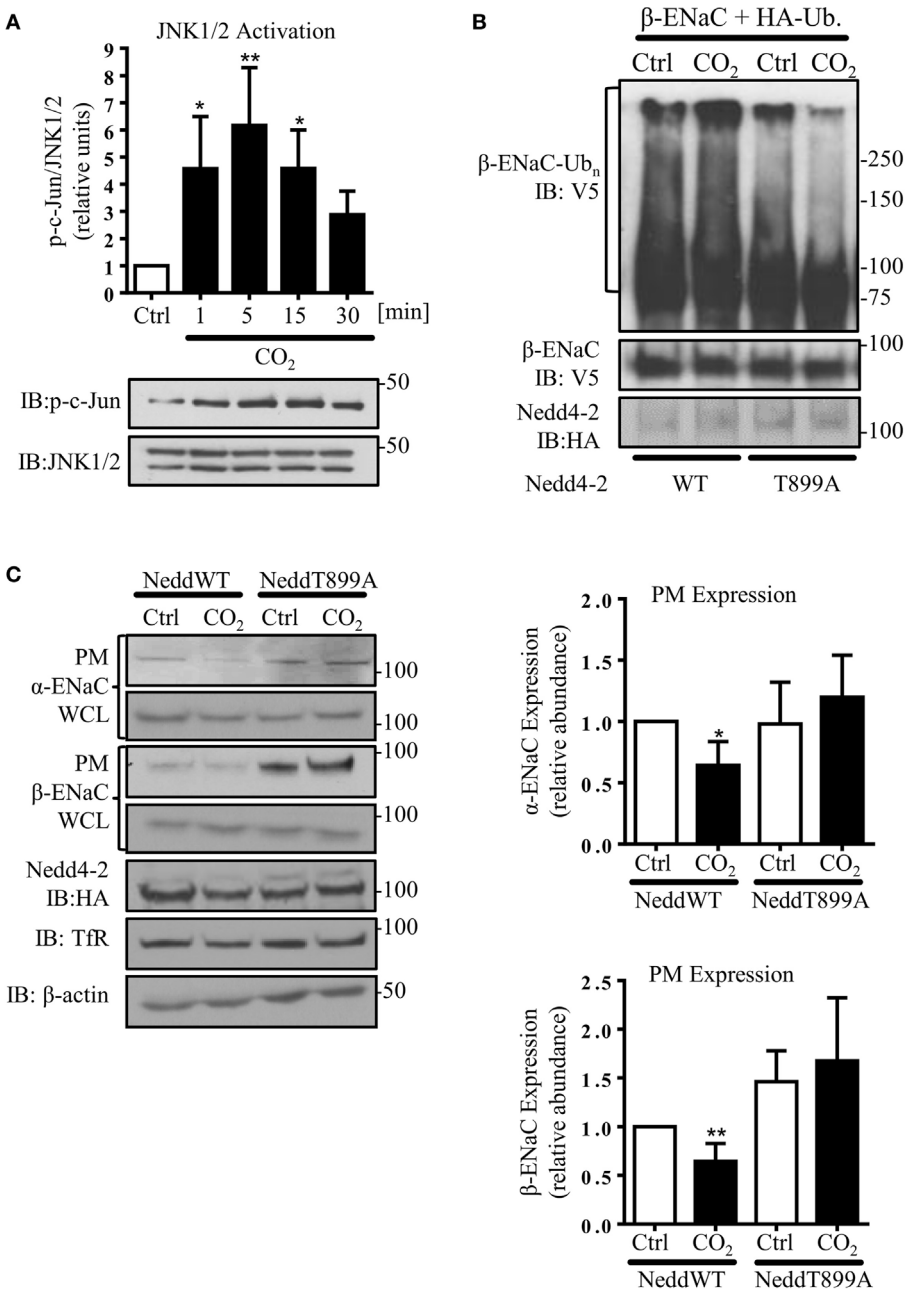

FIGURE 4 | **Continued**

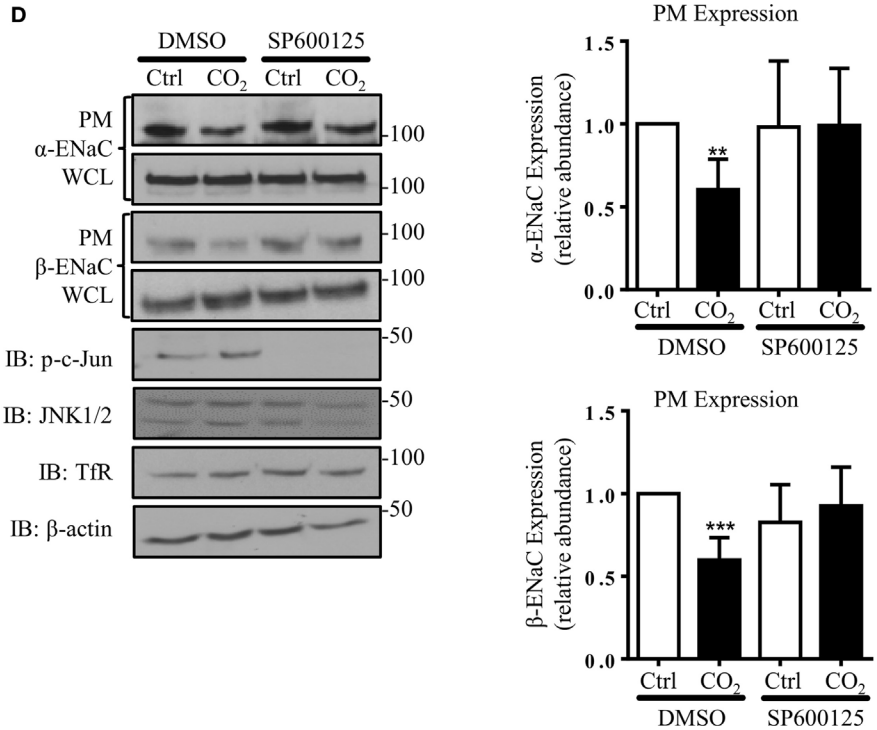

FIGURE 4 | c-Jun N-terminal kinase (JNK)1/2-dependent Nedd4-2 phosphorylation at Thr899 facilitates β-ENaC polyubiquitination and endocytosis. **(A)** A549 cells were exposed to 40 mmHg CO_2 (Ctrl) for 15 min or to 120 mmHg CO_2 (CO_2) for 1 to 30 min at a pH_e of 7.4 and the phosphorylation of c-Jun and the total amount of JNK1/2 were measured by immunoblotting. Graph represents the p-c-Jun/JNK1/2 ratio. Representative immunoblots of p-c-Jun and total JNK1/2 levels are shown. Values are expressed as mean ± SEM ($n = 3$; $^*p < 0.05$; $^{**}p < 0.01$). **(B)** A549 cells were co-transfected with β-ENaC, HA-ubiquitin, and HA-Nedd4-2 wild type (WT) or mutant (T899A). Cells were treated with 40 mmHg CO_2 or 120 mmHg CO_2 for 30 min at a pH_e of 7.4. Total ubiquitinated β-ENaC was detected by immunoblotting with anti-V5 antibody. **(C)** A549 cells were co-transfected with α- and β-ENaC and Nedd4-2 wild type or mutant and were exposed to CO_2 as described above. Epithelial sodium channel (ENaC) at the plasma membrane (PM) was determined by biotin-streptavidin pull-downs and immunoblotting. Representative immunoblots of α- and β-ENaC at the PM, total protein abundance of ENaC, Nedd4-2, and β-actin are shown. Mean ± SEM ($n = 3$; $^*p < 0.05$). **(D)** A549 cells were co-transfected with α- and β-ENaC exposed to 40 mmHg CO_2 or 120 mmHg CO_2 for 30 min at a pH_e of 7.4 in the presence or absence of SP600125 (25 μM, 30 min pretreatment). ENaC at the PM was determined by biotin-streptavidin pull-downs and immunoblotting. Representative immunoblots of α- and β-ENaC at the PM, total protein abundance of ENaC, p-c-Jun, JNK1/2, and β-actin are shown. Bars represent mean ± SEM ($n = 5$; $^{**}p < 0.01$; $^{***}p < 0.001$).

and the basolateral Na,K-ATPase have been identified as key players. Indeed, we have previously shown that the Na,K-ATPase is downregulated by hypercapnia; however, a potential regulation of ENaC by carbon dioxide has not been previously investigated. Various factors have been shown to affect ENaC cell surface abundance and function, including interleukin-1β, interleukin-4, transforming growth factor-β, LPS, or hypoxia (41–45), which similar to hypercapnia are often observed in patients with respiratory failure. As reducing hypercapnia without further damaging the lung is challenging, a better understanding of the molecular patterns initiated by elevated CO_2 levels may help us to interfere with the deleterious signals, thereby rescuing or at least not further aggravating lung damage.

Ubiquitination is a posttranslational modification that regulates trafficking and stability of proteins (46). Numerous studies described that depending on the stimulus ENaC subunits may undergo multimono- or polyubiquitination leading to channel retrieval from the cell surface or degradation of ENaC (47–49). It is also well documented that the phosphorylation status of target molecules and the E3 ubiquitin ligase often play a pivotal role in the initiation of ubiquitination (50, 51). Previous findings established the significance of mitogen-activated protein kinase (MAPK) in the hypercapnia-induced impairment of AFC (7, 33). Moreover, it has also been described that ERK and JNK, two prominent members of the MAPK family, may alter the phosphorylation status of ENaC and the E3 ligase of the channel, Nedd4-2, respectively (31, 37).

Thus, we first investigated whether elevated CO_2 concentrations affect ENaC cell surface stability by a mechanism involving ubiquitination of the channel and whether the MAPK pathway is involved in the hypercapnia-induced signaling events. Of note, a remarkable and rapid increase in polyubiquitination of β-ENaC and a significant reduction of the cell surface abundance of the α/β-ENAC complex were observed in AEC exposed to hypercapnia, as early as 30 min after CO_2 exposure, suggesting that ENaC function is probably sensitive to changes in CO_2 levels. In contrast, in the first half an hour after CO_2 exposure, total intracellular levels of ENaC remained unchanged, suggesting that CO_2 influenced

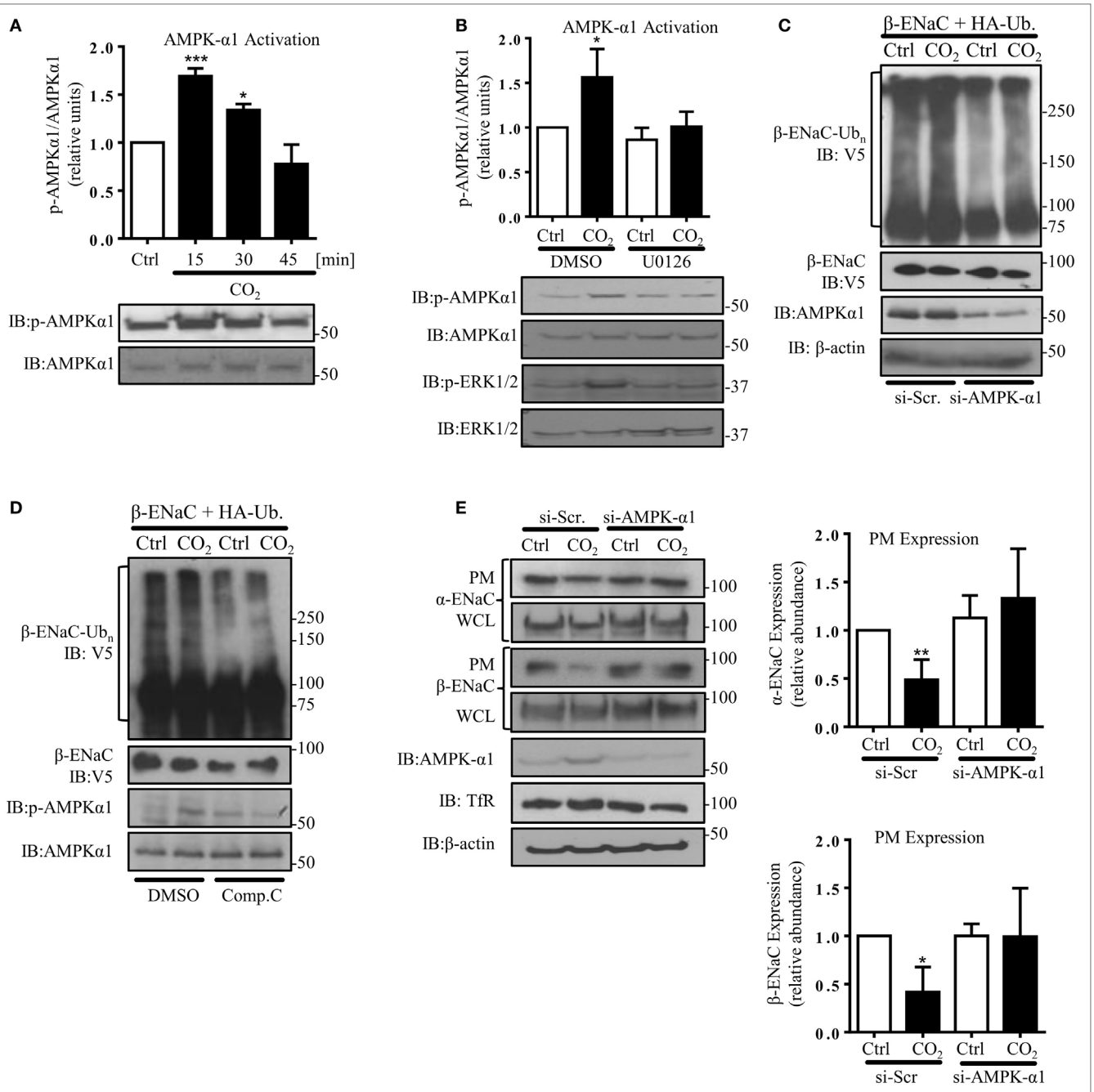

FIGURE 5 | Hypercapnia induces epithelial sodium channel (ENaC) endocytosis *via* extracellular signal-regulated kinase (ERK)1/2-dependent AMPK-α1 activation. **(A)** A549 cells were exposed to 40 mmHg CO_2 (Ctrl) for 15 min or to 120 mmHg CO_2 (CO_2) for 15–45 min at a pH_e of 7.4. The phosphorylation of AMPK-α1 at Thr172 and the total amount of AMPK-α1 were measured by immunoblotting. Graph represents the p-AMPK-α1/AMPK-α1 ratio. Representative immunoblots of p-AMPK-α1 and total AMPK-α1 are shown. **(B)** A549 cells were treated with 40 mmHg CO_2 (Ctrl) or with 120 mmHg CO_2 (CO_2) for 15 min at a pH_e of 7.4 in the presence or absence of 10 μM U0126 (30 min pretreatment). Phosphorylation of AMPK-α1 at Thr172, p-ERK1/2, and the total amount of both proteins were determined by immunoblotting. Graph represents the p-AMPK-α1/AMPK-α1 ratio. Representative immunoblots of p-AMPK-α1, p-ERK1/2 and total level of AMPK-α1 and ERK1/2 are shown. Values are expressed as mean ± SEM ($n = 3$; $^*p < 0.05$; $^{***}p < 0.001$). **(C)** A549 cells were co-transfected with β-ENaC, HA-ubiquitin, and siRNA targeting AMPK-α1 or a scrambled siRNA and were treated with 40 or 120 mmHg CO_2 for 30 min. β-ENaC polyubiquitinated isoforms were determined with anti-V5 antibody. Representative immunoblots of β-ENaC, AMPK-α1, and β-actin are shown. **(D)** A549 cells were co-transfected with β-ENaC and HA-ubiquitin and were treated with 40 mmHg CO_2 or 120 mmHg CO_2 for 30 min at a pH_e of 7.4 in the presence or absence of compound C (20 μM, 30 min pretreatment). Total ubiquitinated β-ENaC was detected as described above. Representative immunoblots of β-ENaC, p-AMPK-α1, and total AMPK-α1 are shown. **(E)** Cells were co-transfected with α- and β-ENaC and siRNA targeting AMPK-α1 or a scrambled siRNA and exposed to 40 mmHg CO_2 or 120 mmHg CO_2 for 30 min at a pH_e of 7.4. Biotinylated ENaC proteins were detected by immunoblotting. Representative western blots of α- and β-ENaC at the plasma membrane (PM) and total protein abundance of ENaC, p-AMPK-α1, and AMPK-α1 are shown. Bars present mean ± SEM ($n = 3$; $^*p < 0.05$; $^{**}p < 0.01$).

the trafficking of the channel rather than protein degradation. Furthermore, no significant changes in the ubiquitination status of α-ENaC have been detected upon hypercapnic treatment, highlighting and further confirming the central regulatory role of β-ENaC in the trafficking of the channel (52).

Our data demonstrate that elevated CO_2 levels cause a rapid and time-dependent ERK1/2 activation followed by phosphorylation of β-ENaC at the Thr615 residue. Moreover, genetic inhibition of Nedd4-2, the E3 ubiquitin ligase that drives ubiquitination of the various ENaC subunits (49), by a specific siRNA reduced β-ENaC polyubiquitination and prevented the hypercapnia-induced redistribution of α- and β-ENaC from the PM to the intracellular store, indicating a central role for Nedd4-2 in ENaC ubiquitination and endocytosis in AEC exposed to hypercapnia. Indeed, ERK1/2 has previously been described as a negative regulator of ENaC. For example, Eaton et al. showed that protein kinase C-δ drives ERK activation leading to ENaC internalization (30). Another study established that the ERK-mediated ENaC downregulation is promoted by phosphorylation β- and γ-ENaC, resulting in enhancement of Nedd4-2/ENaC interaction and thus, decreased Na^+ transport (31). Therefore, hypercapnia by enhancing ERK activity promotes phosphorylation of the ENaC β-subunit, which may increase the affinity of the E3 ubiquitin ligase to ENaC.

We have previously shown that JNK is also implicated in CO_2 responses and that phosphorylation of the kinase is required for the CO_2-induced inhibition of the Na,K-ATPase in the alveolar epithelium (7). The significance of JNK in cellular adaptation to stress has been shown by several studies (53). Of note, the possible role of JNK in modulating Nedd4-2 activity and ENaC current has been reported in polarized kidney epithelial cells (28). Remarkably, this study also showed that the Thr899 residue in the HECT (homologous to the E6-AP carboxyl terminus) domain of Nedd4-2 may be phosphorylated by JNK1, which was required for ubiquitination of α-ENaC (28). To assess the potential involvement of JNK-mediated Nedd4-2 phosphorylation in the hypercapnia-induced downregulation of ENaC, we mutated Thr899 to Ala to prevent phosphorylation of the E3 ligase at this residue. Importantly, this point mutation largely prevented the CO_2-induced polyubiquitination of β-ENaC although activation of JNK was evident and stabilized α- and β-ENaC at the cell surface. Thus, our data together with the previously published literature suggest that phosphorylation of Nedd4-2 by JNK at the Thr899 residue is critical for the hypercapnia-induced ubiquitination of β–ENaC, which drives endocytosis of the ENaC complex from the PM in AEC.

We have previously shown that AMPK, a cellular metabolic sensor that inhibits several ion transporters including the cystic fibrosis transmembrane conductance regulator, Na,K-ATPase, and ENaC, is rapidly activated by hypercapnia (8, 29, 54). Regarding the regulation of ENaC, it has been shown that chemical stimulation of AMPK by 5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranoside inhibited ENaC activity in lung epithelial cells (55). Moreover, enhanced abundance of ENaC channels at the cell surface was reported in the distal airways in AMPK-$\alpha1^{-/-}$ mice (28). Interestingly, AMPK has also been reported to regulate Nedd4-2 activity (26, 29). In the current study, treatment of AEC with a specific siRNA against AMPK-α1 or an AMPK-α inhibitor, compound C markedly decreased CO_2-induced β-ENaC polyubiquitination and endocytosis of α-, and β-ENaC, which is consistent with previous findings showing that in human embryonic kidney cells, AMPK activation promoted Nedd4-2/ENaC association (26). Furthermore, and in line with a previously published study (7), we also show that in the context of hypercapnia, AMPK is an upstream regulator of JNK. Thus, it is probable that the AMPK-regulated effects of CO_2 on Nedd4-2 and ENaC are indirect and mediated by JNK. Moreover, although AMPK is an early element of the CO_2-induced signaling pattern, its activation appears to be downstream of ERK upon hypercapnic exposure. This is of particular importance as ERK appears two have a dual role in the hypercapnia-induced downregulation of ENaC. On the one hand, it rapidly phosphorylates the β-subunit of the channel and by activating AMPK and JNK, it indirectly promotes phosphorylation of the E3 ligase Nedd4-2 as well. Of note, both of these phosphorylation events seem to be critically required for the CO_2-induced ubiquitination and subsequent endocytosis of ENaC, probably by enhancing the association of the E3 ligase and the target molecule.

Our study has some clear limitations. Although we show a rapid activation of ERK and a subsequent phosphorylation of β-ENaC at the Thr615 residue, which is a known target of ERK, we have not investigated the potential rescue of β-ENaC ubiquitination or trafficking of the ENaC complex after preventing phosphorylation at this specific site. A mutation of this residue will be necessary to definitely prove that ERK-promoted phosphorylation of β-ENaC at this residue drives the downregulation of the channel upon hypercapnia. Moreover, the current study was performed exclusively in AECs and further *in vivo* investigations will be necessary to establish the role of the hypercapnia-induced signaling events identified in the current manuscript in ENaC-driven AFC and alveolar epithelial barrier dysfunction in an animal model of hypercapnic acute lung injury.

Taken together, our study shows for the first time that upon exposure to elevated CO_2 levels, ENaC cell surface abundance is rapidly downregulated in a human AEC line by a specific, CO_2-induced and ERK-, AMPK-, and JNK-mediated signaling pathway, which promotes phosphorylation of both β-ENaC and Nedd4-2, leading to ubiquitination of β-ENaC and subsequent internalization of the α/β-ENaC complex. This novel signaling pathway may contribute to the persistence of alveolar edema and thus, interfering with these molecular mechanisms may improve alveolar fluid balance and lead to better outcomes in patients with ARDS and hypercapnia.

AUTHOR CONTRIBUTIONS

Conception or design of the work: PG and IV; acquisition, analysis, or interpretation of data: PG, BB, KM, SH, RM, WS, and IV; drafting the work: PG and IV; revising it critically for important intellectual content: PG, BB, KM, SH, RM, WS, and IV. All the authors approved the final version of the manuscript and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.00591/full#supplementary-material.

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TNF Lectin-Like Domain Restores Epithelial Sodium Channel Function in Frameshift Mutants Associated with Pseudohypoaldosteronism Type 1B

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Previous *in vitro* studies have indicated that tumor necrosis factor (TNF) activates amiloride-sensitive epithelial sodium channel (ENaC) current through its lectin-like (TIP) domain, since cyclic peptides mimicking the TIP domain (e.g., solnatide), showed ENaC-activating properties. In the current study, the effects of TNF and solnatide on individual ENaC subunits or ENaC carrying mutated glycosylation sites in the α-ENaC subunit were compared, revealing a similar mode of action for TNF and solnatide and corroborating the previous assumption that the lectin-like domain of TNF is the relevant molecular structure for ENaC activation. Accordingly, TNF enhanced ENaC current by increasing open probability of the glycosylated channel, position N511 in the α-ENaC subunit being identified as the most important glycosylation site. TNF significantly increased Na^+ current through ENaC comprising only the pore forming subunits α or δ, was less active in ENaC comprising only β-subunits, and showed no effect on ENaC comprising γ-subunits. TNF did not increase the membrane abundance of ENaC subunits to the extent observed with solnatide. Since the α-subunit is believed to play a prominent role in the ENaC current activating effect of TNF and TIP, we investigated whether TNF and solnatide can enhance αβγ-ENaC current in α-ENaC loss-of-function frameshift mutants. The efficacy of solnatide has been already proven in pathological conditions involving ENaC in phase II clinical trials. The frameshift mutations αI68fs, αT169fs, αP197fs, αE272fs, αF435fs, αR438fs, αY447fs, αR448fs, αS452fs, and αT482fs have been reported to cause pseudohypoaldosteronism type 1B (PHA1B), a rare, life-threatening, salt-wasting disease, which hitherto has been treated only symptomatically. In a heterologous expression system, all frameshift mutants showed significantly reduced amiloride-sensitive whole-cell current compared to wild type αβγ-ENaC, whereas membrane abundance varied between mutants. Solnatide restored function in α-ENaC frameshift mutants to current density levels of wild type ENaC or higher despite their lacking a binding site for solnatide, previously located to the region between TM2 and the C-terminus of

the α-subunit. TNF similarly restored current density to wild type levels in the mutant αR448fs. Activation of βγ-ENaC may contribute to this moderate current enhancement, but whatever the mechanism, experimental data indicate that solnatide could be a new strategy to treat PHA1B.

Keywords: lectin-like domain of tumor necrosis factor, TIP peptides, solnatide (AP301), amiloride-sensitive epithelial sodium channel, pseudohypoaldosteronism type 1B

INTRODUCTION

Tumor necrosis factor (TNF) is a mammalian inflammatory cytokine, which exerts a plethora of effects primarily aimed at defending the host against invading pathogens. Apart from mediating its activities through cross-linking with specific receptors on the surface of mammalian cells (1), TNF participates in innate immune functions through a lectin-like (TIP) domain, spatially distinct from the TNF-receptor binding site (2–4). The lectin-like domain of TNF recognizes and interacts with specific oligosaccharide moieties, in particular *N*,*N*′-diacetylchitobiose (5). TNF is crucially involved in the control of *Trypanosoma brucei brucei* and *T. cruzei* infections, through the trypanolytic effect triggered by interaction of its lectin-like domain with the N-linked *N*,*N*′-diacetylchitobiose core of the variant surface glycoproteins (VSG) of these organisms (1, 2, 6–14). Another effect of the TNF TIP domain observed in early work was the amiloride-sensitive increase in membrane conductance in microvascular endothelial cells (MVECs) (4) and alveolar epithelial cells (15), an effect which we now know is due to activation of the amiloride-sensitive epithelial sodium channel (ENaC) (16). The potential physiological role of the lectin-like domain of TNF in resolution of alveolar edema has been demonstrated in various rodent models of flooded lungs (15, 17, 18). Furthermore, transgenic mice expressing a mutated TNF lectin-like domain are more prone to develop lung edema than their wild-type (WT) counterparts when challenged with the bacterial toxin pneumolysin (PLY) (19).

The synthetic, cyclic, 17-residue peptide, solnatide, mimics the lectin-like domain (TIP) of human TNF (2). Like TNF, TIP peptide can influence regulation of alveolar fluid balance. Solnatide has been shown to activate fluid reabsorption in *in situ* and *in vivo* flooded rat lung models (18) and a mouse version the TIP peptide, mTIP, decreased pulmonary edema in isolated, endotoxin-injured rabbit lung (20). Moreover, solnatide, instilled intratracheally into rats prior to lung transplantation, significantly improved lung function, indicating its use as a potential therapy for ischemia reperfusion injury associated with lung transplantation (21). Inhalation of nebulized solnatide in a porcine bronchoalveolar lavage (BAL) model of acute lung injury (ALI) resulted in an increased PaO_2/FiO_2 ratio and reduced extravascular lung water index (EVLWI) (22). More recently, solnatide demonstrated profound therapeutic activity in a rat model of pulmonary edema induced by acute hypobaric hypoxia and exercise (23).

Solnatide activates both endogenously and heterologously expressed ENaC by increasing the open state probability, P_o, of the channel (16, 24, 25). The oligosaccharide-binding property of the TIP domain of TNF plays an important role in the mechanism by which TNF and solnatide interact with and activate ENaC, although the exact nature of this interaction is not yet understood. Elimination of the Na^+ current-enhancing effect of solnatide following PNGase F-mediated deglycosylation of A549 and H441 cells or of HEK-293 cells heterologously expressing human ENaC suggested that TIP interacts with carbohydrate groups on the extracellular loop of ENaC subunits (16, 19). Proof of the importance of interaction with glycosylated residues in the extracellular loop of ENaC for TIP potentiation of Na^+ current was obtained from studies with heterologously expressed ENaC in which the five Asn glycosylation sites in the extracellular loop of alpha ENaC had been removed, singly or multiply, by mutation to Gln (26).

The current-potentiating effect of solnatide not only manifests itself in channel kinetics but also in abundance of ENaC subunits at the membrane. We have observed a temporary increase in abundance of α-, β-, γ-, and δ-ENaC 5 and 10 min after prior exposure of HEK-293 cells transiently expressing ENaC to solnatide, but after 1 h, levels return to those seen in the absence of solnatide (16, 26). The solnatide-induced increase in membrane abundance of pore-forming α- and δ-ENaC subunits is significant statistically whereas that of β- and γ-ENaC subunits only slight (26).

In this study, we explore the mechanism of TIP activation of ENaC in electrophysiological and Western blotting experiments using TNF and solnatide. Direct interaction of TNF with ENaC has hitherto not been reported, and so its physiological role in alveolar liquid clearance (ALC) during lung inflammation has been largely inferred from numerous studies with solnatide. A recent study, which sought to determine the precise mechanism by which solnatide stimulated Na^+ uptake in the presence or absence of PLY, demonstrated that TIP activates ENaC through binding to the carboxyl-terminal domain of the α subunit (19). In the present study, we investigated how native TNF affects Na^+ current and membrane abundance of ENaC subunits in cells heterologously expressing WT hENaC and mutant hENaC and compared and contrasted these observations to our findings with solnatide.

Surprisingly, solnatide rescues the loss-of-function phenotype in ENaC mutants carrying mutations at conserved positions in α-, β-, and γ-ENaC known to cause pseudohypoaldosteronism type 1B (PHA1B), restoring current levels in these mutant ENaC-expressing cells to WT levels or even higher (27). PHA1B is a very rare inherited disease caused by mutations in the genes encoding the α (SCNN1A), β (SCNN1B), or γ (SCNN1G), subunit of ENaC, resulting in defective transepithelial sodium transport (28). PHA1B usually manifests itself in the neonatal period with life-threatening salt loss, hyperkalemia, acidosis, and elevated aldosterone levels due to end-organ resistance to aldosterone.

Patients suffering from PHA1B are at risk from life-threatening, salt-losing crises, combined with severe hyperkalemia and dehydration throughout their entire lives (29, 30). There is as yet no definitive treatment for PHA1B other than supportive management aimed to reduce sodium wasting and hyperkalemia and to restore water–electrolyte and acid–base balance.

In the work reported here, we investigate whether α-ENaC frameshift mutants known to cause PHA1B are also rescued by solnatide, even though they lack the carboxyl-terminal domain of α-ENaC previously postulated to be the site of interaction of solnatide with ENaC (19, 31).

MATERIALS AND METHODS

Cell Culture

Human alveolar epithelial A549 cells (ATCC no. CCL-185) in passages 80–97 and human embryonic kidney HEK-293 cells (ATCC no. CRL-1573) in passages 3–25 were seeded in Dulbecco's modified Eagle medium/F12 nutrient mixture Ham plus L-glutamine (DMEM/F-12; Gibco™ by Life Technologies, LifeTech Austria), supplemented with 10% fetal bovine serum (FBS; Gibco™ by Life Technologies, LifeTech Austria) and 1% penicillin–streptomycin (Sigma-Aldrich, Vienna, Austria). Cells were maintained at 37°C with 5% CO_2 in a humidified incubator.

Molecular Biological Methods

cDNAs encoding α-, β-, and γ-hENaC were a kind gift from Dr. Peter M. Snyder (University of Iowa, Carver College of Medicine, Iowa City, USA). cDNA-encoding δ-hENaC was a kind gift from Dr. Mike Althaus (Justus-Liebig University, Giessen, Germany).

Site-Directed Mutagenesis

Point mutations of α-, β-, γ- and δ-hENaC and PHA1B frameshift mutations of α-hENaC were prepared with the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, CA, USA). Mutagenic primers were designed individually with the Primer Design Program provided on the producer's website or for the frameshift mutations the same base changes as reported in patients (see **Table 2**) were performed. Primers were ordered from Sigma-Aldrich, Vienna, Austria.

Mutant strand synthesis, digestion of template, and transformation were performed according to the manufacturer's protocol, and plasmid DNA was extracted from *Escherichia coli* (*E. coli*) cells using the GeneJET Plasmid Miniprep Kit (Thermo Scientific, Loughborough, UK). The mutant DNA was checked by sequencing from LGC Genomics GmbH, Berlin, Germany.

Larger amounts of DNA were provided by amplifying WT or mutant α-, β-, γ-, or δ-hENaC in DH5α competent cells (Invitrogen by Thermo Fisher Scientific, CA, USA) and then extracting DNA using the Plasmid Midi Kit (QIAGEN GmbH, Hilden, Germany).

Transfection

HEK-293 cells were transfected 1 day after cell seeding using X-tremeGENE HP DNA transfection reagent (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. A set of WT or mutant αβγ- or δβγ-hENaC, or α-, β-, γ-, or δ-hENaC alone was used, and the ratio of DNA to transfection reagent was 1:3. The expression was highest 48–72 h after transfection.

Cell Surface Biotinylation and Western Blotting

Cell surface biotinylation was performed as previously described (26). In brief, A549 cells or transiently transfected HEK-293 cells were grown in 10 cm dishes in 37°C, 5% CO_2 incubator in DMEM medium supplemented with 5% FBS. Cells were treated with 40 nM TNF or 200 nM solnatide for 5, 10, or 30 min when 90% confluency had been reached. Medium was aspirated, and then cells were washed twice with 10 ml ice-cold phosphate-buffered saline (PBS), covered with 2.5 mg EZ-Link Sulfo-NHS-SS-Biotin (Thermo Scientific, Rockford, USA), dissolved in 10 ml ice-cold PBS, and incubated at 4°C with gentle agitation for 30 min. Fifty milliliters of quenching solution were added to cells; then cells were scraped in solution and transferred to fresh 50 ml tube. Cell suspension was centrifuged at 500 × *g* for 3 min. Supernatant was discarded, and 5 ml of Tris-buffered saline (TBS) was added to the cell pellet. The cell pellet was resuspended and centrifuged at 500 × *g* for 3 min. Supernatant was discarded and cell pellet was resuspended in lysis buffer containing protease inhibitor cocktail (10 μM pepstatin A, 10 μM phenylmethylsulfonyl fluoride, and 10 μM leupeptin) and transferred to fresh 1.5 ml centrifuge tube. Cell pellet was then homogenized on ice by ultrasonication using 1 s bursts and incubated on ice for at least 30 min. Intact cells and nuclei were pelleted by centrifugation at 10,000 × *g* for 2 min under cooling conditions. Pellet was then discarded and supernatant was transferred to fresh tube, incubated overnight with 0.5 ml NeutrAvidin Agarose under gentle rotation at 4°C and centrifuged at 500 × *g* for 5 min under cooling conditions. Supernatant was then discarded and the pellet washed twice with 200 μl lysis buffer. The biotinylated proteins were eluted with 100 μl sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tris, pH 6.8, 1% SDS, 10% glycerine, 50 mM dithiothreitol) containing 10 μM E64 at 65°C for 10 min. Sample was then centrifuged at 500 × *g* for 2 min. Pellet was discarded and supernatant subjected to protein electrophoresis and immunoblotting. The biotinylated proteins were separated under reducing conditions by SDS-PAGE using 7.5% SDS gel along with prestained protein marker (cat. #12949 from Cell Signaling). Proteins were then transferred onto a nitrocellulose membrane (UltraCruzTM 0.45 mm, Santa Cruz Biotechnology, TX, USA) by semi-dry blotting at 25 V for 30 min. Unspecific binding sites were blocked by incubating the membrane overnight at 4°C with 3% FBS in PBS supplemented with 0.02% sodium azide. Membrane was then incubated for 90 min with primary antibody (anti-α-hENaC, anti-δ-hENaC, and anti-β-actin from Sigma Aldrich; anti-β- and anti-γ-hENaC from Santa Cruz Biotechnology). Membrane was washed 5× with 10 ml PBS containing 0.1% Tween-20 (PBST), and corresponding horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) were applied. After 90-min incubation, membrane was washed 3× with PBST and once with PBS. Enhanced chemiluminescence (ECL) substrate (Amersham

ECL Plus Western Blotting Detection Reagent, GE Healthcare, Vienna, Austria) was used for visualization. Following incubation for 2 min, membranes were exposed to X-ray films (Amersham Hyperfilm ECL, GE Healthcare). Exposed films were scanned and quantified using ImageJ (NIH, MD, USA).

Electrophysiology

Electrophysiological experiments were performed as described in detail by Shabbir et al. (16). Briefly, effects of TNF and solnatide on WT and mutated hENaC were studied on transfected HEK-293 cells at room temperature (19–22°C) 24–48 h after plating. Currents were recorded with the patch clamp method in the whole-cell mode. The chamber contained 1 ml of the bath solution of the following composition (in mM): 145 NaCl, 2.7 KCl, 1.8 $CaCl_2$, 2 $MgCl_2$, 5.5 glucose, and 10 HEPES, adjusted to pH 7.4 with 1 M NaOH solution. Micropipettes were pulled from thin-walled borosilicate glass capillaries (Harvard Apparatus, Holliston, MA, USA) with a DMZ Zeitz Puller to obtain electrode resistances ranging from 2 to 5 MΩ. The pipette solution contained (in millimolars): 135 potassium methane sulfonate, 10 KCl, 6 NaCl, 1 Mg_2ATP, 2 Na_3ATP, 10 HEPES, and 0.5 EGTA, adjusted to pH 7.2 with 1 M KOH solution. Chemicals for pipette and bathing solutions were supplied by Sigma-Aldrich (Vienna, Austria). Electrophysiological measurements were carried out with an Axopatch 200B patch clamp amplifier (Axon Instruments, CA, USA). Capacity transients were canceled, and series resistance was compensated. Whole-cell currents were filtered at 5 kHz and sampled at 10 kHz. Data acquisition and storage were processed directly to a PC equipped with pCLAMP 10.2 software (Axon Instruments, CA, USA). After GΩ-seal formation, the equilibration period of 5 min was followed by control recordings at a holding potential of −100 mV. Then, aliquots of a stock solution, which was prepared with distilled water, were cumulatively added into the bath solution. The wash-in phase lasted about 1–5 min. After steady-state had been reached, the same experimental protocol was applied for each concentration of TNF and solnatide as well as during control recordings.

Statistical Analysis

Data were analyzed with OriginPro 2017 (OriginLab, Northampton, MA, USA) and figures were edited with CorelDRAW X7 (Corel Corporation, Ottawa, ON, Canada). Data are represented as mean ± SEM of at least three independent biological replicates/experiments. Significant differences of two independent values were evaluated by unpaired Student's *t*-test. Whereas one-way ANOVA followed by Tukey's *post hoc* test was used when groups of data were compared with each other. The type of statistical test is indicated in the figure legends. In case no specific test is mentioned, ANOVA was performed.

Test Compounds

Tumor necrosis factor (CAS Registry Number 94948-59-1, Sigma-Aldrich, Austria) and the TNF lectin-like domain derived peptide solnatide, also known as AP301 and called TIP peptide [CAS Registry Number: 259206-53-6; CA Index Name: L-cysteine, L-cysteinylglycyl-L-glutaminyl-L-arginyl-L-.alpha.-glutamyl-L-threonyl-L-prolyl-L-.alpha.-glutamylglycyl-L-alanyl-L-.alpha.-glutamyl-L-alanyl-L-lysyl-L-prolyl-L-tryptophyl-L-tyrosyl-, cyclic (1.fwdarw.17)-disulfide], with the amino acid sequence CGQRETPEGAEAKPWYC were tested for their ability to activate wild-type and mutant ENaC. Synthesis and description of solnatide is reported in detail by Hazemi et al. (24).

RESULTS

Electrophysiological TNF–ENaC Interaction

In a recent study by Czikora et al. (19) the authors postulated for the first time a direct interaction between the cytokine TNF and the amiloride-sensitive sodium ion channel in a multiple step manner, starting with the interaction with glycosylated membrane components, followed by caveolae-dependent uptake and finally binding to the carboxyl-terminal domain of the α-subunit. This mode of action would suggest a physiological role of the lectin-like domain of TNF in ALC. However, in Czikora's study, using a cyclic peptide which mimics the lectin-like domain of TNF, it was not demonstrated directly that native TNF can also activate ENaC *via* these proposed mechanisms.

In previous experiments with A549 cells that endogenously express α-, β-, γ-, and δ-subunits, we could demonstrate a current activating effect by both TNF as well as the TIP peptide solnatide (24), and this increase in current by TNF (**Figure 1**) and solnatide (16) was confirmed in heterologously expressed αβγ-ENaC and individual ENaC subunits. The onset of action

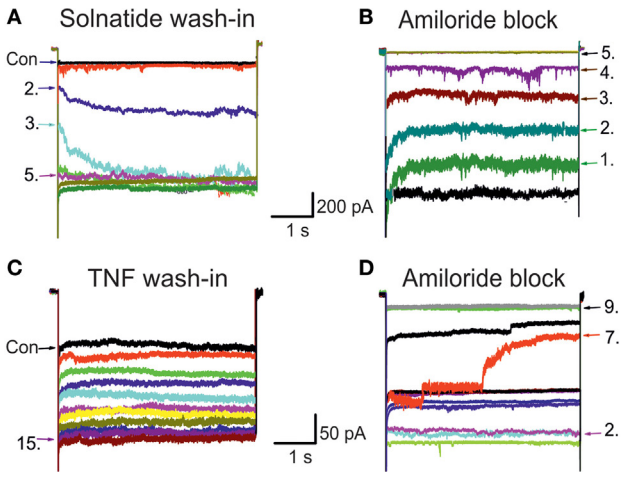

FIGURE 1 | Original traces of wild-type (WT) epithelial sodium channel (ENaC) showing solnatide and tumor necrosis factor (TNF) wash-in and amiloride block. The control whole-cell current (Con; untreated) of HEK-293 cells transiently transfected with WT αβγ-ENaC. Each set of traces represents current measured when cells were clamped at −100 mV, using 20 s pulse intervals. Pulse numbers are indicated to show the time course of wash-in and amiloride block. **(A)** Typical 200 nM solnatide wash-in; 5th pulse showed the steady-state level. **(B)** Typical 10 μM amiloride block of 200 nM solnatide-induced current; 5th pulse showed full block of inward sodium current. **(C)** Typical 20 nM TNF wash-in; 15th pulse showed the steady-state level. **(D)** Typical 10 μM amiloride block of 20 nM TNF-induced current; 9th pulse showed full block of inward sodium current.

was slower with TNF compared to solnatide and was blocked by 10 μM amiloride within a few pulses (**Figure 1**). Therefore, as a next step, we studied the effect of native TNF on hENaC in more detail.

To study single subunits and mutant ENaC HEK-293 cells were used, as no mRNA encoding ENaC subunits has been found in untransfected HEK-293 cells indicating no endogenous expression of ENaC (40). Only cells with a clear amiloride response and with significantly higher current than non-transfected (16) and mock-transfected (27) HEK-293 cells were used for data analysis. In αβγ-ENaC heterologously expressed in HEK-293 cells, TNF enhanced amiloride-sensitive sodium current with approximately 8-fold higher potency (EC_{50}: 6.7 ± 2.1 nM) than solnatide (EC_{50}: 54.7 ± 2.2 nM), and TNF was even about 13-fold more effective than solnatide in α- and δ-ENaC subunits (**Table 1**). Notably, however, the maximal steady-state current level of the TNF-activated current in αβγ-ENaC was significantly ($p < 0.001$, $n = 7$) lower than that of solnatide-induced current (**Table 1**). The main targets of TNF were the pore-forming subunits α- and δ-ENaC, similar to what has been shown previously for solnatide (16) (**Table 1**). Interestingly, compared to solnatide, the current-activating effect of TNF was more pronounced in the individual subunits, but without statistically significant difference. The weakest current increase by TNF was found in the γ-subunit, so that no reliable EC_{50} value could be estimated.

In A549 cells single channel open probability, mean open time, number and duration of bursts were significantly increased by TNF and solnatide without affecting conductivity of the channel, and this increase was completely abolished in PNGase F pretreated cells (16). To verify which of the putative *N*-linked glycosylation sites participate in binding of TNF to the extracellular loop we generated single N (asparagine) to Q (glutamine) mutants in the human α-subunit at each potential glycosylation site (N232, N293, N312, N397, N511) and co-expressed these mutated α-subunits in HEK-293 cells along with βγ-subunits. Similar to solnatide, but less pronounced, we could show that each *N*-glycan was involved in TNF-induced increase in current with position αN511 being the most important glycosylation site. For comparison, the maximal TNF-induced current of 162.5 ± 7.5 pA in αβγ-ENaC ($n = 7$) was significantly lower in αN232Qβγ-ENaC with 84.7 ± 8.8 pA ($p < 0.001$, $n = 5$), and attenuation of TNF-induced current was most pronounced in the mutant αN511Qβγ-ENaC with 50.7 ± 5.3 pA ($p < 0.001$, $n = 4$).

TNF and Membrane Abundance of ENaC

In A549 cells (**Figure 2B**), as well as in heterologously expressed α- and δ-ENaC, treatment with 40 nM TNF caused a significant, transient increase in membrane abundance of α- and δ-subunits after 10 min ($p < 0.01$, $n = 4$), while the increase of the β- and γ-subunit was not significant. The increased expression of α- and δ-subunits returned to control values after 30 min (**Figure 2B**). These results confirm data obtained in presence of solnatide (26) with the only difference that for TNF a longer incubation time of 10 min was needed to observe an increase in membrane expression of α- and δ-subunits. Furthermore, our data with TNF on the membrane expression level also underline the importance of the *N*-linked glycosylation sites for the interaction of the cytokine with the ion channel. WT and single or quintuple (αM5) α-subunit mutants (N232Q, N293Q, N312Q, N397Q, N511Q) were transfected along with βγ-hENaC in HEK-293 cells. Expression levels of αN232Q mutant in presence of TNF were comparable to WT, whereas in αN511Q and α-ENaC lacking all five glycosylation sites an increase of membrane abundance of α-ENaC by 40 nM TNF was inhibited (**Figure 2A**). These results indicate that in the α-subunit, position N511 plays a prominent role in the interaction of TNF with ENaC (**Figure 2A**). As Czikora et al. (19) postulated that the carboxyl terminal of α-hENaC is essential for the interaction with the lectin-like domain of TNF, we deleted the carboxyl-terminal domain by introducing stop codons at L576 in α-hENaC to generate αL576X, as well as in the δ-subunit at position D522 to create δD522X. In these mutants, no increase in membrane abundance could be observed in presence of TNF (**Figure 3**), which again confirms the data with solnatide (26).

α-ENaC Frameshift Mutations

Unexpectedly, solnatide rescues the loss-of-function phenotype in ENaC mutants (27) carrying mutations at conserved positions in α-, β-, and γ-ENaC known to cause PHA1B. Since the α-subunit is supposed to play a prominent role in the ENaC current activation by TNF and TIP peptide, we investigated whether TNF and solnatide can also enhance αβγ-ENaC current in α-ENaC loss-of-function frameshift mutants, i.e., αI68fs, αT169fs, αP197fs, αE272fs, αF435fs, αR438fs, αY447fs, αR448fs, αS452fs, and αT482fs (**Table 2**), which have been reported to cause PHA1B. These frameshift mutants lack the carboxyl-terminal domain of α-ENaC previously postulated

TABLE 1 | Comparison of the effect of tumor necrosis factor (TNF) and solnatide on amiloride-sensitive Na^+ current.

hENaC subunit(s)	Amiloride-sensitive control current (pA)	Maximal induced current (pA)		EC_{50} (nM)	
		Tumor necrosis factor (TNF) ($n = 5$)	Solnatide[a]	TNF ($n = 5$)	Solnatide[a]
αβγ	75.8 ± *4.5*	162.5 ± *7.5****	953.2 ± *11.5*	6.7 ± *2.1****	54.7 ± *2.2*
α	55.3 ± *5.5*	48.1 ± *5.0****	11.3 ± *6.2*	4.2 ± *1.9****	57.8 ± *3.4*
β	11.5 ± *3.7*	18.5 ± *5.5*	n.d.	18.8 ± *2.9*	n.d.
γ	14.0 ± *5.1*	8.0 ± *3.3*	n.d.	n.d.	n.d.
δ	60.6 ± *2.5*	27.8 ± *2.1****	15.6 ± *7.9*	5.0 ± *0.3****	63.5 ± *9.9*

[a]*Data from Ref. (16).*

****p < 0.001, t-test, significant difference between maximal TNF- and solnatide-induced current and significant difference between EC_{50} values for TNF and solnatide, respectively.*

n.d., no detectable current.

Values are given as mean ± S.E.

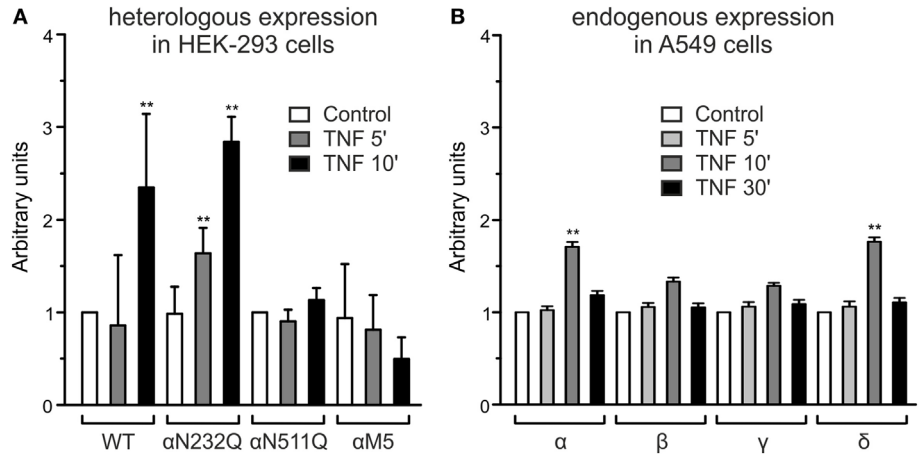

FIGURE 2 | Effect of tumor necrosis factor (TNF) on membrane abundance of N-linked glycosylation site mutations in the extracellular loop of α-epithelial sodium channel (ENaC) and of single subunits of ENaC. **(A)** A complex of wild-type (WT) αβγ- or single αN232Q and αN511Q mutants as well as quintuple α-ENaC mutant (N232Q, N293Q, N312Q, N397Q, N511Q) combined with WT βγ-ENaC was heterologously expressed in HEK-293 cells, untreated (control) or treated with 40 nM TNF for 5 or 10 min. Biotinylated surface proteins were analyzed using Western blot; the expression of α-ENaC was normalized compared to β-actin and set in relation to WT control (=1). Significant differences are indicated, $^{**}p < 0.01$ ($n = 3$). **(B)** Biotinylated surface proteins from A549 cells untreated or after 5, 10, or 30 min treatment with 40 nM TNF, heterologously expressing αβγδ-ENaC, were analyzed with anti-α-, β-, γ-, or δ-ENaC antibodies. The expression was normalized to β-actin and set in relation to its respective control. Significant differences are indicated, $^{**}p < 0.01$ ($n = 3$).

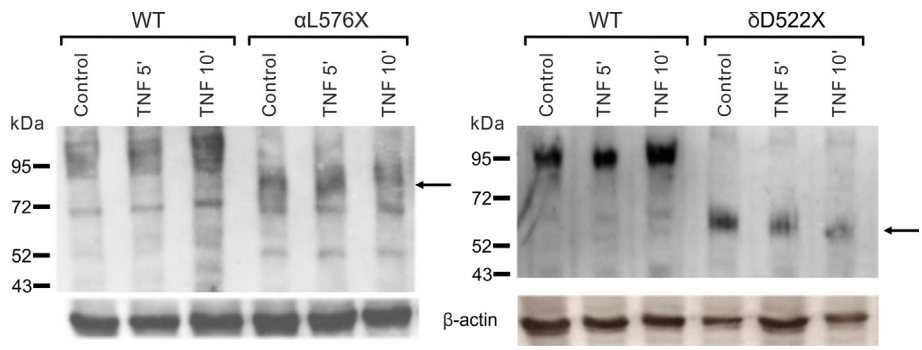

FIGURE 3 | Effect of tumor necrosis factor (TNF) on membrane abundance of αL576X and δD522X mutants. Mutant αL576X (left blot) or δD522X (right blot) was co-expressed with wild-type (WT) βγ-hENaC in HEK-293 cells. WT αβγ- or δβγ-epithelial sodium channel (ENaC) was used as reference. Cells were treated with 40 nM TNF for 5 or 10 min, as indicated, or untreated (control). Biotinylated surface proteins were blotted and visualized with anti-α-ENaC (left blot) or anti-δ-ENaC (right blot) antibodies. WT α- and δ-ENaC show a band at about 95 kDa, whereas the truncated mutants are shorter (the relevant bands are indicated by arrows). A representative blot out of three independent biological replicates is shown in each case.

to be the site of interaction of solnatide with ENaC (19, 31). Apart from αI68fs all studied frameshift mutants originate in the extracellular loop of α-ENaC predominantly clustering in the thumb. Worth mentioning, all described frameshift mutations have the WT sequence before the mutation and some random amino acids after the mutations, until a stop codon occurs. The theoretical total length of the truncated proteins is indicated in **Table 2**.

Solnatide Restores Amiloride-Sensitive Sodium Current in Frameshift Mutations of α-ENaC

To determine whether TNF and solnatide not only activate Na^+ current in WT αβγ-ENaC but also in PHA1B-causing α-ENaC frameshift mutations, experiments were performed by transfecting mutant α-ENaC together with WT βγ-subunits into HEK-293 cells. The macroscopic amiloride-sensitive Na^+ currents of all investigated α-ENaC frameshift PHA1B mutants were significantly ($p < 0.001$, for number of experiments see **Table 3**) decreased compared to WT control level (**Figure 4A**). Remarkably, solnatide was able to activate the reduced current in all studied frameshift mutants up to or even higher than WT control current in absence of solnatide (**Figure 4B**), even though these mutants lack the carboxyl-terminal domain of α-ENaC previously postulated to be the site of interaction of solnatide with ENaC (19, 31). A maximum level of concentration-dependent current activation was reached at 200 nM with EC_{50} values as

TABLE 2 | Total protein length and affected regions of α-frameshift mutations that are verified to occur in PHA1B patients.

Mutant (protein)	Truncated protein length (AA)	Affected region	Domain location in homology model of mouse α-ENaC (32)	Mutation in patient (DNA)	First published in
αI68fs	142	Exon 2 cytoplasmic	Intracellular	203delTC	(33)
αT169fs	203	Exon 3 extracellular loop	Finger	505delAC	(34)
αP197fs	204	Exon 3 extracellular loop	Finger	587-588insC	(35)
αE272fs	309	Exon 4 extracellular loop	Finger	814-815insG	(36)
αF435fs	480	Exon 8 extracellular loop	Thumb	1305delC	(34)
αR438fs	480	Exon 8 extracellular loop	Thumb	1311delG	(36)
αY447fs	458	Exon 8 extracellular loop	Thumb	1340insT	(37)
αR448fs	459	Exon 8 extracellular loop	Thumb	1342-1343insTACA	(35)
αS452fs	480	Exon 8 extracellular loop	Thumb	1356delC	(38)
αT482fs	495	Exon 10 extracellular loop	Palm	1449delC	(39)

TABLE 3 | EC_{50} values of pseudohypoaldosteronism type 1B frameshift mutants for solnatide.

Construct	EC_{50}	*n*
Wild-type (WT)	54.7 ± 2.2	11
αI68fs	73.4 ± 13.4	9
αT169fs	58.3 ± 5.2	9
αP197fs	84.0 ± 4.9***	5
αE272fs	75.6 ± 5.4***	5
αF435fs	64.6 ± 8.7	7
αR438fs	56.6 ± 8.0	7
αY447fs	57.2 ± 4.8	7
αR448fs	50.8 ± 2.4	3
αS452fs	68.5 ± 4.1**	5
αT482fs	50.8 ± 5.5	5

*Significant difference compared to WT was calculated with the unpaired Student's t-test, **p < 0.01, ***p < 0.001.*

indicated in **Table 3**. TNF was also able to activate current in mutant ENaC; in case of αR448fs (**Figure 5**) up to WT control current without treatment (compare with **Figure 4**). Similar to WT ENaC the maximal TNF-induced current is lower than the solnatide-induced current in αR448fs mutant (compare **Figure 5** and **Table 1**), but the approximately 3.5-fold increase (TNF-induced/amiloride-sensitive current) in mutant ENaC exceeds the 2-fold current activation in WT ENaC after treatment with TNF. Mutant solnatide (T6A, E8A, E11A), which had no current-activating effect on WT ENaC (24), also had no effect on PHA1B mutants αF435fs and αR448fs.

Varied Effect of Solnatide on Membrane Abundance of α-ENaC Frameshift Mutants

To study α-ENaC protein abundance in plasma membrane cell surface, biotinylation of HEK-293 cells transiently transfected with WT or different PHA1B mutants was followed by SDS-PAGE and immunoblotting. Expression of frameshift mutants varied markedly. For example, expression of αF435fs, αY447fs, αR448fs, and αT482fs was highly significantly ($p < 0.001$, $n = 4$), and αP197fs was significantly ($p < 0.01$, $n = 4$) increased compared to WT. Notably, expression of the two mutants αF435fs and αT482fs was strikingly increased although the amiloride-sensitive Na^+ current was significantly ($p < 0.001$, $n = 7$ and $p < 0.001$, $n = 5$, respectively) attenuated (**Figure 4A**). Expression of αR438fs and αS452fs was comparable to WT ENaC, whereas expression of αI68fs, αT169fs, and αE272fs was significantly ($p < 0.001$, $n = 4$) decreased compared to expression of WT ENaC. Treatment of HEK-293 cells expressing WT or mutant frameshift α-ENaC with solnatide led to a transient and significant increase in membrane abundance of α-ENaC (**Table 4**).

Deglycosylation of α-ENaC Frameshift Mutations

We have previously shown that glycosylation of the extracellular loop of ENaC is one of the prerequisites of solnatide-induced ENaC activation (26). To validate the role of glycosylation in TNF- and solnatide-induced amiloride-sensitive Na^+ current activation in frameshift mutants, cell surface expression and patch-clamp experiments were performed following PNGase F treatment of αR448fsβγ as an example. As shown in **Figure 5**, no current could be induced by TNF or solnatide in αR448fs ($n = 3$) mutants when preincubated with PNGase F.

Taken together, these results indicate that frameshift mutation αR448fsβγ requires glycosylation of extracellular sites of ENaC for solnatide and TNF-induced activation of amiloride-sensitive sodium current.

For studies on the role of glycosylation in expression, the two mutants, αR448fs and αT482fs, were chosen, because they showed a marked increase in membrane expression in presence of solnatide. As illustrated in **Figures 6** and **7**, the solnatide induced increase in membrane abundance was completely abolished in deglycosylated mutants.

Role of β- and γ-ENaC in α-ENaC Frameshift Mutations

Lucas et al. (31) identified positions V567, E568, and E571 in the α-subunit as the crucial sites for binding of the lectin-like domain of TNF. They generated alanine replacement mutants in this region of α-ENaC and examined its interaction with the TIP peptide. In triple V567A/E568A/E571A and double V567A/E568A mutants, they found reduced binding capacity of the TIP peptide. Despite the absence of these relevant binding sites in our studied α-frameshift mutants, solnatide caused a significant current increase. As solnatide showed a small current increase in WT βγ-ENaC, we created alanine mutants (βM2γM2) in equivalent residues of β(E539A, E542A)- and γ(E548A, E551A)-ENaC. E539 and E542 in β-ENaC and E548 and E551 in γ-ENaC

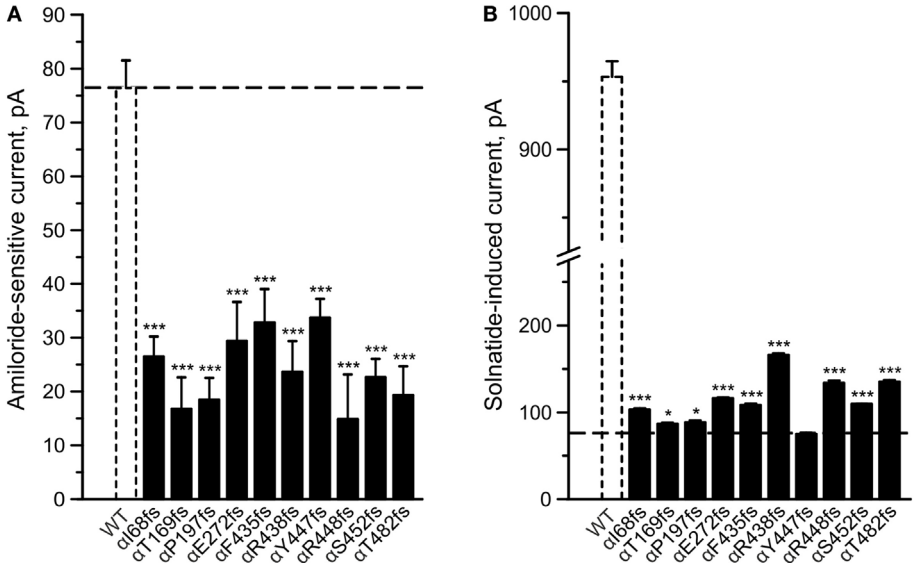

FIGURE 4 | Amiloride-sensitive sodium current and solnatide-induced current in pseudohypoaldosteronism type 1B (PHA1B) frameshift mutants. Wild-type (WT) or mutant α-epithelial sodium channel (ENaC) was co-expressed with βγ subunits in HEK-293 cells. Cells were patched in the whole-cell mode, and the inward current was elicited at −100 mV. The 10 μM amiloride-sensitive current **(A)** and 200 nM solnatide-induced current **(B)** of 10 frameshift mutations in α-ENaC associated with PHA1B (black bars) are shown in relation to WT (white, broken bar). For comparison the amiloride-sensitive current of WT αβγ-ENaC is indicated as broken line. Significant difference compared to WT control was calculated using one-way ANOVA followed by Tukey's *post hoc* test, $^{*}p < 0.05$, $^{***}p < 0.001$ ($n = 3$–11).

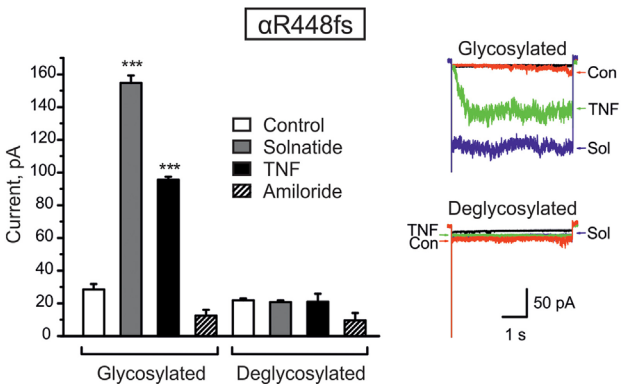

FIGURE 5 | Deglycosylation of R448fs with PNGase F abolished both solnatide- and tumor necrosis factor (TNF)-induced activation. Mean values of 200 nM solnatide- and 20 nM TNF-induced inward currents in control (glycosylated) and 100 units PNGase F treated (deglycosylated) αR448fsβγ (left), $^{***}p < 0.001$ compared with control as determined by unpaired Student's *t*-test, $n = 3$. Typical solnatide- and TNF-induced current traces of αR448fsβγ in control and PNGase F (100 U) treated transiently transfected HEK-293 cells. For comparison, original traces from separate solnatide and TNF experiments are superimposed (right).

are homologous to E568 and E571 in the α-subunit, whereas the V567 residue of α-ENaC is I538 in β-ENaC and I547 in γ-ENaC. Solnatide, however, still increased the amiloride-sensitive sodium current in these mutants, which implies that these regions in β- and γ-ENaC do not play any role in the current-activating effect of α-frameshift mutations.

TABLE 4 | Effect of solnatide on membrane abundance of α-epithelial sodium channel frameshift mutations.

Mutation	Control	Solnatide	
		5 min	10 min
Wild-type	1	1.48 ± 0.12***	1.40 ± 0.09***
αI68fs	0.15 ± 0.06	0.25 ± 0.07*	0.32 ± 0.11**
αT169fs	0.54 ± 0.06	0.80 ± 0.03***	0.46 ± 0.09
αP197fs	1.18 ± 0.05	1.33 ± 0.07**	0.48 ± 0.09***
αE272fs	0.35 ± 0.06	0.72 ± 0.03***	0.64 ± 0.05***
αF435fs	2.37 ± 0.15	3.71 ± 0.20***	2.82 ± 0.17**
αR438fs	0.90 ± 0.07	1.39 ± 0.14***	0.87 ± 0.10
αY447fs	1.27 ± 0.10	1.57 ± 0.13***	1.89 ± 0.16***
αR448fs	1.32 ± 0.10	5.88 ± 0.32***	1.30 ± 0.12
αS452fs	1.11 ± 0.25	1.36 ± 0.11***	1.94 ± 0.08***
αT482fs	4.67 ± 0.27	6.23 ± 0.24***	4.58 ± 0.30

$^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, significant difference from respective control values (one-way ANOVA, Tukey's post hoc test).

DISCUSSION

We have previously shown that the synthetic cyclic peptide solnatide, which mimics the lectin-like domain of TNF, requires one of the two pore-forming α- and δ-ENaC subunits to induce its maximum amiloride-sensitive sodium current-activating effect (16). Loss-of-function mutations in ENaC genes translate into the salt-wasting genetic disease PHA1B (33, 41). We have also shown that loss-of-function point mutations of ENaC found in PHA1B patients conduct significantly low current when transfected along with WT β- and γ-subunits (27). Remarkably, amiloride-sensitive

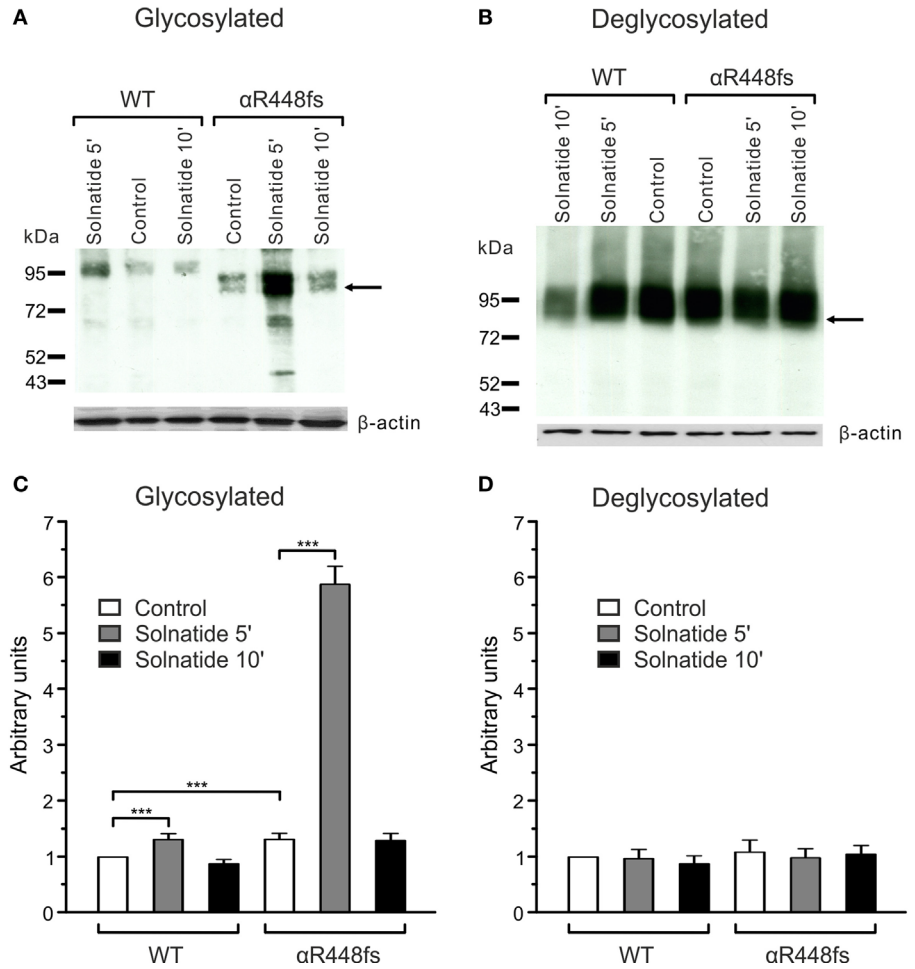

FIGURE 6 | **Effect of solnatide on the membrane abundance of αR448fs without and with PNGase F treatment**. Biotinylated surface proteins of HEK-293 cells heterologously expressing WT αβγ-epithelial sodium channel (ENaC) or αR448fsβγ-ENaC treated with 200 nM solnatide at indicated time points and/or 100 units PNGase F were blotted and analyzed with anti-α-ENaC antibody. One representative blot out of four independent biological replicates is shown before [**(A)**; glycosylated] and after PNGase F treatment [**(B)**; deglycosylated]. Wild-type (WT) α-ENaC shows a band at about 95 kDa and for mutant α-ENaC the protein band, which was used for quantification is indicated by arrows. α-ENaC expression was normalized to β-actin and set in relation to WT control (= 1). The membrane abundance of glycosylated αR448fs-ENaC is highly increased after 5 min of solnatide treatment **(C)**, whereas after PNGase F treatment no differences can be observed [**(D)**; deglycosylated]. Significant differences are indicated, ***$p < 0.001$ ($n = 4$).

currents were restored to WT control levels by solnatide and its congener, AP318 (27). In the present study, experiments were performed to elucidate the effect of the TNF lectin-like domain, both as an integral part of the TNF molecule as well as represented by solnatide, on PHA1B frameshift mutations.

Lectin-Mediated Activation of ENaC by TNF

The mechanism of TNF-induced ion channel modulation has been intensively studied and, in particular, TNF in combination with other cytokines could drive a pathological condition to a more aggressive state (42, 43). However, as a possible therapeutic molecule, the machinery of TNF-induced activation of ion channels is still largely unknown (44). TNF exhibits a dual role of action in pathological conditions; specifically, TNF has been shown to contribute to the pathogenesis and development of pulmonary edema, through binding to TNF receptors and consequent initiation of the inflammatory cascade. However, some studies have demonstrated surprisingly that TNF can also promote alveolar fluid reabsorption *in vivo* and *in vitro*, a protective effect mediated by the lectin-like domain of the cytokine, which is spatially distinct from the TNF-receptor binding sites (45).

The current-enhancing effect of TNF on different ion channels including ENaC has been documented (15, 46). We have previously shown that solnatide, mimicking the lectin-like domain of TNF, can activate WT ENaC channels (16, 24, 26), as well as ENaC carrying PHA1B-causing mutations (27). In the present study, our data provide evidence for a mechanism of lectin-like domain-mediated, TNF-induced activation of ENaC carrying PHA1B-causing frameshift mutations. Our data demonstrate that TNF has to bind to ENaC glycosylation

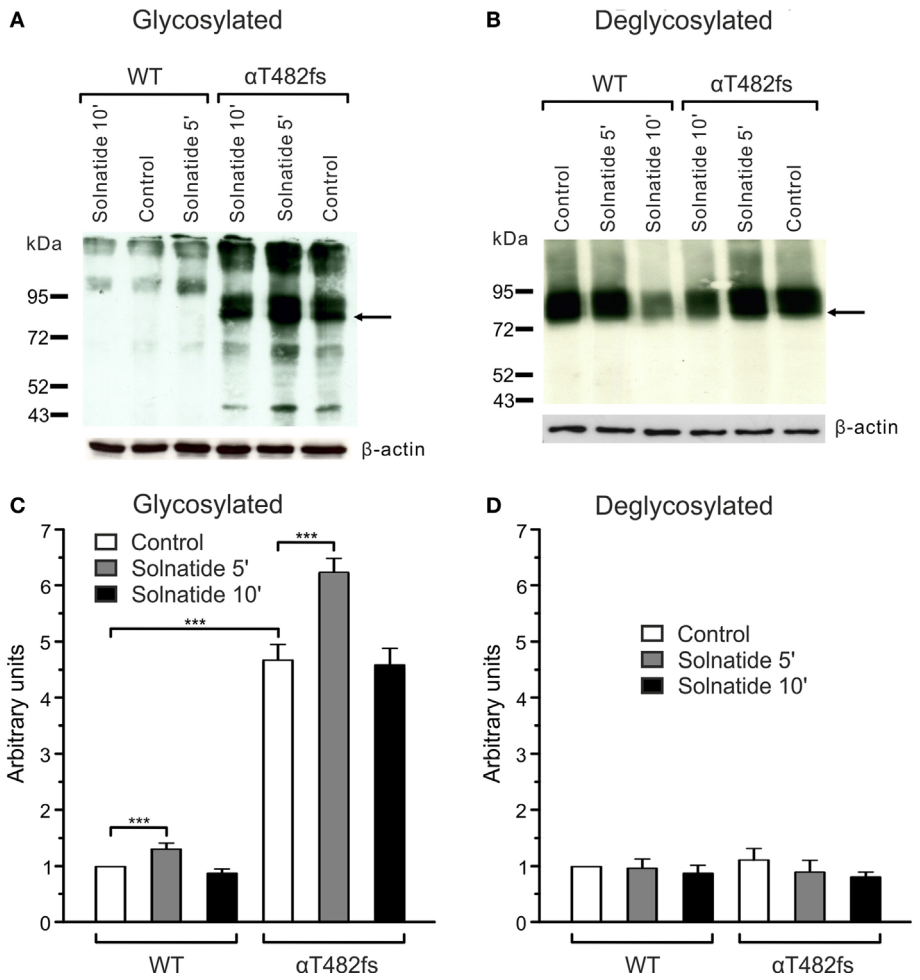

FIGURE 7 | Effect of solnatide on the membrane abundance of αT482fs without and with PNGase F treatment. Biotinylated surface proteins of HEK-293 cells heterologously expressing wild-type (WT) αβγ-epithelial sodium channel (ENaC) or αT482fsβγ-ENaC treated with 200 nM solnatide at indicated time points and/or 100 units PNGase F were blotted and analyzed with anti-α-ENaC antibody. One representative blot out of four independent biological replicates is shown before [**(A)**; glycosylated] and after PNGase F treatment [**(B)**; deglycosylated]. WT α-ENaC shows a band at about 95 kDa and for mutant α-ENaC the protein band which was used for quantification is indicated by arrows. α-ENaC expression was normalized to β-actin and set in relation to WT control (=1). The membrane abundance of glycosylated αT482fs-ENaC is already increased without solnatide (control) compared to WT and even more after 5 min of solnatide treatment **(C)**, whereas after PNGase F treatment no differences can be observed [**(D)**; deglycosylated]. Significant differences are indicated, ***$p < 0.001$ ($n = 4$).

sites of the extracellular loop through its lectin-like domain in order to exert its ENaC-activating effect as well as to increase translocation of newly synthesized channels to the plasma membrane. Notably, one TNF molecule, which exists as a stable homotrimer (47) contains three lectin-like domains which make the TNF a highly potent activator of ENaC compared to solnatide (a single lectin-like domain mimicking molecule); see EC_{50} values in **Table 1**. In contrast, the maximal stimulatory effect of solnatide was greater and was reached more rapidly after ~2-min exposure, compared to that of TNF, which was reached after ~5 min (**Figure 1**). The maximum induced current for solnatide was 953.2 ± 11.5 pA compared to 162.5 ± 7.5 pA induced by TNF (**Table 1**). The reason for slower time course of activation and smaller current induced by TNF could be that TNF is a bulkier, larger molecule (the mature TNF trimer has a molecular mass of approximately 52 kD) than solnatide (17-mer cyclic peptide, molecular mass 1.9 kD) and hence occupies more space around the extracellular loop of ENaC, around which in comparison more molecules of solnatide, could be accommodated and simultaneously engage with glycosylation or other sites of interaction.

An alternative interpretation of slower TNF time course of activation of ENaC compared to solnatide could be that TNF and solnatide interact with both the extracellular and intracellular domains of ENaC; the time required to activate ENaC would simply reflect the necessity of TNF and solnatide to penetrate the plasma membrane. Binding of the triad of lectin-like domains at the tip of the native TNF homotrimer to glycosylation sites on the ENaC heterooligomer might hinder further folding and subsequent penetration of the TNF molecule across the plasma

membrane. We have previously shown that solnatide required αβγ-ENaC or δβγ-ENaC to show its maximum stimulatory effect (16). To our surprise, in single subunit experiments, TNF-induced current was higher than solnatide-induced current (**Table 1**).

Direct interaction of TNF with ENaC has hitherto not been reported and so its physiological role in improving ALC during lung inflammation has been largely inferred from numerous studies with solnatide and other TIP peptides (18, 20–23). A recent study which sought to determine the precise mechanism by which solnatide stimulated Na^+ uptake in the presence or absence of PLY, demonstrated that TIP activates ENaC through binding to the carboxyl-terminal domain of the α-subunit (19). Using heterologously expressed WT ENaC we show in the present study that native TNF enhances Na^+ current, although the maximum TNF-activated current is less than with solnatide.

Tumor necrosis factor, like solnatide, also requires the intracellular carboxyl-terminal region of α- or δ-ENaC to exert its effect of bringing about an increase in membrane abundance of the respective subunits (**Figure 3**). Thus, with the mutants αL576Xβγ-ENaC and δD522Xβγ-ENaC, which lack the region between TM2 and the carboxyl terminus of α- or δ-hENaC, respectively, the increase in membrane abundance seen with WT α- or δ-ENaC was not observed (**Figure 3**). These data are in agreement with our previously published reports (19, 26, 31) that the carboxyl-terminal domain of α- or δ-ENaC is an essential motif for TNF lectin-like domain induced activation of the channel.

The possibility exists that TNF binds to the cell membrane surrounding or in the vicinity of oligomeric ENaC in a general, non-specific manner, thereby altering the disposition of ENaC in the bilayer and resulting in a conformation with a higher P_o. Such an affect would still be amiloride-sensitive if the amiloride-binding sites in ENaC subunits were intact and accessible and would be eliminated by addition of amiloride. A precedent for such non-specific membrane insertion of TNF at low pH has been documented (48). Specifically, a role has been suggested for residues in the lectin-like domain of TNF in membrane insertion. The lectin-like domain occupies residues Cys101-Glu116 of human TNF (2), located in triplicate in the highly flexible loop region at the apex of the bell-shaped native TNF trimer (49). Trp114, the first-ordered residue after the apical flexible loop (47), is buried at pH 7.4 but could readily become exposed to an aqueous milieu upon protonation of nearby residues (e.g., Glu116, a salt-bridge participant), resulting in increased surface hydrophobicity and a tendency for insertion into the lipid bilayer by hydrophobic interactions (48). Moreover, membrane penetration has been shown to stabilize the low pH conformation of TNF, and membrane inserted TNF exhibits a native trimeric structure (48). The corresponding bulky, hydrophobic region of TIP peptides (Trp15 in solnatide) has been shown to be one of the essential characteristic features required for the Na^+ current-potentiating effect of these peptides (24). In the case of solnatide, the cyclic peptide is totally exposed to the aqueous environment leading one to question whether Trp15 would similarly lend to the peptide the tendency for membrane insertion by hydrophobic interaction. Earlier studies with artificial lysosomes, however, could produce no evidence of direct membrane interaction of this TIP peptide (50), leading the authors to conclude that interaction of the TIP domain *via* an ion channel or other membrane protein was required for its current-potentiating effect.

Restoration of ENaC Current in Frameshift Mutants by TNF and Solnatide

Previously it has been shown that frameshift mutations in pacemaker channels (*HCN4*) do produce a functional channel, which shows normal intracellular trafficking and membrane integration, when transfected in mammalian cells (51), whereas in the case of the cardiac sodium channel (*SCN5A*), a complete loss-of-function phenotype was reported (52).

Computational and site-directed mutagenesis approaches have shown that the lectin-like domain of TNF and solnatide, the synthetic peptide which mimics it, exert their ENaC-activating effect through binding with glycosylation sites of extracellular loops of ENaC (26, 53). Because solnatide has been shown to directly bind with glycosylation sites of ENaC, we treated αR448fsβγ-ENaC with PNGase F prior to testing in a patch clamp assay with solnatide and TNF. Convincingly, neither TNF nor solnatide potentiated amiloride-sensitive current in αR448fsβγ-ENaC following PNGase F treatment, contrary to the activation observed without prior PNGase F treatment (**Figure 5**). PNGase F treatment also abolished the increase in membrane abundance observed with mutants αR448fs and αT482fs in the presence of solnatide (**Figures 6** and **7**). These results indicate and are consistent with our previous results that glycosylation sites on

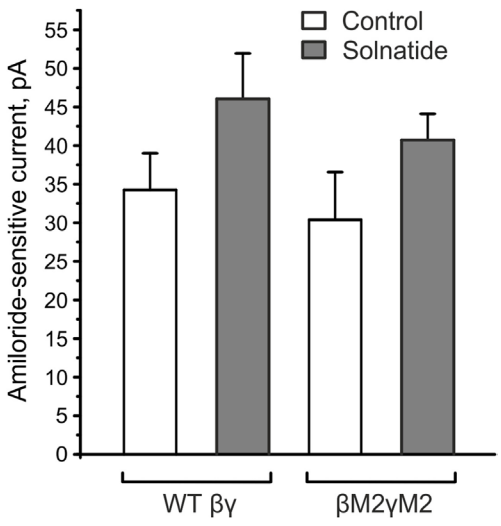

FIGURE 8 | βE539AE542AγE548AE551A mutants (βM2γM2) did not affect the solnatide-induced activation of βγ-epithelial sodium channel (ENaC). HEK-293 cells transiently transfected with wild-type (WT) βγ- or mutant βM2γM2-ENaC were patched in the whole-cell mode. The inward current at −100 mV was measured in absence (control) and presence of 200 nM solnatide and the 10 μM amiloride-sensitive current was calculated. E539 and E542 in β-ENaC and E548 and E551 in γ-ENaC are homologous to the postulated solnatide-binding sites (V567), E568 and E571 in α-ENaC (31), but solnatide was still able to activate βM2γM2-ENaC (not significant) to an equal extent as WT βγ-ENaC ($n = 3$).

the extracellular loop of ENaC are essential for solnatide-induced activation of ENaC (16, 26).

All the frameshift mutants described in the current work lack the amiloride-binding site of the α-subunit, located in TM2, but retain amiloride-binding sites in the co-expressed WT β- and γ-subunits. The amiloride-binding site of α-ENaC is at S556, the position corresponding to Gly439 in ASIC1 (54) and located in the middle of TM2. Amiloride-binding sites occur at equivalent positions in the β- and γ-ENaC subunits (54, 55). The amiloride sensitivity shown by the PHA1B frameshift mutants described in the present work must therefore be due to amiloride binding to sites in the β- and γ-subunits.

In the present study we analyzed frameshift mutations of α-ENaC which produce a truncated ENaC α-subunit. We found that these frameshift mutants can generate amiloride-sensitive current, but it is significantly lower than WT ENaC (**Figure 4A**). Remarkably, solnatide restored the amiloride-sensitive current in all these frameshift mutants to WT or higher levels (**Figure 4B**). As shown in **Table 2**, these mutants generate a truncated α-ENaC of different lengths ranging from 142 (I68fs) to 495 (T482fs) amino acid residues. The αI68fs mutation results in production of a truncated α-subunit comprising a polypeptide chain of 142 amino acid residues, of which residues 1–67 are WT and 68–142 are non-native due to the shift in the reading frame of the mRNA transcript by two nucleotide positions. Analysis of the 142 mutant amino acid sequence with the TMPred bioinformatics tool for prediction of membrane-spanning regions, failed to detect any TM regions, whereas in WT α-hENaC, TM1 is located between residues F86-F110 by sequence comparison with the ENaC homolog, ASIC1 (56). The 142-residue polypeptide resulting from the αI68fs mutation is unlikely to penetrate the membrane, but may associate intracellularly with β- and γ-subunits and thus be detectable in the biotinylated membrane protein fraction.

Previous work of others had shown that the PHA1B mutant αI68fs conducts 0.1% current compared with WT when co-expressed with rat βγ-ENaC in *Xenopus* oocytes (57). Surprisingly, solnatide induced a current increase in αI68fsβγ-ENaC, which lacks both TM regions and all hitherto known or hypothesized binding motives for solnatide activation, namely: glycosylation sites in the extracellular loop (26), carboxyl-terminal domain of α-ENaC (19); V567 and E568 in TM2, residues found to be critical for solnatide and TNF binding (31).

The solnatide-induced activation of αI68fsβγ-ENaC could be in part due to the presence of βγ-ENaC subunits co-transfected with mutant αI68fsENaC. In fact, this applies to all the frameshift mutants examined here. To solve this puzzling discrepancy, we analyzed solnatide activation of ENaC comprising the β- and γ-subunits only. As shown in **Figure 8**, solnatide could activate the inward sodium current through βγ-ENaC channels to a level comparable to that observed for αI68fsβγ-ENaC (**Figure 4B**). These data indicate and are in agreement with our previously published results (16), namely that solnatide can activate βγ-ENaC marginally. Solnatide has been shown to activate ENaC by binding critical residues located in TM2 of α-hENaC (31). Lucas et al. (31) found that double (V567A,E568A) and triple (V567A, E568A, E571A) α-ENaC mutants showed reduced binding capacity to solnatide and TNF, resulting in an abolition of the increase in P_o usually observed with WT ENaC in the presence of solnatide, although membrane expression was the same as WT. To explore the possibility that the observed potentiation of Na^+ current in the αI68fsβγ-ENaC mutant could be due to binding of solnatide or TNF to residues of βγ-ENaC equivalent to E568 and E571, two of the three residues in TM2 of α-ENaC studied by Lucas et al. (31), we generated point mutations of β-ENaC: E539A, E542A and γ-ENaC: E548, E551 (**Figure 8**). A small increase in the amiloride-sensitive Na^+ current was still observed with βE539A, E542A, γE548A, E551A-ENaC in the presence of solnatide (**Figure 8**). These results indicate that some other mechanism is responsible for solnatide-induced potentiation of the Na^+ current, albeit small, in these βγ-ENaC TM2 mutants, which lack α-ENaC and therefore the crucial residues V567A, E568A in TM2, as well as residues in β- and γ-ENaC, E539, E542 and E548, E551, respectively, equivalent to E568 and E571 in α-ENaC. Such a mechanism could explain the Na^+ current-potentiating effect of solnatide on the αI68fsβγ-ENaC and the other frameshift mutants examined in the present study.

Langloh et al. (58) also studied the effects of mutating residues in TM2 of α-hENaC, but unlike the alanine mutants described above, they mutated highly conserved glutamic acid residues to arginine, E568R, E571R, and D575R, thus reversing the charge at these important positions in TM2. Whole-cell amiloride-sensitive current recorded from oocytes injected with the α-ENaC mutants along with WT β- and γ-ENaC, was low compared with the WT channel, but plasma membrane abundance of the mutant channels was the same as that of WT. The mutations decreased channel conductance but did not affect Na^+:K^+ permeability.

Results of earlier experiments conducted by our group with mutants αL576Xβγ-ENaC and δD522Xβγ-ENaC, which lack the region between TM2 and the carboxyl terminus of α- or δ-hENaC (26), had indicated a residual albeit non-significant increase in the amiloride-sensitive current in the presence of solnatide. A channel lacking all carboxyl termini, namely αL576XβD546XγD556X, showed an even slighter, non-significant increase of current after treatment with solnatide in preliminary experiments. This small increase was not seen in the case of αL576Xβγ-ENaC and δD522Xβγ-ENaC in which the glycosylation sites in the extracellular loop had been removed by mutation (26). Thus, apart from the requirement for an intact carboxyl-terminal region in the α-subunit, some other unknown glycosylation-mediated mechanisms seem to play a minor role in TIP activation of ENaC.

A striking feature of the frameshift mutations examined in this work is their non-random distribution in the 3D molecular structure of the α-ENaC subunit. Specifically, of the 10 reported mutations in the current report, five (50%) are located in the thumb domain (**Table 2**) according to the domain nomenclature established for the ENaC homolog, ASIC1 (56). Of the remaining mutations, three are located in the finger domain, one in the palm domain, and one intracellularly. Another α-ENaC frameshift mutation causing PHA1B previously investigated by our group, S243fs (27, 39), is located in the finger domain. Although we cannot purport to have investigated all known PHA1B mutations (some of which have not been reported in the literature), there does seem to be a trend for the thumb domain of α-ENaC to

manifest only frameshift mutations, since all PHA1B-causing mutations so far located to the thumb domain of α-ENaC are frameshift mutations (unpublished findings) translated from exon 8 of the mRNA transcript. All frameshift mutations result in truncated polypeptide chains that contain the α-ENaC amino terminal native sequence preceding the mutation followed by a sequence of non-native residues of varying length, depending on the position of the mutation and length of the out-of-frame mRNA before a stop codon is encountered (**Table 2**). At the gene level, a mutational hotspot resulting in insertion or deletion of nucleotide base pairs might be the cause of such clustering of mutations in exon 8. The results presented here suggest that at the protein level, since α-subunits are detected by surface biotinylation, a channel with severely reduced Na^+ conducting capacity is produced, apparently comprised of truncated α-subunit and full-length wild-type β- and γ-subunits. Alternatively, truncated polypeptide chains are trafficked to the membrane, but Na^+ conducting channels, albeit of severely compromised activity, are assembled from β- and γ-subunits only.

The effect of solnatide on increasing membrane abundance in the frameshift mutants was extremely varied and no trend could be discerned, other than that the effect is transient with a peak around 5−10 min of exposure to solnatide, suggesting that solnatide exerts its effect by increasing trafficking of mutant α-ENaC to the membrane. Some mutants, specifically αF435fs and αT482fs, were characterized by a markedly increased membrane abundance of the truncated subunit compared to WT ENaC in the absence of solnatide, the membrane abundance increasing even further following exposure to solnatide (**Table 4**). All frameshift mutants described here lack the "PPxY" and "YXXΦ" motifs located in the intracellular carboxyl-terminal region and required for ubiquitination and endocytosis (59, 60), and in the absence of which, mutant subunits would accumulate at the cell surface. This could explain the significantly higher abundance in the membrane of some of the frameshift mutants compared to WT ENaC. The increase in abundance of mutant subunits compared to WT does not seem to correlate with higher amiloride-sensitive current either without or in the presence of solnatide (**Figure 4**, **Table 4**), suggesting that these mutant subunits are mostly dysfunctional proteins. Nevertheless, mutant subunits do increase in abundance in response to solnatide and this effect, combined with the increase in P_o brought about by the lectin-like domain interacting with mutant α-subunits and possibly with WT β- and γ-subunits results in solnatide rescuing these PHA1B frameshift mutants and restoring amiloride-sensitive Na^+ current to physiological levels.

Concluding Remarks

The results presented here validate the use of TIP peptides as experimental models for the TNF lectin-like domain, previously assumed in numerous studies (19, 21, 31, 45, 61). Although the PHA1B frameshift mutants investigated in the present study lack features shown in earlier studies to be critical for TNF lectin-like domain interaction with ENaC, the fact that solnatide potentiates amiloride-sensitive Na^+ current to physiological levels, rescuing the mutants, indicates that some additional glycosylation-dependent mechanism, possibly involving β- and γ-ENaC, contributes to the solnatide-induced amiloride-sensitive Na^+ current. Consequently, as we previously reported for point mutations causing PHA1B (27), TIP peptides would seem to be good candidates for lead compounds in the drug development process for treatment of this life-threatening hereditary disease caused by loss-of-function mutations in ENaC.

AUTHOR CONTRIBUTIONS

AW gave substantial contribution to the design of the work, performed experiments, analyzed and interpreted data, and drafted the work. MA performed experiments, analyzed and interpreted data, and drafted the work. ST gave substantial contributions to the conception and design of the work, interpretation of data, and drafted the work. DEM, FP, and SI performed electrophysiological experiments, analyzed data, and drafted the work. ALW, BU, DG, and DM performed Western blot experiments, analyzed data, and drafted the work. BF, HF, and HP gave contribution to the conception of the work and revised it critically. IC and RL interpreted data and revised the work critically for important intellectual content. RL-G gave substantial contribution to the conception and design of the work, interpretation of data, and drafted the work. WS gave substantial contribution to the design of the work, performed experiments, analyzed data, and drafted the work. All the authors approved the version to be published and agree to be accountable for the content of the work.

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Epithelial Sodium Channel-α Mediates the Protective Effect of the TNF-Derived TIP Peptide in Pneumolysin-Induced Endothelial Barrier Dysfunction

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Background: *Streptococcus pneumoniae* is a major etiologic agent of bacterial pneumonia. Autolysis and antibiotic-mediated lysis of pneumococci induce release of the pore-forming toxin, pneumolysin (PLY), their major virulence factor, which is a prominent cause of acute lung injury. PLY inhibits alveolar liquid clearance and severely compromises alveolar–capillary barrier function, leading to permeability edema associated with pneumonia. As a consequence, alveolar flooding occurs, which can precipitate lethal hypoxemia by impairing gas exchange. The α subunit of the epithelial sodium channel (ENaC) is crucial for promoting Na^+ reabsorption across Na^+-transporting epithelia. However, it is not known if human lung microvascular endothelial cells (HL-MVEC) also express ENaC-α and whether this subunit is involved in the regulation of their barrier function.

Methods: The presence of α, β, and γ subunits of ENaC and protein phosphorylation status in HL-MVEC were assessed in western blotting. The role of ENaC-α in monolayer resistance of HL-MVEC was examined by depletion of this subunit by specific siRNA and by employing the TNF-derived TIP peptide, a specific activator that directly binds to ENaC-α.

Results: HL-MVEC express all three subunits of ENaC, as well as acid-sensing ion channel 1a (ASIC1a), which has the capacity to form hybrid non-selective cation channels with ENaC-α. Both TIP peptide, which specifically binds to ENaC-α, and the specific ASIC1a activator MitTx significantly strengthened barrier function in PLY-treated HL-MVEC. ENaC-α depletion significantly increased sensitivity to PLY-induced hyperpermeability and in addition, blunted the protective effect of both the TIP peptide

and MitTx, indicating an important role for ENaC-α and for hybrid NSC channels in barrier function of HL-MVEC. TIP peptide blunted PLY-induced phosphorylation of both calmodulin-dependent kinase II (CaMKII) and of its substrate, the actin-binding protein filamin A (FLN-A), requiring the expression of both ENaC-α and ASIC1a. Since non-phosphorylated FLN-A promotes ENaC channel open probability and blunts stress fiber formation, modulation of this activity represents an attractive target for the protective actions of ENaC-α in both barrier function and liquid clearance.

Conclusion: Our results in cultured endothelial cells demonstrate a previously unrecognized role for ENaC-α in strengthening capillary barrier function that may apply to the human lung. Strategies aiming to activate endothelial NSC channels that contain ENaC-α should be further investigated as a novel approach to improve barrier function in the capillary endothelium during pneumonia.

Keywords: epithelial sodium channel, non-selective cation channel, TNF, pneumonia, pneumolysin, endothelial barrier function

INTRODUCTION

Pulmonary permeability edema is a life-threatening complication of severe pneumonia and acute respiratory distress syndrome (ARDS), characterized by impaired alveolar liquid clearance (ALC) and alveolar–capillary hyperpermeability (1). Antibiotic treatment of patients infected with *Streptococcus pneumoniae* significantly reduces bacterial load, but it can also cause massive release of bacterial toxins in the lung compartment (2). The 53-kDa pneumococcal pore-forming virulence factor pneumolysin (PLY) was shown to be an important mediator of permeability edema, due to its capacity to impair both endothelial (3, 4) and epithelial barrier function (5). Although pneumococci release sufficient amounts of PLY to perforate the host cell plasma membrane, this does not necessarily cause immediate cell death, since membrane segments harboring toxin-induced pores can be either internalized or eliminated by microvesicle shedding. Dysregulation of cellular homeostasis secondary to transient pore formation/elimination is likely responsible for the damaging actions of PLY (6). To date, no proven treatment exists for increased pulmonary permeability edema, apart from ventilation strategies. Hence, the search for novel therapeutic agents that have the ability to restore both endothelial barrier function and ALC capacity is warranted.

Apart from impairing barrier function, PLY has also been shown to decrease the activity of the epithelial sodium channel (ENaC) (7), which is expressed on the apical side of alveolar epithelial cells and which, together with the basolaterally expressed Na^+–K^+-ATPase (8, 9), represents the primary mediator of Na^+ uptake and liquid clearance in the alveolar compartment. In its native form, ENaC consists of three subunits, α, β, and γ (10, 11), but also a fourth δ subunit has been described, which can substitute for the α subunit (12). ENaC activity is defined as the product of its surface expression *N*, which is at least partially determined by Nedd-4-2-dependent ubiquitination (13) and its open probability *P*o, the latter of which is significantly increased by the formation of a complex comprised of ENaC subunits with MARCKS and PIP_2 (14). In order to be fully functional, ENaC has to interact with the actin cytoskeleton and in particular with the actin-binding protein, filamin A (FLN-A) (15).

We recently demonstrated that the 17 residue circular TIP peptide (sequence: CGQRETPEGAEAKPWYC), which mimics the lectin-like domain of TNF, directly binds to two domains within the crucial α subunit of ENaC (16–18). The TIP peptide, through binding to residues Val567 and Glu568 increases the channel's open probability time by promoting complex formation between human ENaC-α and MARCKS (18). In addition, the peptide augments ENaC-α surface expression in PLY-treated H441 cells, by means of reducing the subunit's ubiquitination (18). This activity requires the presence of *N*-glycosylated Asn residues in the extracellular loop of the subunit (17). The presence of the TIP peptide has been shown to increase ALC and to ameliorate acute lung injury *in vivo* in several species (16, 19–23). The TIP peptide is well tolerated, and no significant side effects have been reported upon inhalation in healthy male volunteers (24). The TIP peptide is emerging as a potential therapeutic candidate for improving lung function. Data from two phase IIa clinical trials with inhalation of TIP peptide (a.k.a. AP301 and solnatide) in acute lung injury patients, the majority of which had severe pneumonia, and another trial in patients with primary graft dysfunction upon lung transplantation (www.ClinicalTrials.gov, Identifier NCT01627613 and NCT02095626, respectively) document efficacy. Both of these pathologies are characterized by capillary endothelial dysfunction.

Although originally thought to mainly constitute the rate-limiting entry step in Na^+ reabsorption across lung, kidney, and colon epithelia, it has become clear in recent years that ENaC may also play an important role in the vasculature. In large vessels, ENaC is expressed in both endothelial and vascular smooth muscle cell compartments, where it operates as a mechano-sensitive channel, exposed to varying rates of blood flow and laminar shear stress (25). In contrast to large vessels, the presence or role of ENaC in the microvasculature, such as in the capillaries in the lung, remains understudied and represents the primary focus of this study. The TIP peptide was

shown to increase Na^+ uptake in pulmonary microvascular endothelial cells (26) and to restore impaired endothelial barrier function in the presence of the pore-forming toxins PLY and listeriolysin-O (3, 27).

In view of the previously observed protective activities of the TNF-derived TIP peptide on capillary barrier function in the presence of bacterial toxins, in this study, we investigated the role of its binding partner—ENaC-α—in microvascular endothelial cell barrier function. Our objective was to identify those common signaling molecules modified by bacterial toxins that are involved in both endothelial barrier impairment and ENaC dysfunction.

MATERIALS AND METHODS

Cells

Human lung microvascular endothelial cells (HL-MVEC) were grown in complete EBM-2 medium (Lonza, Walkersville, MD, USA) at 37°C and 5% CO_2. Experiments with PLY were performed in serum-free medium, since the toxin's activity is neutralized by cholesterol.

PLY Purification

Pneumolysin was purified from a recombinant *Listeria innocua* 6a strain expressing LPS-free PLY. The batch of PLY used in this study had a specific activity of 1.25×10^7 hemolytic units per milligram.

Biochemicals

Rabbit polyclonal anti-ENaC-α (59), β (60), and γ (2102) antibodies were generated in the laboratory of D.C.E (14), anti-human FLN-A, anti-human phospho-FLN-A, anti-human CaMKII, anti-human phospho-CaMKII, and anti-Actin HRP were from Cell Signaling Technology (Danvers, MA, USA). Rabbit anti-human ENaC-α was from Novus Biologicals (Littleton, CO, USA), a rabbit anti-human ASIC1 for IP was from EMD Millipore (Temecula, CA, USA), and a rabbit anti-hASIC1 antibody for WB was a kind gift from Dr. John Wemmie, University of Iowa. Goat anti-rabbit secondary antibodies conjugated to HRP were from Cell Signaling Technology (Danvers, MA, USA). MitTx was purchased from Alomone (Jerusalem, Israel), CaMKII inhibitor XII was from EMD Millipore (Billerica, MA, USA), and the TIP peptide was custom-ordered and purchased from AMBIOPHARM (North-Augusta, SC, USA).

Depletion of ENaC-α or Acid-Sensing Ion Channel 1a (ASIC1a) in HL-MVEC

Human lung microvascular endothelial cells were treated with a pool of target-specific 19–25 nt siRNAs designed to knock down either ENaC-α conducting subunit or ASIC1a gene expression, and non-specific, non-targeting siRNA were obtained from Ambion (Grand Island, NY, USA). All siRNA's were received in lyophilized form. HL-MVEC were transfected at 70–80% confluence with 50–75 nM final concentration of siRNA using siPORT™ Amine transfection reagent (Ambion, Life Technologies, Grand Island, NY, USA) and used for further experiments at 48 h post transfection.

Immunoprecipitation

Human lung microvascular endothelial cells were grown in 60-mm culture flasks and were washed with PBS, scraped, and lysed in 400 µl of 20 mM Tris–HCl, pH 7.4 buffer containing 0.15 M NaCl, 1% non-idet P-40, 2 mM EDTA, as well as protease inhibitors. Lysates were incubated with empty beads in order to remove the non-specific binding partners (preclearing step) and subsequently with ASIC1 antibody for 1 h at 4°C. The mixture of the antibody and the precleared whole cell lysate was then incubated with agarose G magnetic beads overnight at 4°C, followed by three washing steps with PBS containing 2% BSA and eluted in 150 µl of Laemmli buffer. The resulting supernatants were analyzed by western blotting with ENaC-α antibody.

Immunoblotting Procedure

Immediately after treatment, HL-MVEC were washed twice with ice-cold PBS and lysed with RIPA buffer containing a phosphatase and a protease inhibitor mixture. After centrifugation, clear supernatants were mixed with SDS sample buffer and boiled for 5 min. Protein extracts were separated on SDS/PAGE, transferred to a nitrocellulose membrane, incubated with primary antibodies, and subsequently after washing with HRP-conjugated secondary Ab. Immunoreactive proteins were visualized with Clarity solution (Bio-Rad, Hercules, CA, USA) and were then captured using ChemiDoc system (Bio-Rad). The relative intensity of each protein band was quantified using the ImageLab software (Bio-Rad).

NanoPro Technology

Immediately after treatment, cells were washed and lysed with buffers from ProteinSimple (Santa Clara, CA, USA) as described previously (28). Preparation of cell lysates for size-based assay, using the Peggy system, was performed as described by the manufacturer (ProteinSimple).

Measurement of Transendothelial Electrical Resistance

Transendothelial electrical resistance in HL-MVEC monolayers [electrical cell-substrate impedance sensing (ECIS) system 1600R; Applied Biophysics, Troy, NY, USA] was measured as described previously (3).

Statistical Analysis

All experimental data are presented as mean ± SD. Control samples and those obtained upon various stimuli were compared by unpaired Student's *t*-test. For multiple group comparisons, one-way ANOVA was used. Also, $p < 0.05$ was considered statistically significant.

RESULTS

HL-MVEC Express All ENaC Subunits

We previously demonstrated, using whole cell voltage-clamped patch clamp, that TIP peptide increased amiloride-sensitive Na^+ currents in freshly isolated mouse MVEC (26). Here, we

investigated whether HL-MVEC express the three ENaC subunits. Immunoblotting analysis revealed the presence of both uncleaved and mature ENaC-α, β, and γ subunits in human lung MVEC (**Figure 1**). The immunoreactive bands of ENaC-α at 95, 75, and 65 kDa represent different forms of the subunit, resulting from posttranslational modifications (e.g., glycosylation) and proteolytic processing.

ENaC-α Expression Strengthens Barrier Function in PLY-Treated HL-MVEC Monolayers

The association of ENaC with the cytoskeletal network at the apical membrane is required to help maintain its presence at this site and to prevent its removal by endocytosis (30, 31). It has not yet been investigated whether activation of the channel also affects endothelial barrier function. We therefore investigated whether ENaC expression in HL-MVEC monolayers affects barrier function, by depleting ENaC-α using specific siRNA, employing scrambled non-specific siRNA as a control. The efficacy of the siRNA-mediated depletion is shown in **Figure 2A**. As shown in **Figure 2B**, transfection with ENaC-α siRNA significantly reduced expression of ENaC-α in HL-MVEC, using the prominent 75 kDa band corresponding to the mature subunit for quantification. Depletion of ENaC-α significantly increased sensitivity of HL-MVEC to PLY at 30 min post addition of the toxin (**Figure 2C**). This was measured as normalized monolayer resistance, using ECIS (ECIS1600R, Applied Biophysics, Troy, NY, USA), in cells treated with 60 ng/ml PLY for 30 min. Silencing of ENaC-α did not affect basal barrier function (data not shown). The protective action of the TIP peptide (50 μg/ml) in PLY-induced barrier dysfunction, which we reported previously (3) and which was also observed in the presence of scrambled siRNA, was eliminated after depleting ENaC-α (**Figure 2C**). These results suggest an important role for ENaC-α in restoring capillary barrier function in the face of PLY challenge.

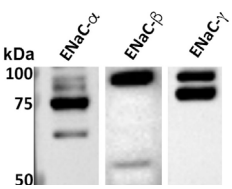

FIGURE 1 | Representative western blot of basal expression of α, β, and γ epithelial sodium channel (ENaC) subunits in human lung microvascular endothelial cells. The immunoreactive bands at 95, 75, and 65 kDa are different forms of the subunits resulting from posttranslational modifications (e.g., glycosylation) and proteolytic processing. We previously confirmed the identity of similar bands excised from Coomassie stained gels after immunoprecipitation using the ENaC-α 59 and ENaC-β 60 antibodies and then performing LC/MS. We further corroborated the specificity of these antibodies by performing competition experiments using the recombinant fusion proteins that were used as the immunogens to generate the ENaC-α 59 and ENaC-β 60 antibodies (14). The characterization of the ENaC-γ 2102 antibody was described elsewhere (29). Although these ENaC antibodies cross-react with mouse, rat, and human species, we do expect there to be some variations in the previously reported sizes of the bands for ENaC expressed in the kidneys and, as shown here, in the lungs. These variations might be due to differences in proteases within the kidneys and lungs that cleave ENaC.

ENaC-α Stimulation Blunts PLY-Induced CaMKII Activation and FLN-A Phosphorylation

Apart from its role in endothelial barrier function demonstrated above, ENaC-α has also been shown to be crucial for ALC (32), since mice lacking the subunit die shortly after birth with flooded lungs. ALC is, in part, modulated by ENaC activity. As such, we wanted to identify an interacting partner that binds to ENaC-α and that regulates both barrier function and Na^+ uptake. The actin-binding protein FLN-A, in its non-phosphorylated form, is a prominent regulator of endothelial barrier function, since it prevents stress fiber formation (33). FLN-A directly associates with ENaC subunits and promotes their association with the chaperone protein MARCKS, thereby inducing complex formation of the channel with PIP_2 (15). This complex formation is crucial for regulating the open probability of ENaC (14). Increased intracellular Ca^{2+} levels mobilize calmodulin, which in turn activates calmodulin-dependent kinase II (CaMKII), which then

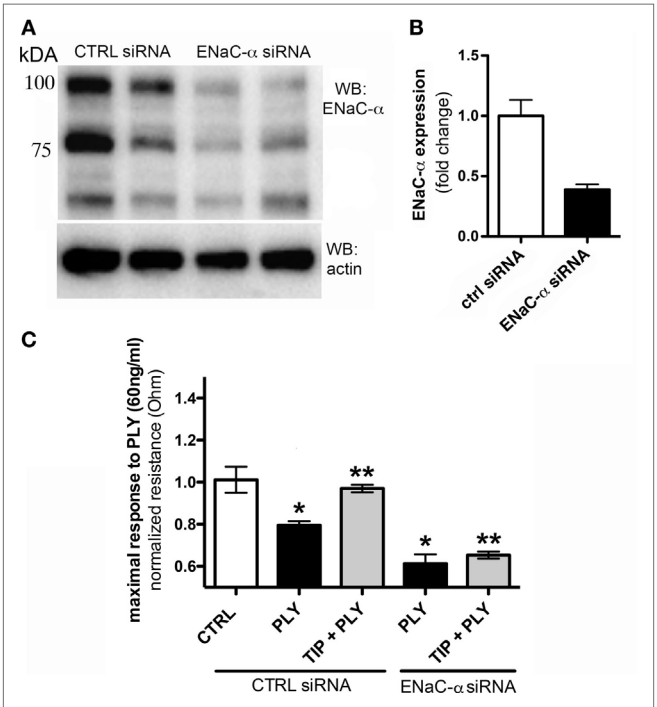

FIGURE 2 | **(A)** Representative western blot of epithelial sodium channel (ENaC)-α expression in human lung microvascular endothelial cells (HL-MVEC), transfected with either scrambled siRNA (control) or ENaC-α siRNA. **(B)** Efficacy of siRNA-mediated depletion of ENaC-α, relative protein expression in HL-MVEC. **(C)** Transendothelial resistance (measured in electrical cell-substrate impedance sensing 1600R) in HL-MVEC, transfected with scrambled siRNA or ENaC-α siRNA and treated for 30 min with 60 ng/ml of PLY (corresponding with the maximal drop in resistance), in the presence or absence of TIP peptide (*$p < 0.05$ versus ctrl, **$p < 0.05$ versus PLY).

phosphorylates its substrate, FLN-A (15, 33). Phosphorylated FLN-A blunts the association of ENaC with MARCKS, and impairs ENaC activity (15).

Pneumolysin (100 or 200 ng/ml) induces FLN-A phosphorylation from as early as 15 min and persisting for at least 60 min (**Figure 3A**). TIP peptide (50 μg/ml), as well as the CaMKII inhibitor XII (1mM) inhibits FLN-A phosphorylation induced by PLY (60 ng/ml) (**Figures 3B,C**). As shown in **Figure 4A**, PLY-treatment (90 ng/ml) induces CaMKII activation in HL-MVEC within 10 min. Thus, both PLY-induced phosphorylation of FLN-A and CaMKII can be partially inhibited by the TIP peptide or by a CaMKII inhibitor (**Figures 3A,B** and **4A,B**). Taken together, these data indicate that PLY, whose deleterious actions on barrier function in HL-MVEC monolayers are at least partially dependent on promoting Ca^{2+} influx (3), has the capacity to activate CaMKII, which in turn increases phosphorylation of FLN-A. TIP peptide binding to ENaC-α at least partially blunts these events.

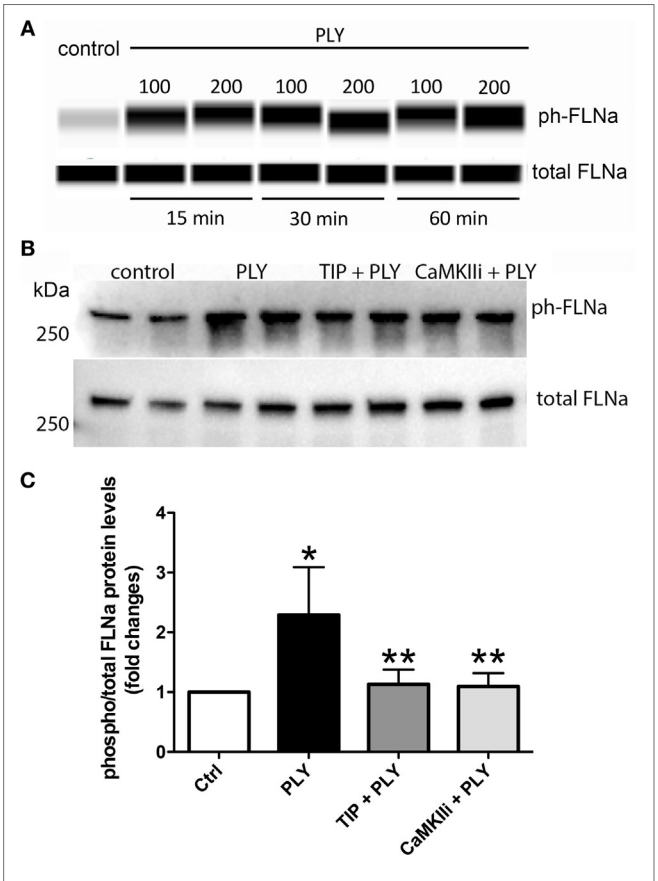

FIGURE 3 | **(A)** Time-dependent representative nanopro technology-based western blot of PLY-induced filamin A (FLN-A) phosphorylation in human lung microvascular endothelial cells (HL-MVEC) (100 and 200 ng/ml), as described (28). **(B)** Representative western blot. **(C)** Quantification of phospho- over total protein ratio of PLY (60 ng/ml)-mediated FLN-A phosphorylation in HL-MVEC after 20 min. Cells were either pretreated with TIP peptide (50 μg/ml) or the CaMKII inhibitor (1 mM) for 15 min. Values are presented as means ± SD of three independent experiments in duplicates (*$p < 0.05$ versus ctrl, **$p < 0.05$ versus PLY).

The Hybrid ENaC-α/ASIC1a Non-Selective Cation Channel Mediates Barrier Protection from PLY

In order to address the apparent discrepancy between our results with the TIP peptide, which improves barrier function in HL-MVEC, and results obtained by others demonstrating that aldosterone-induced activation of ENaC leads to stiffening in large vessel endothelial cells (34), we investigated the potential implication of other, non-selective cation channels (NSC) in the ability of the TIP peptide to preserve barrier function in PLY-treated HL-MVEC monolayers. Indeed, ENaC-α is not only a subunit of ENaC but also a component of hybrid NSC channels, where it forms a complex with the ASIC1a subunit (35–37). These hybrid NSC channels, when expressed in type 2 alveolar epithelial cells, were recently shown to contribute significantly to ALC (37).

MitTx (20 nM), an activator of ASIC1a and of NSC (38, 39), significantly reduced PLY-mediated (60 ng/ml) barrier dysfunction in HL-MVEC, to the same extent as the TIP peptide (50 μg/ml) (**Figure 5A**). siRNA-mediated depletion of ENaC-α abrogated the protective effect of both MitTx and TIP peptide in PLY-treated HL-MVEC monolayers (**Figure 5A**). Moreover, siRNA-mediated depletion of ASIC1a, which is expressed in HL-MVEC (**Figure 5D**) abrogated the inhibitory effect of the TIP peptide on PLY-induced FLN-A phosphorylation (**Figures 5B,C**). Of note, PLY induced significantly higher FLN-A phosphorylation in cells lacking ASIC1a (**Figure 5B**). These results indicate that NSC channels at least partially

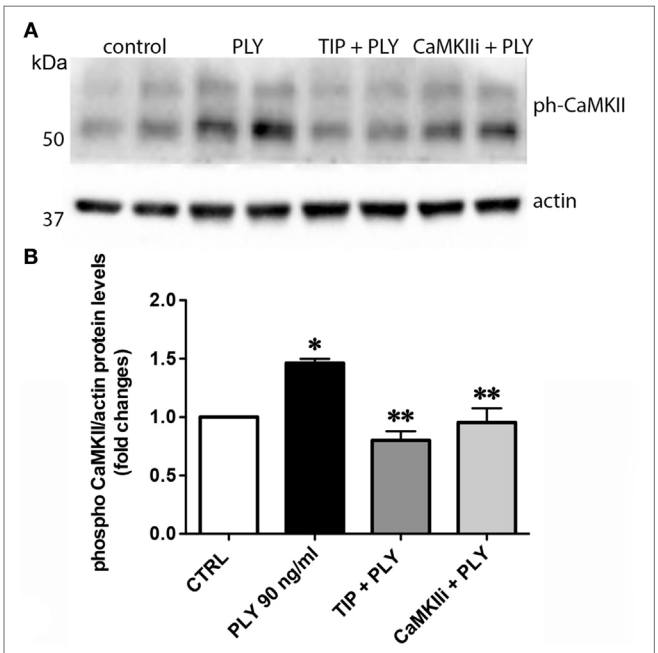

FIGURE 4 | **(A)** Representative western blot and **(B)** quantification of phospho-CaMKII over actin ratio of PLY (90 ng/ml)-mediated CaMKII activation in human lung microvascular endothelial cells after 20 min. Cells were either pretreated with TIP peptide (50 μg/ml) or the CaMKII inhibitor XII (1 mM) for 15 min. Values are presented as means ± SD of three independent experiments in duplicates (*$p < 0.05$ versus ctrl, **$p < 0.05$ versus PLY).

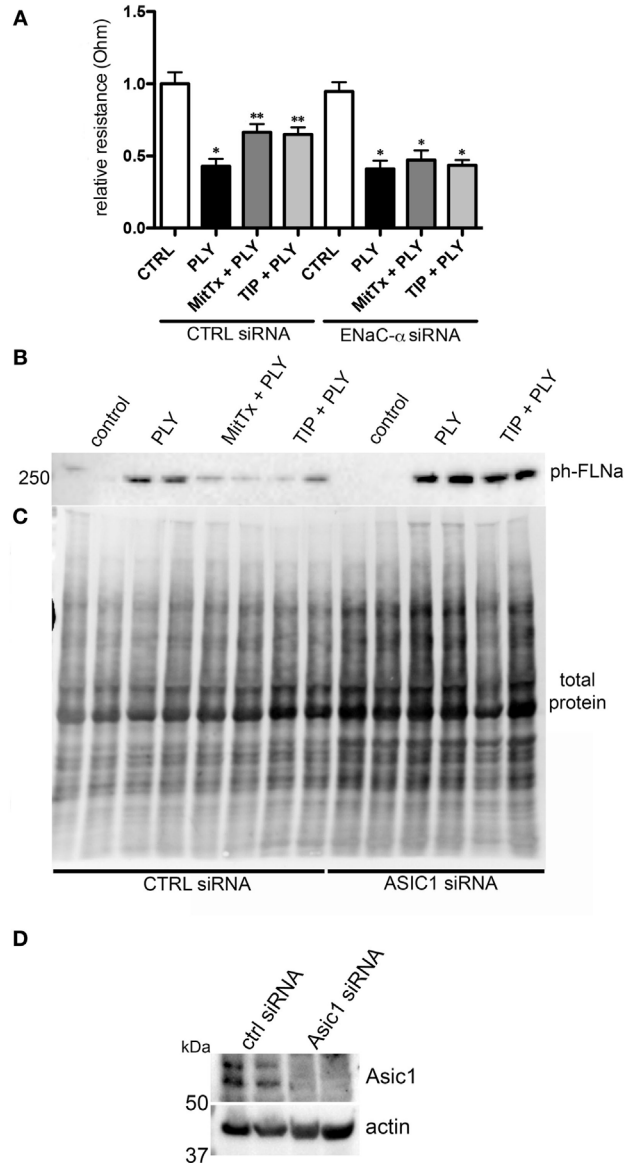

FIGURE 5 | **(A)** Transendothelial resistance (measured in electrical cell-substrate impedance sensing 1600R) in human lung microvascular endothelial cells (HL-MVEC), transfected with scrambled siRNA or epithelial sodium channel (ENaC)-α siRNA and treated for 30 min with 60 ng/ml of PLY, in the presence or absence of TIP peptide (50 μg/ml) or MitTx (20 nM) ($n = 3$, SEM) (*$p < 0.05$ versus ctrl, **$p < 0.05$ versus PLY). **(B)** Representative western blot and quantification of phospho- over total protein ratio of PLY (60 ng/ml)-mediated filamin A (FLN-A) phosphorylation in HL-MVEC after 20 min. Cells were either pretreated with TIP peptide (50 μg/ml) or MitTx (20 nM) for 15 min. **(C)** Representative stain-free blot showing total protein transferred to the nitrocellulose membrane. **(D)** siRNA-mediated acid-sensing ion channel 1a (ASIC1a) silencing in HL-MVEC.

mediate barrier protection against PLY in HL-MVEC and that both ENaC-α and ASIC1a subunits are crucial for this activity. To confirm this interaction, we performed a co-IP experiment using ASIC1a antibody as bait. We detected two bands corresponding to uncleaved (95 kDa) and mature (around 75 kDa) ENaC-α in the immunoprecipates (**Figure 6**).

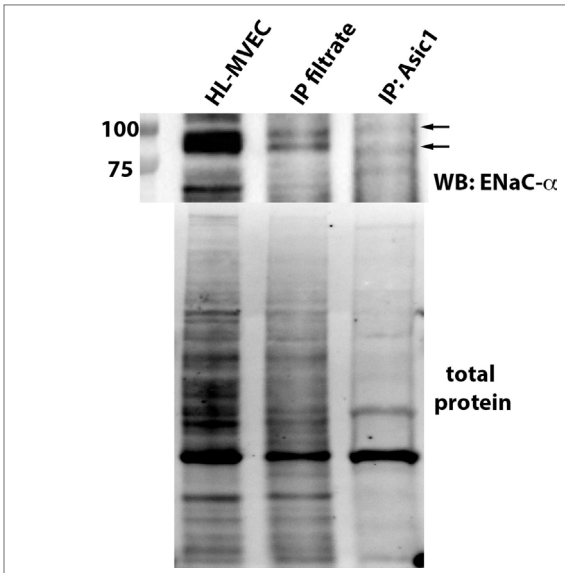

FIGURE 6 | Representative immunoprecipitation experiment assessing binding of epithelial sodium channel (ENaC)-α (indicated by arrows) to native ASIC1 in human lung microvascular endothelial cells (HL-MVEC) and representative stain-free blot showing the total protein transferred to the nitrocellulose membrane before the co-IP experiment (whole HL-MVEC lysate—first lane), the filtrate after the co-IP (second lane), and the eluent (eluted from the ASIC1 antibody-decorated magnetic beads—third lane).

DISCUSSION

Decreased lung capillary barrier function represents one of the major complications of severe pneumonia and ARDS and promotes the development of permeability edema. Upon autolysis or antibiotic-induced lysis, the G^+ pathogen *S. pneumoniae*, the main etiological agent of community acquired pneumonia in the US, releases the cholesterol-binding and pore-forming toxin PLY.

Pneumolysin-induced Ca^{2+}-influx, which is blunted by lanthanum chloride, is crucial for the ability of the toxin to induce hyperpermeability in human lung MVEC (3). PLY reduces endothelial barrier function in part by means of activating protein kinase C-α, which in turn impairs NO generation by endothelial nitric oxide synthase (eNOS) (3, 4), which was shown to be required for basal barrier function (40). Increased Ca^{2+} influx can also mobilize calmodulin, which activates CaMKII. Activated CaMKII phosphorylates the actin-binding protein FLN-A. Although the non-phosphorylated form of FLN-A prevents stress fiber formation, increased Ca^{2+} influx promotes the shift to its phosphorylated form, which is incapable of supporting barrier integrity (15).

Apart from preventing stress fiber formation, FLN-A also promotes the interaction between the chaperone protein MARCKS and ENaC subunits (15), which in turn increases the open probability time of the channel. As summarized in **Figure 7**, our findings suggest that ENaC-α, as a subunit of NSC, can be activated by the TIP peptide, whereas the ASIC1a subunit of NSC is activated by MitTx. Both of these mechanisms of NSC activation promote barrier protection, by means of reducing PLY-induced activation of CaMKII, FLN-A phosphorylation, and finally lung capillary barrier dysfunction, respectively. A role for ENaC-α in epidermal barrier protection

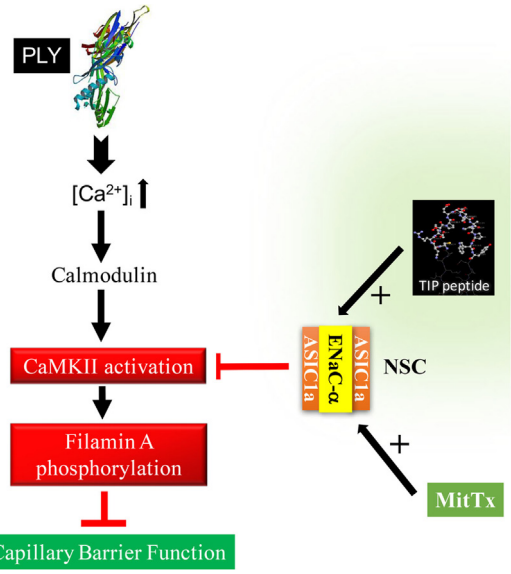

FIGURE 7 | Proposed sequence of events in the role of epithelial sodium channel (ENaC)-α in barrier protection in pneumolysin (PLY)-treated human lung microvascular endothelial cells. PLY, upon pore formation, increases Ca^{2+}-influx (3), which in turn mobilizes calmodulin. Calmodulin activates CaMKII, which in turn phosphorylates its substrate filamin A (FLN-A) (15). Phosphorylated FLN-A promotes stress fiber formation and increases capillary permeability. Activation of NSC, by either TIP peptide (binding to ENaC-α) or MitTx [binding to acid-sensing ion channel 1a (ASIC1a)], abrogates PLY-mediated CaMKII activation and protects as such from PLY-induced hyperpermeability.

was shown previously (41). Of note, a recent study demonstrated that both the β1 subunit of the Na^+–K^+-ATPase as well as the α subunit of ENaC strengthen capillary endothelial barrier function in mice in the presence of LPS (42). These data together with those presented here suggest an important role for ENaC-α in protecting endothelial barrier function in the presence of bacterial toxins.

Our results with the TNF-derived TIP peptide, which directly binds to ENaC-α and which has the capacity to increase both expression and open probability of ENaC in the presence of PLY, are in sharp contrast to the suggested role of ENaC in aldosterone-induced vascular stiffening and eNOS dysfunction in large vessel endothelial cells (34). Although it cannot be excluded that aldosterone, apart from activating ENaC also activates other pathways possible leading to endothelial dysfunction (43), and that large vessel endothelial cells, as investigated in the Kusche-Vihrog studies, are phenotypically different from microvascular endothelial cells, our results indicate that ENaC-α participates in barrier strengthening in HL-MVEC at least partially in the context of a complex different from classical ENaC.

Acid-sensing ion channels represent a family of proteins activated upon extracellular acidification (35). Although primarily found in neurons, ASIC1 expression was also demonstrated in type 2 alveolar epithelial cells, where they play an important role in vectorial Na^+ transport-mediated ALC (37), as well as in cerebral arteries (44) and in pulmonary arterial smooth muscle cells (45). We demonstrate here that human pulmonary microvascular endothelial cells also express ASIC1a. MitTx, an ASIC1 and NSC activator, which does not interact with ENaC, strengthens barrier function in PLY-treated HL-MVEC. However, the protective effects of MitTx are blunted in cells in which ENaC-α was depleted, indicating that its actions are not mediated by the typical ASIC1a channel complex, but rather by an ASIC1a/ENaC-α hybrid. Moreover, the inhibitory effect of the TIP peptide on PLY-induced FLN-A phosphorylation is abrogated in cells in which ASIC1a was depleted, indicating that the classical ENaC channel complex is not sufficient to mediate its effect. These results indicate that, rather than the classical ASIC1a and ENaC channels, a hybrid NSC channel, consisting of both ASIC1a and ENaC-α, is likely to mainly mediate the protective effects of both the TIP peptide and MitTx in lung capillary endothelial cells. This mechanism could be especially relevant in conditions of acidification, as can be found during bacterial pneumonia. Acidification of exhaled breath condensate in ventilated acute lung injury and ARDS patients was shown to correlate with local pulmonary inflammation (46). As such, it seems plausible that under these conditions, ASCIC1a, as well as NSC can be activated.

In conclusion, our data indicate that the barrier protective effect of ENaC-α in PLY-treated HL-MVEC monolayers is at least partially mediated by NSC channels in these cells. As such, the TIP peptide, which has the capacity to activate both ALC across alveolar epithelium and endothelial barrier function in the presence of bacterial toxins, could represent a therapeutically promising candidate to tackle pulmonary permeability edema associated with bacterial pneumonia. *In vivo* studies will be needed to further test this mechanism under pathologically relevant conditions.

AUTHOR CONTRIBUTIONS

Conception or design of the work: IC, AAA, MM, DF, TC, DE, and RL; acquisition, analysis, or interpretation of data: IC, SS, BG, HP, MH, BB, MR, and JG: drafting the work: IC and RL; revising it critically for important intellectual content: AAA, MR, GW, YH, YS, AV, DF, and DE. All authors approved the final version of the manuscript and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Involvement of Cytokines in the Pathogenesis of Salt and Water Imbalance in Congestive Heart Failure

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Congestive heart failure (CHF) has become a major medical problem in the western world with high morbidity and mortality rates. CHF adversely affects several systems, mainly the kidneys and the lungs. While the involvement of the renin–angiotensin–aldosterone system and the sympathetic nervous system in the progression of cardiovascular, pulmonary, and renal dysfunction in experimental and clinical CHF is well established, the importance of pro-inflammatory mediators in the pathogenesis of this clinical setting is still evolving. In this context, CHF is associated with overexpression of pro-inflammatory cytokines, such as tumor necrosis factor-α, interleukin (IL)-1, and IL-6, which are activated in response to environmental injury. This family of cytokines has been implicated in the deterioration of CHF, where it plays an important role in initiating and integrating homeostatic responses both at the myocardium and circulatory levels. We and others showed that angiotensin II decreased the ability of the lungs to clear edema and enhanced the fibrosis process *via* phosphorylation of the mitogen-activated protein kinases p38 and p42/44, which are generally involved in cellular responses to pro-inflammatory cytokines. Literature data also indicate the involvement of these effectors in modulating ion channel activity. It has been reported that in heart failure due to mitral stenosis; there were varying degrees of vascular and other associated parenchymal changes such as edema and fibrosis. In this review, we will discuss the effects of cytokines and other inflammatory mediators on the kidneys and the lungs in heart failure; especially their role in renal and alveolar ion channels activity and fluid balance.

Keywords: heart failure, alveolar epithelium, renal cells, inflammation, cytokines, alveolar fluid clearance

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INTRODUCTION

Congestive heart failure (CHF) has recently become a major medical problem in the developed countries with increased rates of mortality and morbidity, particularly among the elderly population. CHF constitutes an enormous economic burden on health service because of the expensive costs of the various therapeutic modalities, frequent hospital admissions, and poor quality of life. In the developed countries, it is estimated that up to 2% of the adult population suffers from this syndrome; whereas, in patients ≥65 years of age, the prevalence surges to more than 10% (1). The pathophysiologic conditions of CHF are various and include either decreased cardiac

output due to loss of cardiac muscle tissue as it is observed in myocardial infarction, myocarditis and dilated cardiomyopathy; or increased filling pressures of the heart as it is evident in hypertension, hypertrophic and restrictive cardiomyopathies, and certain valvular diseases. CHF can also develop due to a volume overload deriving from arteriovenous shunts or fistulas and administration of fluid excess.

Understanding the underlying mechanisms leading to the development of CHF and its complications is therefore essential for optimizing the treatment of CHF and exploring novel therapies that aim to improve the outcome of the disease (2). Since the early 1980s, the importance of vasoconstrictor neurohormonal systems in the pathogenesis of CHF has been increasingly recognized. Numerous studies in patients and in experimental models of CHF have established the important role of the renin–angiotensin–aldosterone system (RAAS) and the sympathetic nervous system (SNS) in the progression of cardiovascular and renal dysfunction in CHF. It is now accepted that excessive neurohormonal activation may adversely affect cardiac function and the hemodynamic condition by enhancement of systemic vasoconstriction and promoting salt and water retention by the kidney. In addition, prolonged activation of the SNS and RAAS may have direct deleterious actions on the myocardium, independent of their systemic hemodynamic effects (3–5). However, generally, inflammation plays an important role in most cardiac diseases, and receptor-mediated innate immunity is primarily investigated with respect to toll-like receptors. However, the role of the innate immune system in heart failure has been controversial (6).

Cytokines that are composed of a vast array of relatively low molecular weight, pharmacologically active proteins; have been implicated in the progression of CHF. The most important cytokines are tumor necrosis factor-α (TNF-α), interleukin (IL) 1β, and IL-6. These cytokines share some of their major characteristics (redundancy), and all act in a pro-inflammatory sense (7). Adhesion molecules, autoantibodies, nitric oxide (NO), and endothelin-1 are also thought to be relevant to the pathogenesis of CHF (8).

Recently, it was shown in patients with acute decompensated heart failure (ADHF) that following standard treatment of ADHF, the monocyte profile and circulating inflammatory markers (C-reactive protein and IL-6) shifts to more closely resemble those of healthy controls, suggesting the contribution of systemic inflammation to the pathophysiology of ADHF (9). We and others have shown the deleterious consequences of heart failure on the lungs and the kidneys; therefore, we decided in this review to focus on the effects of cytokines and other inflammatory mediators on the lungs and the kidneys in heart failure; especially their role in renal and alveolar ion channels activity and fluid balance.

THE CONTRIBUTION OF THE IMMUNE SYSTEM TO HEART FAILURE

There are several theories regarding the activation of the immune system in heart failure (10). One hypothesis is based on the consequences of heart failure, that is, systemic venous congestion including the mesenteric venous system with consequent bowel edema and increased permeability that leads to bacterial translocation, endotoxin release and resultant activation of the immune system (11). The second theory is related to the ability of the failing heart to produce cytokines; Torre-Amione et al. have shown that TNF-α mRNA and TNF-α protein were present in the explanted hearts from dilated cardiomyopathy and ischemic heart disease patients but not in non-failing hearts (12).

In the third hypothesis, the state decreased cardiac output in heart failure causes systemic tissue hypoxia with subsequent systemic inflammation, which in turn may be the primary stimulus for increased TNF-α production (13).

The heart undergoes extensive structural and functional remodeling in response to injury, central to which is the hypertrophy of cardiac myocytes, with excessive deposition of extracellular matrix (14). Myocardial fibrosis is commonly categorized as one of two types: reactive fibrosis or replacement fibrosis. Reactive fibrosis occurs in perivascular spaces and corresponds to similar fibrogenic responses in other tissues; replacement fibrosis occurs at the site of myocyte loss.

Myocardial fibrosis is attributed to cardiac fibroblasts, which resides in the myocardium and is confirmed to be abundant (15). Following myocardial injury, all types of fibroblasts proliferate and differentiate into myofibroblasts, a process that is orchestrated by classic mediators such as TGF-β1, endothelin-1, and angiotensin II (Ang II). Notably, fibrosis is accelerated as result of intercellular interaction and cross talk; in this case, between activated fibroblasts and cardiomyocytes (16).

The effects of fibrosis on the heart muscle are various and include impairment of cardiac function, both systolic and diastolic. It also caused electrical instability and the development of fatal ventricular arrhythmias. This arrhythmogenic activity occurs in areas that couple fibroblasts and cardiomyocytes due to discontinuous slowing of conduction and consequent arrhythmia (17).

The CORONA study that included 1,464 patients with chronic ischemic systolic HF demonstrated that serum levels of TNF-α, soluble TNF receptors type I and II (sTNF-RI and sTNF-RII), and the chemokines monocyte chemoattractant protein-1 and interleukin-8 (IL-8) were independent predictors of all endpoints (all-cause mortality, cardiovascular mortality, and worsening heart failure). After further adjustment for estimated glomerular filtration rate (GFR), the ApoB/ApoA-1 ratio, NT-proBNP, and high-sensitivity C-reactive protein, only IL-8 remained a significant predictor of all endpoints (except the coronary endpoint), while sTNF-RI remained independently associated with CV mortality (18). Recently, in concordance of this study, it was reported IL-8 was negatively correlated with the left ventricular end-diastolic diameter and positively with left ventricular systolic volume (19). However, it should be emphasized that the elevated levels of cytokines in general and in heart failure, in particular, may not be responsible for tissue injury, rather it may reflect a concomitant phenomenon where cytokines could be used as biomarkers for heart failure but not effectors.

Pulmonary System

The alveoli are composed of thin layer of epithelial cells; alveolar epithelial cells type I and type II (AECI and AECII, respectively) that occupy together 99% of surface area of the lungs and play a crucial role in breathing and preserving lung homeostasis. There are also alveolar residential macrophages that protect the lungs from pathogens and regulate lung immune response (20).

Alveolar macrophages—AMφ comprise 95% of bronchoalveolar lavage and are part of cellular compartment of innate immunity that has an essential role in pathogen defense. Another type of macrophages is interstitial macrophages or bone-marrow derived macrophages that are also involved in the process of lung defense (21).

Alveolar Fluid Clearance (AFC)

Active sodium (Na^+) transport across the alveolar-capillary barrier is important in keeping the airspaces free of fluid in healthy conditions and for the resorption of lung edema in pathologic conditions. Briefly, Na^+ enters the alveolar epithelial cells through apical amiloride sensitive Na^+ channels (ENaC), and by a process that consumes energy is pumped out of the cell by the Na,K-ATPase located in the basolateral membrane in exchange for potassium entry on a ratio of 3:2 Na^+–K^+ against their chemical gradient (20, 22–27) (**Figure 1**). ENaC constitutes the rate limiting step for sodium absorption in epithelial cells of various sites including distal renal tubule, distal colon, exocrine glands, and lungs. Concerning the latter, ENaC plays a critical role in AFC. A support for this notion was derived from Hummler et al. who demonstrated that AFC in knockout mice to ENaC was severely attenuated with resultant fatal respiratory distress (27). Notably, non-selective Na^+ channels (NSC) and cyclic nucleotide-gated channel have been shown to be involved in the process of AFC, however to a lesser extent than ENaC (28). In addition, K^+ ions are recycled by basolateral K^+ channels, which also participate in the control of Na^+ and fluid absorption (26). It has been shown that AFC is modulated by several pharmacologic modalities and interventions; such as catecholamines, angiotensin, vasopressin, endothelin, gene therapy, hypercapnia, hyperoxia, sepsis, and others (20, 29–33). Notably, Ang II decreased AFC *via* c-AMP–Na, K-ATPase pathway. Whereas, it was reported that Ang II plays a role in

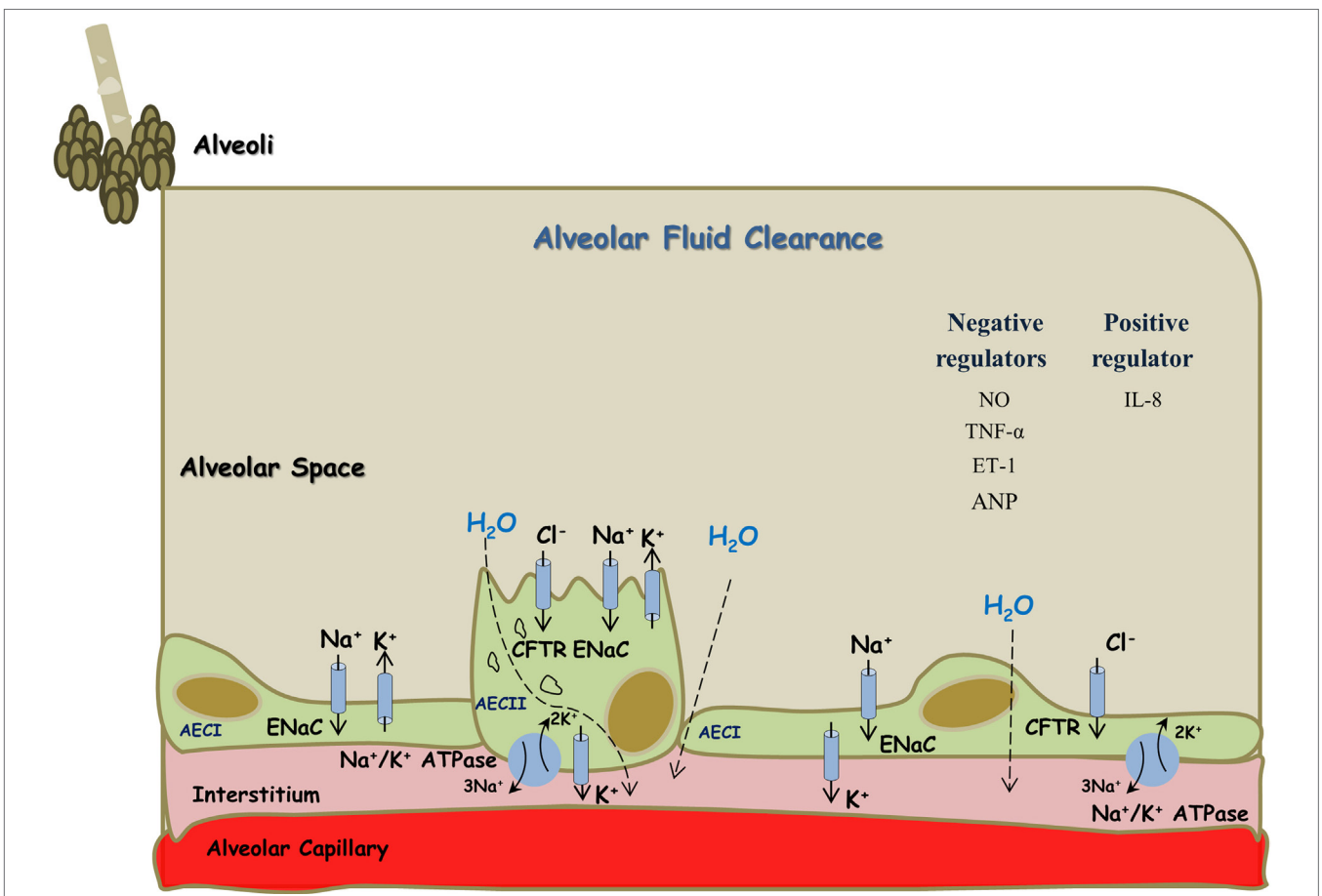

FIGURE 1 | Alveolar fluid clearance process in the lung epithelium. Sodium is actively transported from alveolar space to the lungs' interstitium and blood vessels; achieved mainly by apical ENaC and basolateral Na^+/K^+ ATPase located at AECI and AECII. This results in the formation of osmotic gradient, which drives transcellular and paracellular movements of water molecules. Some regulators, including cytokines, negatively affect this process while others appear to be with positive effects. AECI, alveolar epithelial cells type I; AECII, alveolar epithelial cells type II.

lung fibrosis by phosphorylating p38 and p42/44 kinases (also called extracellular signal-regulated protein kinases, ERK 1/2) (31). Ang II-induced mitogen-activated protein kinase (MAPK) activation has been implicated in myocardial hypertrophy, inflammation and neurotransmitter catecholamine synthesis, and release in the brain (34–36). These two kinases play a distinct role in the induction and signaling of pro-inflammatory cytokines. Specifically, fibroblasts stimulated with Ang II showed a strong time-dependent expression of COX-2 protein. The p38 MAPK inhibitor SB203580 but not the p42/44 MAPK-inhibitor PD98059 suppressed Ang II-induced COX-2 protein expression, a pro inflammatory enzyme (37). Likewise, blockade of Ang II receptors type I and II (AT1 and AT2, respectively) reduced the levels of TNF-α and its damage on renal tubular cell injury, thus exerting cytoprotective effects (38). Concerning the interaction between the RAAS and CNS systems, Wei et al. demonstrated that Ang II stimulates MAPK to upregulate brain AT1 receptors in rats with HF (39). Similarly, these authors demonstrated that Ang II-activated MAPK signaling pathways contribute to sympathetic excitation in HF (40). Specifically, intracerebroventricular administration of two selective p44/42 MAPK inhibitors, PD98059 and UO126, induced significant decreases in mean arterial pressure, heart rate, and renal sympathetic nerve activity in rats with HF but did not affect these parameters in sham controls. In addition, MAPK can be activated by other factors, such as pro-inflammatory cytokines and reactive oxygen species (41, 42), which are known to increase during inflammatory, pulmonary, and cardiac diseases. ERK1 and ERK2 play a crucial role in the pathogenesis of cardiac and vascular diseases. In this context, it was found that ERK1/2 and p38 MAPK activation occurred within 10 min of transverse aortic constriction, a model of pressure load heart failure (43). Similarly, activation of ERK, Jun kinase (JNK), and p38 MAPK has been demonstrated in other clinical and experimental heart failure (44).

The ability of the lungs to clear edema is impaired in acutely increased left atrial pressure (45–48). The underlying mechanisms are not fully understood; it has been assumed that NO synthesized in the alveolar endothelial cells attenuated the ability of the lungs to clear fluids *via* alveolar endothelial–epithelial interactions (45). The addition of Ang II to cultured vascular smooth muscle cells did not induce neither nuclear factor kappa B (NF-κB) activation nor iNOS or VCAM-1 expression. However, when added together with IL-1β, Ang II, through activation of the (AT1) receptor, inhibited iNOS expression and enhanced VCAM-1 expression induced by the cytokine. The inhibitory effect of Ang II on iNOS expression was associated with a downregulation of the sustained activation of extracellular signal-regulated kinase (ERK) and NF-κB by IL-1β, whereas the effect on VCAM-1 was independent of ERK activation. The effect of Ang II on iNOS was abolished by inhibition of p38 MAPK with SB203580. The authors concluded that Ang II, by a mechanism that involves p38 MAPK, differentially regulates the expression of NF-κB-dependent genes in response to IL-1β stimulation by controlling the duration of activation of ERK and NF-κB (49).

In chronic heart failure, however, the ability of the lungs to clear edema is increased particularly in compensated CHF (50, 51). Verghese et al. have shown that in most of the patients with hydrostatic pulmonary edema, AFC is intact or even increased. Notably, in this population, there was a trend though insignificant toward better outcomes (52).

De Vito reported that Na^+/H^+ Exchanger isoform 1 might be a possible mediator of immunity involved in cytoplasmic pH (pH_i) homeostasis and expression of cytokines and chemokines (53). Our laboratory is currently investigating the expression pattern of Na^+/H^+ Exchanger (NHE) isoforms in alveolar epithelial cells and to evaluate their involvement in AFC process in both control and heart failure rats. CHF was induced by the placement of arteriovenous fistula between the abdominal aorta and vena cava (50). Notably, one should bear in mind that many of the immune cell functions are coupled with pH_i modification. Specifically, an increase in pH_i represents an important signal for cytokine and chemokine release, whereas a decrease in phagosomal pH can induce an efficient antigen presentation (53). Thus, our hypothesis in this regard speculates a potential role of one of the NHE isoforms in the inflammatory aspect of heart failure in general and pulmonary system in particular.

The Effects of Cytokines on AFC

The effects of cytokines on AFC were examined on a variety of acute lung injury (ALI) models and found to play a controversial role (18, 19, 54).

The role of the immune system in patients with acute respiratory distress syndrome (ARDS) and ALI is well known; briefly, soon after lung injury, endothelial cells are damaged with gap formation that allows fluid permeability, activation, and migration of neutrophils with activation of pro-inflammatory cytokines such as TNF-α, IL-1β, and the transcriptional regulatory NF-κB. Notably, in response to stimuli, such as infection, NF-κB is activated with consequent cellular responses that lead to pulmonary edema due to ALI/ARDS (55). Peteranderl et al. recently demonstrated that in mice lungs infected with influenza A (IAV), the rate of AFC was decreased *via* inhibiting the recruitment of Na,K-ATPase α subunit to the plasma membrane (54). It was demonstrated that this process was mediated by a paracrine cross talk between the infected and non-infected AEC and alveolar macrophages. The mediators that were involved in this interaction were principally interferon α and to lesser degree IFNβ and an IFN-dependent elevation of macrophage TNF-related apoptosis-inducing ligand. Interestingly, interruption of this cellular cross talk accelerates the rate edema resolution, which is of biologic and clinical importance to patients with IAV-induced lung injury (56).

TNF-α levels are known to be increased in heart failure. It was shown that LV ejection fraction was depressed in transgenic mice overexpressing TNF-α in cardiomyocytes; this effect was dependent on TNF-α gene dosage (57). However, the knowledge regarding the role of cytokines on AFC in the context of heart failure is limited. Rezaiguia et al. have shown that TNF-α instilled in normal rats increased alveolar liquid clearance by 43% over 1 h compared with control rats; conceivably, due to ENaC stimulation. TNF-α, which is secreted from alveolar macrophages binds to TNF receptors located on alveolar epithelial cells, where it induces its effects probably *via* upregulating of G proteins

coupled ENaC. This effect is mediated *via* the lectin-like domain of TNF-α (58, 59). Another suggested mechanism is recruitment of ion channels to the cell membranes (60, 61). Moreover, it was demonstrated that in a model of ischemia–reperfusion in rats; AFC was upregulated, at least partly *via* TNF-α-dependent mechanism (62). On the other hand, it was reported that treating alveolar epithelial cells with TNF-α, the mRNA expression of ENaC subunits was decreased with compatible decrease in activity (63, 64). Therefore, these studies demonstrated that exposure to TNFα decreases ENaC mRNA and protein expression, as well as ENaC function both in alveolar type II cells and in injured lungs.

In models of ALI, it was demonstrated that IL-8 mediated injury to both the endothelium and epithelium, with consequent high permeability edema formation and decreased AFC (54). In addition, pretreatment with anti-IL-8 antibodies successfully restored the rate of AFC to normal probably by attenuating injury to the epithelium (18, 19).

The Effect of NO and Endothelin on AFC

Endothelin-1 (ET-1), a potent vasoactive peptide produced by endothelial cells and released during injurious stimuli such as pulmonary hypertension and heart failure. It has been shown that elevated concentrations of ET-1 predict mortality and hospitalizations in HF patients (65). It is noteworthy that ET-1 has an inhibitory effect on lung edema clearance *via* an endothelial epithelial interaction. The underlying mechanism involves activation of endothelial ETB receptors and NO generation leading to alveolar epithelial Na,K-ATPase downregulation in a cyclic guanosine monophosphate (cGMP)-independent manner (32).

Kaestle et al. have explored the role of NO in both acute and chronic heart failure. They have shown that in isolated mouse lungs, hydrostatic edema formation was attenuated by NO synthase (NOS) inhibition. Similarly, edema formation was decreased in isolated mouse lungs of endothelial NOS-deficient mice. Whereas, in chronic heart failure model; AFC was preserved as a result of endothelial dysfunction and decreased NO generation. This effect is mediated by endothelial-derived NO acting as an intercompartmental signaling molecule at the alveolo-capillary barrier (45) (**Figure 1**).

Renal System

Kidney dysfunction is common in heart failure and is associated with an increased risk of mortality. The interaction between the heart and kidney in this setting is complex, involving multiple interdependent mechanisms including hemodynamic alterations and activation of multiple neurohormonal as well as pro-inflammatory systems (**Figure 2**).

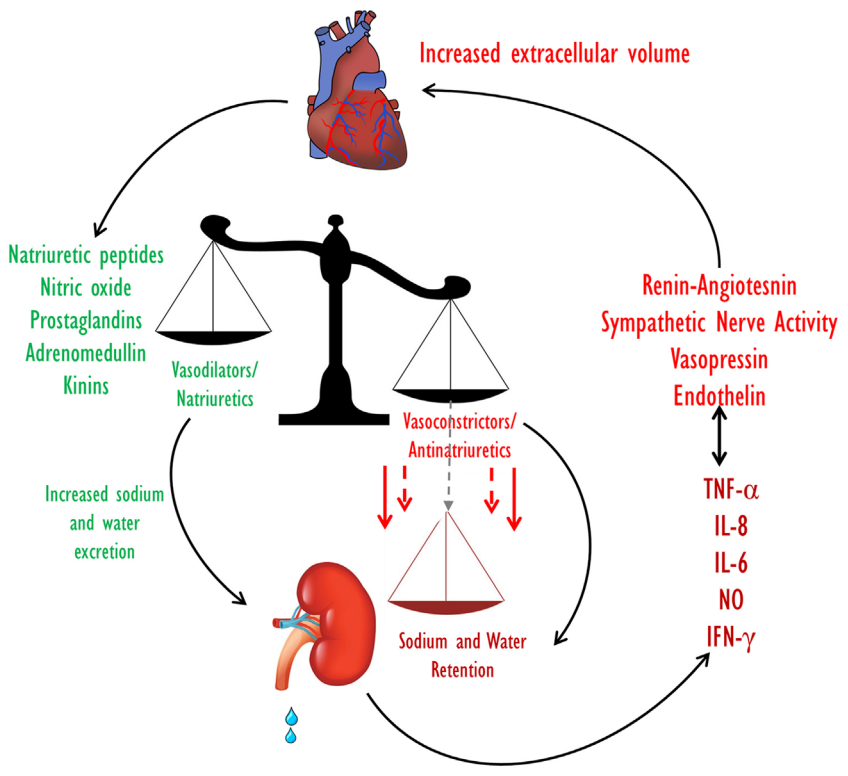

FIGURE 2 | Extracellular fluid volume control in CHF. Volume homeostasis in CHF is determined by the balance between the natriuretic and the anti-natriuretic arms. In decompensated CHF, enhanced activities of the Na^+-retaining systems along with activation of pro inflammatory substances overcome the effects of the vasodilatory/natriuretic systems, leading to a net reduction in Na^+ excretion and eventually to an increase in ECF volume. CHF, congestive heart failure; ECF, extracellular fluid.

Effects of Cytokines on Renal Handling of Water and Salt

The kidney is a major target organ of various hormones, and paracrine and autocrine substances. Many of the latter belong to the cytokines family, and some of the hormones that act on the kidney possess pro inflammatory properties. CHF is known to cause amplification of several pro-inflammatory mediators that can be detected at high concentrations in several vital organs and blood stream. The biologic sources of this chronic inflammatory state in CHF are not fully recognized. However, the heart and the kidneys produce a wide range of pro-inflammatory cytokines in response to activation of various neurohormonal systems and endotoxin accumulation as described below. Moreover, the hypo perfusion of the kidney during cardiorenal syndrome (CRS) results in sodium and water retention with further venous congestion. The biomechanical stretch of the vascular endothelium stimulates cytokine production (66).

The Renin–Angiotensin System

The RAAS plays a major role in the pathogenesis of heart failure and responsible for the cardiovascular and renal manifestations of this disease (67–69). At the initial phase of CHF, the RAAS exerts beneficial effects aimed at BP maintenance by direct systemic vasoconstriction, or indirectly *via* augmentation of the SNS activity and by promoting renal sodium retention. However, as CHF progress, the biological activities of the RAAS turn to be deleterious and contribute significantly to the disease aggravation (67). The main active substances of the RAAS are Ang II and aldosterone, which play a key role in the adverse cardiac and renal manifestations of severe CHF (3). Concerning the kidney, both Ang II and aldosterone act directly on the proximal tubule and collecting dust where they enhance Na^+ reabsorption *via* NHE3 and ENaC, respectively (**Figure 3**). Specifically, two-thirds of filtered sodium is reabsorbed in the proximal tubule *via* cotransporters along amino acid, glucose, phosphor as well as NHE3. Water follows sodium *via* aquaporin 1. At the distal tubule, sodium is reabsorbed by Na, K-cotransporter sensitive to thiazide. In the collecting ducts, a minimal amount of sodium (2–3%) is reabsorbed *via* amiloride sensitive ENaC that is upregulated by aldosterone. Water is reabsorbed in the collecting ducts *via* aquaporin 2 induced by vasopressin (70, 71). Furthermore, it is conceivable to reason that the anti-natriuretic effect of the RAAS is counterbalanced by the natriuretic/vasodilatory effect of atrial natriuretic peptide (ANP) on the kidney, thereby leading to substantial urinary retention of sodium and water retention with resultant edema formation (72, 73). In addition, increased activity of the RAAS contributes to the attenuated endothelial-dependent renal vasodilatation and the development of endothelial dysfunction characterizing CHF (74). Concerning the latter, it is attributed to several factors including the immune system. In support of this notion, it has been reported that T cells and various T cell-derived cytokines play a role in the pathogenesis of fluid/salt imbalance and elevated vascular resistance. For instance, various stimuli including Ang II, aldosterone, and catecholamines, which are known to be activated in CHF and hypertension increase the count of effector like T cells, which infiltrate the renal tissue in the perivascular regions of both arteries and arterioles (66, 75). There is also accumulation of monocyte/macrophages in these vascular beds (75). Both cell types release several cytokines including IL-17, IFN-γ, tumor necrosis factor-α (TNF-α), and IL-6, which cause renal damage and vascular dysfunction, resulting in avid sodium retention and elevated vascular resistance (75). By applying MI model induced by left coronary artery ligation in rats, Cho et al. demonstrated elevated activated monocytes (CC chemokine receptor 2^+ ED-1^+) in peripheral blood, along with the infiltration of ED-1^+ macrophages and the increment of nuclear p65 in the kidney of MI rats, suggesting the contribution of NF-κB-mediated inflammation in the development of type I CRS. The inflammatory cytokines, IL-6, and tumor necrosis factor-α (TNF-α) mRNA expression, as well as microvascular endothelial permeability and

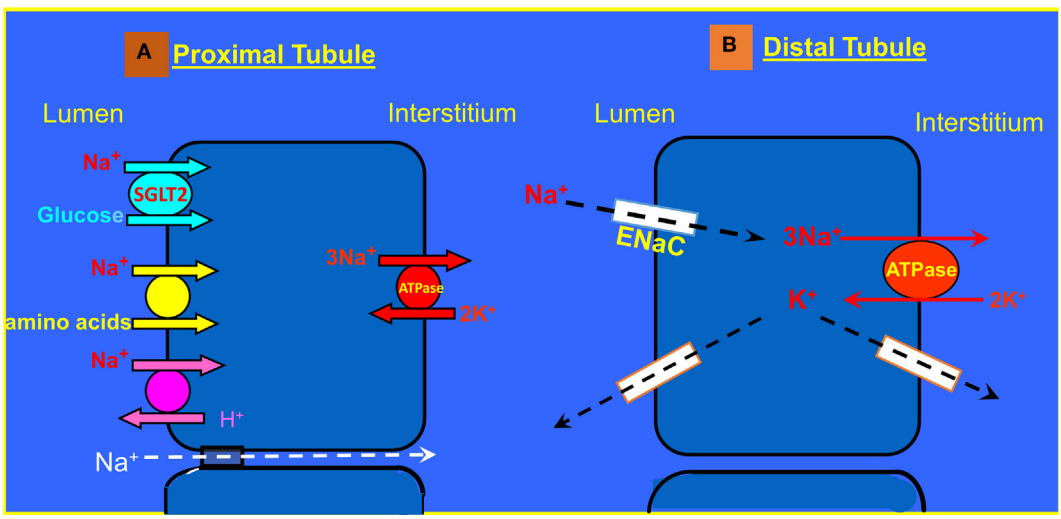

FIGURE 3 | **(A)** Sodium transport in the proximal tubule. **(B)** Reabsorption of Na^+ and K^+ transport in the principle cells of the collecting duct.

tubular cell apoptosis, significantly enhanced in the kidneys of MI rats. These findings support the involvement of the immune activation/inflammation in the pathophysiology of CRS besides the hemodynamic alteration, pathological compensatory neurohormonal activation and oxidative stress (76).

As mentioned above, the RAAS activates the immune system but also the immune system activates certain components of RAAS (8). In this context, TNF-α and IL-6 stimulate the generation of angiotensinogen, exaggerate sodium retention and enhance renal fibrosis (77). One of the most famous representatives of the adverse cytokines in CHF is TNF-α, whose circulatory levels increase in correlation with the severity of the disease (66). In line with the deleterious pro-inflammatory role of Ang II in CHF, pharmacological blockade of the AT1 receptors in this clinical setting decreased the levels of pro-inflammatory cytokines including TNF-α (78, 79). Similarly, *in vivo* studies demonstrated that Ang II enhanced the expression of both TNF-α and IL-6 in the cardiomyocytes and in renal cortical and tubular cells (78, 80). These findings support the notion that the RAAS, especially Ang II, triggers the production of pro-inflammatory molecules in CHF.

Finally, several studies provided a keen linkage between oxidative stress and immune activation in several cardiovascular diseases including CHF and hypertension (81, 82). Interestingly, long-term activation of the RAAS impairs mitochondrial function, and increase oxidative stress burden which in turn can lead to renal injury and sodium and water retention (81). The major prooxidative stress stimulator is Ang II, where administration of the latter enhances renal mitochondrial oxidative stress and reduces GFR in rats with heart failure.

Sympathetic Nervous System

Activation of SNS is one of the hallmarks of CHF. It is well established the SNS mediates system and renal vasoconstrictor and salt retaining biological actions (83). However, experimental studies have demonstrated the involvement of pro-inflammatory cytokines TNF-α, IL-6, and IL-1β in CHF rats that were exposed to chronic stimulation of β-adrenergic stimulation with isoproterenol for 12 weeks as manifested by increased mRNA expression of these cytokines in cardiomyocytes and cardiac blood vessels. Nevertheless, the mRNA expression of NO was not increased. Thus, it is presumed that one mechanism underlying the beneficial effects of β-adrenergic blockade in heart failure may involve attenuation of TNF-α and IL-1β expression independent of iNOS and NO. In this context, β-blockade with metoprolol causes significant decline in TNF-α and IL-1β, but not IL-6 expression in the myocardium (84). It should be emphasized that the kidneys are preferentially innervate by sympathetic nerve fibers and the activity of renal sympathetic nerve is markedly increased in CHF. Stimulation of the renal nerve augments sodium reabsorption, decreases renal blood flow (RBF) *via* renal artery constriction, and stimulates renin release through β1 receptors on the juxtaglomerular apparatus (85).

Whether enhanced circulatory levels or locally produced cytokines in response to SNS or RAAS activation in CHF mediate some of the adverse renal actions of these systems remained largely to be elucidated. However, previous studies have shown that pro-inflammatory mediators such as TNF-α, IL-6, and CRP play a role in the pathophysiology of progressive renal injury and probably in salt and water imbalance characterizing various acute and chronic kidney diseases including CRS. As mentioned above, volume expansion associated with CHF promotes secretion of cytokines by endothelial cells. In this context, it was reported that TNF-α caused renal dysfunction as was evident by intravascular volume expansion due to salt and water retention (86). Likewise, oxidative stress enhances NaCl absorption by the thick ascending limb through activation of protein kinase C (87). Additional explanation to the avid sodium retention and water imbalance in heart failure is renal epithelial and endothelial damage, which leads to a loss of endo–epithelial barrier integrity and function. In this respect, Cho et al. have shown that CRS type I and II was associated with increased tubular damage marker as was evident by elevated levels of NGAL and tubular cell apoptosis. Moreover, macrophage infiltration and inflammatory cytokine expression possibly mediated by the NF-κB pathway, and microvascular endothelial damage increased significantly in kidneys at 3 days post-induction of MI, suggesting the important contribution of inflammation in the pathogenesis of type I CRS. These changes ultimately led to renal interstitial fibrosis along with chronically decreased heart function (type II CRS) (76). Although the direct effects of cytokines on ion transports in the kidney were no studied yet, the observation that heart failure is associated with enhanced renal TNF-α and IL-6 expression along tubular cell apoptosis supports such a role. In addition, microvascular endothelial injury characterized by endothelial cell apoptosis, alteration of actin cytoskeleton, or increased expression of leukocyte adhesion molecules has mediated the early phase of renal injury following ischemia and by facilitating leukocyte transmigration; it substantially contributes to tissue inflammation. In the long run, activation of the Ang II and SNS along renal hypoperfusion causes further activation of pro-inflammatory cytokines, which in their turn enhance neurohormonal activation thus creating a vicious cycle (88).

Endothelin

ET peptides are synthesized as preproETs in endothelium, heart, and kidney, processed into a big precursor and then converted into biologically active peptides such as ET-1 by 2 ET-converting-enzyme isoforms. Active ET-1 binds to ETA and ETB receptors (ETAR/ETBR) expressed in the kidney, lung, brain and cardiovascular system. ETAR activation generally causes vasoconstriction, while ETBR produces vasodilation. The status of the major components of the ET system in the kidney during CHF has been subject to intensive investigation. As outlined above, the endothelin system is over activated in CHF as evident by elevated levels of ET-1 in the circulation, cardiac and renal tissues as well as urinary excretion of this peptide (89–92). The deleterious and beneficial renal and cardiac ET effects are usually attributed to ETAR and ETBR, respectively (93, 94). However, ETAR blockade failed to improve cardiovascular outcomes and caused edema in clinical trials (95–98).

The heart and kidney are both important sources and key targets for ET. Cardiac myocytes and the renal glomerulus, vessels, and tubular epithelium each express ETA and ETB receptors (91).

Elevated ET-1 levels correlate with CHF, hemodynamic dysfunction, and symptom severity (15, 99) and result in cardiac ETA upregulation along ETB downregulation (100–102). ET receptor antagonists attenuate experimental cardiac pathophysiology (103–107). ET-mediated renal pathology during CHF is an area of debate, but the potent reductions in RBF, GFR, natriuresis, and urine volume when ET-1 is increased to CHF levels supports a deleterious role for ET (108). ET levels also strongly correlate with renal dysfunction in patients with CRS (109, 110). However, a sustained cortical vasoconstriction and transient medullary vasodilation indicate renal responses to ET-1 are complex (108, 111). ET also produces dose-dependent changes in renal Na^+ and water excretion, with high levels causing anti-natriuretic and antidiuretic effects, due to reduced GFR and RBF. In contrast, lower doses or local tubular *in situ* epithelial delivery produced ET-1 decrease tubular salt and water reabsorption, which are blocked by ETB antagonists (112).

Collectively, these findings suggest that ET signaling contributes to cardiac and renal dysfunction. Besides its endocrine/paracrine role in the regulation of the cardio vascular and renal hemodynamic and salt balance, ET-1 possesses pro-inflammatory and pro-fibrotic properties in pulmonary, cardiac, and renal diseases. Under these disease conditions, increases in ET-1 are critically involved in initiating and maintaining inflammation and injury, thereby promoting perturbations among the rest of salt and water balance. At the renal level, ET-1 stimulates the aggregation and accumulation of neutrophils, thus propagating glomerular inflammation, a process that can be inhibited by ETA receptor blockade (113).

Natriuretic Peptides (NPs)

This family consists of three members of the NPs: ANP, brain natriuretic peptide (BNP), and C-natriuretic peptides (114, 115). ANP and BNP are secreted mainly from the atria and ventricles, respectively, upon atrial distention and volume overload (116, 117). By binding to the NPR-A receptor, ANP and BNP induce the production of cGMP, which in turn promotes vasodilation, diuresis, natriuresis, and prevent cardiac remodeling, thus playing a major role in the homeostasis of blood pressure as well as of water and salt balance.

Interestingly, clinical and experimental heart failure are associated with high levels of circulating NPs, and today these peptides serve as biomarkers for HF. However, few studies have demonstrated that *de novo* synthesis of NPs in the renal tissue constitutes an essential pathway for maintaining normal blood pressure and fluid balance besides the NPs of cardiac origin. Ritter et al. were the first to report that primary cultures of neonatal and adult rat kidney cells produce and secrete ANP-like prohormone (118). Soon after, by using immunohistochemical staining, Greenwald et al. detected proANP predominantly in the distal cortical nephron (119). It was later that Ramirez et al. reported that all forms of proANP/ANP were found in the kidney, mainly in the proximal and distal nephron (120). ANP and their receptor, NPR-A, were highly expressed as well in these parts of the tubule. The natriuretic and diuretic effects of ANP are attributed to its stimulatory effect on GFR but also to its inhibitory action on ENaC in the collecting duct (121).

Interestingly, several studies demonstrated that ANP also act as autocrine/paracrine factor where it modulates various immune functions (122). There is keen evidence that ANP is locally produced by several immune cells, which also present specific natriuretic receptors. For instance, ANP stimulates the phagocytosis of macrophage and killing activity by ROS production, thus improving the innate immunity (123). Moreover, ANP inhibits lipopolysaccharide-induced NO release by macrophage cells and promotes the inactivation of NF-κB *via* cGMP (53, 123). In a recent study by Mitaka et al. (124), ANP pretreatment prevented kidney–lung cross talk in a rat model of renal ischemic reperfusion injury. Interestingly, this group has also shown that ANP post-treatment ameliorated injuries in kidney and lung by direct tissue protective effect and anti-inflammatory effects, which potentially inhibited interorgan cross talk (125). Zhu et al. have shown that ANP reduced the levels pro-inflammatory cytokines such as IL-1β, IL-6, IL-10, and TNF-α in rats with oleic acid-induced ALI (126). In agreement with its anti-inflammatory properties, ANP interferes with the expression of adhesion molecules such as ICAM-1 and E-selectin (66, 77).

Finally, in critically ill patients, BNP and NT-proBNP levels correlated with inflammatory markers such as CRP and leukocyte count (127). Likewise, patients with septic shock had elevated BNP concentration regardless of the presence of CHF condition. Although the involvement of these anti-inflammatory effects of NPs in the pathogenesis of renal function in CHF has not been completely understood, it may represent a counterbalance compensatory response to the activation of the adverse neurohormonal systems including RAAS, SNS, and ET-1.

NO System

Renal NO is a molecule synthesized from its precursor, L-arginine by the enzyme, NOS; this process takes place in several sites, mainly in the endothelial cells of the renal blood vessels but also in the tubular epithelial and mesangial cells. Notably, three different isoforms of NOS; NOS 1 (bNOS), NOS 2 (iNOS) and NOS 3 (eNOS). NO plays an important role in the regulation of renal hemodynamics and excretory function. Specifically, locally produced NO is involved in the regulation RPF, salt excretion, and renin release. The action of NO is mediated by activation of a soluble guanylate cyclase in adjacent vascular smooth muscle cells, thereby increasing intracellular levels of its second messenger, cGMP (128, 129).

It has been shown that iNOS has been implicated in many human diseases associated with inflammation *via* the activation of the c-JNK, p42/44 MAPK, and p38 kinase pathways (130, 131). This isoform of NOS is responsible for the generation of excessive amounts of NO, which leads to tissue injury due to exaggerated generation of oxidative radicals such peroxynitrate. For instance, iNOS is overexpressed in the venous endothelial cells harvested from patients with decompensation CHF (132). Likewise, excessive NO in the heart leads to myocardial depression and reduced contractility in patients and experimental animals with CHF (133).

These findings lead to the "cytokine hypothesis," which suggests that cytokines play an important pathogenic role in development of HF. This notion is further supported by two studies

demonstrating that iNOS knockout mice display less cardiac dysfunction after myocardial infarction than wild-type controls (134). Interestingly, there is negative interplay between iNOS and ANP, where the latter *via* cGMP production increases intracellular calcium levels in murine macrophages resulting in decreased iNOS expression (135).

SUMMARY AND CONCLUSION

In summary, inflammation and neurohormonal systems appear to interplay one with each other leading to worsening cardiac, pulmonary, and renal functions, which negatively affect patients' outcome. While the adverse role of the RAAS, SNS, and ET-1 in the pathogenesis of CHF is well established, the involvement of the innate and adaptive immune in the cardiac, renal, and pulmonary manifestations of CHF is still evolving. Although we have shown that the inflammatory system plays a substantial direct and indirect role in heart failure, this observation does not prove unequivocally a causal role in CHF. Therefore, another possibility should be considered, such as that these cytokines are elevated in heart failure in response to the underlying injury, and that they may serve as markers rather than drivers of the disease process. The therapeutic interventions aimed at reducing the activation of the immune cells or blockade of certain cytokines were unsatisfactory. Milestones studies that investigated the effect of TNF-α antagonists, etanercept (136), and infliximab (137) on the composite clinical outcomes of mortality and worsening heart failure in the range of several weeks, in patients with chronic systolic heart failure failed to show any benefit. Infliximab failed to show beneficial effect; this was related possibly to the short term treatment. The RENEWAL investigators suggested several possibilities for the lack of benefit of etanercept; among them, lower investigated doses of etanercept, cytokines may not play an important role in heart failure or alternatively, there is a need to simultaneously target several inflammatory mediators and the predisposition for infection due to etanercept. A recent review of these studies argued that the unfavorable outcomes might be attributed to the population cohorts that were mostly severe with advanced heart failure, toxicity of the treatment, and genetic polymorphism (138). Recently, it was shown in a small cohort of 30 patients with systolic heart failure and acute decompensation; the administration of IL-1 blocker, anakinra reduced the inflammatory burden as shown by reduced C-reactive protein levels within 72 h. However, this study did not address clinical outcomes (139). Cavalli et al. have reported that in a patient who developed fulminant myocarditis with biventricular failure and cardiogenic shock, the administration of anakinra restored cardiac function with clinical improvement (140). Yet, we should await more well-designed studies that may prove to be beneficial in reducing target organ damage and preventing congestion characterizing heart failure.

In light of the limited therapeutic tools of congestion of pulmonary and cardiac etiologies, anti-inflammatory treatment strategies may turn to be a novel approach with promising prognostic consequences in the CRS. Yet, further research is required to understand in depth the interaction between the classic neurohormonal systems and the inflammatory ones, the sources of the latter in the CRS, and their effect on the specific mediators of salt and water transporters at the pulmonary and renal tissues.

AUTHOR CONTRIBUTIONS

ZSA and ZA conceived and designed the manuscript structure. ZSA, SK, FB, RI-B, EK, and ZA contributed to writing and reviewing the paper. SK, ZSA, and ZA contributed in preparing the figures.

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Cytokine-Regulation of Na^+-K^+-Cl^- Cotransporter 1 and Cystic Fibrosis Transmembrane Conductance Regulator—Potential Role in Pulmonary Inflammation and Edema Formation

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Pulmonary edema, a major complication of lung injury and inflammation, is defined as accumulation of extravascular fluid in the lungs leading to impaired diffusion of respiratory gases. Lung fluid balance across the alveolar epithelial barrier protects the distal airspace from excess fluid accumulation and is mainly regulated by active sodium transport and Cl^- absorption. Increased hydrostatic pressure as seen in cardiogenic edema or increased vascular permeability as present in inflammatory lung diseases such as the acute respiratory distress syndrome (ARDS) causes a reversal of transepithelial fluid transport resulting in the formation of pulmonary edema. The basolateral expressed Na^+-K^+-$2Cl^-$ cotransporter 1 (NKCC1) and the apical Cl^- channel cystic fibrosis transmembrane conductance regulator (CFTR) are considered to be critically involved in the pathogenesis of pulmonary edema and have also been implicated in the inflammatory response in ARDS. Expression and function of both NKCC1 and CFTR can be modulated by released cytokines; however, the relevance of this modulation in the context of ARDS and pulmonary edema is so far unclear. Here, we review the existing literature on the regulation of NKCC1 and CFTR by cytokines, and—based on the known involvement of NKCC1 and CFTR in lung edema and inflammation—speculate on the role of cytokine-dependent NKCC1/CFTR regulation for the pathogenesis and potential treatment of pulmonary inflammation and edema formation.

Keywords: lung inflammation, pulmonary edema, CFTR, NKCC1, cytokines

INTRODUCTION

Pulmonary edema, defined as excessive fluid accumulation in the interstitial and air spaces of the lungs, is a life-threatening condition leading to impaired gas exchange and respiratory failure. Depending on the underlying cause, pulmonary edema is distinguished into two types; hydrostatic and permeability-type edema. The most common form of hydrostatic edema is cardiogenic edema which occurs as major complication of left-sided heart failure and is characterized by increased transcapillary hydrostatic pressure gradients between pulmonary vasculature and interstitial space

resulting in interstitial lung edema and flooding of the alveoli with protein-poor fluid. Permeability-type lung edema, also referred to as non-cardiogenic pulmonary edema, is defined by an exudation of protein-rich fluid into the alveoli and develops characteristically in the process of inflammatory lung diseases such as the acute respiratory distress syndrome (ARDS) (1, 2). Two processes are critical for the accumulation of protein-rich fluid in the alveolar space: (1) the disruption of endothelial and epithelial barriers leading to increased vascular permeability and (2) the dysregulated expression or impaired function of ion channels in alveolar epithelial cells limiting fluid removal from the distal airspaces. As such, repair of the epithelial cell barrier and effective clearance of fluid from air spaces are essential prerequisites for resolution of pulmonary edema.

Several transporters (specified in the Section "Fluid Transport of Alveolar Epithelium"), expressed on alveolar type I (ATI) and type II (ATII) cells, are involved in active transport of salt and water through the epithelial barrier leading to alveolar fluid clearance (AFC). AFC is impaired in more than 80% of ARDS patients and associated with increased morbidity and mortality (3). Therefore, manipulation of alveolar fluid transport could represent a suitable therapeutically target. However, molecular mechanism resulting in impaired epithelial fluid transport remains unclear. Inflammatory responses involving upregulation of pro-inflammatory cytokines including interleukin-1β (IL-1β), IL-8, tumor necrosis factor-α (TNF-α), and transforming growth factor-β (TGF-β) and their accumulation in BALF and edema fluid are a critical hallmark of ARDS (4–7). In addition to their role in immune responses, these pro-inflammatory mediators are considered to inhibit alveolar fluid transport by regulation of sodium and chloride transporter (7). However, present understandings of mechanisms by which cytokines regulate ion transport are far from complete, with previous work having focused on the regulation of Na^+ transport via epithelial Na^+ channel (ENaC) and the Na^+/K^+-ATPase. In this review, we propose that cytokine-dependent regulation of Na^+-K^+-$2Cl^-$ cotransporter 1 (NKCC1) and Cl^- channel cystic fibrosis transmembrane conductance regulator (CFTR) may play a critical role in lung edema formation and pulmonary inflammation. To this end, we first outline the general principles of alveolar fluid transport and the role of inflammatory cytokines in lung edema formation, then focus specifically on the role of NKCC1 and CFTR in pulmonary edema and inflammation, and their regulation by cytokines, and finally conclude by proposing a critical role for cytokine-dependent regulation of NKCC1 and CFTR as a novel concept in the pathogenesis of pulmonary edema.

REGULATION OF ACTIVE SALT AND WATER TRANSPORT AND EDEMA FORMATION

Fluid Transport of Alveolar Epithelium

The alveolar epithelium forms a tight barrier between vasculature and air-filled compartment to control movement of protein and fluid under physiological conditions. It comprises ATI cells, which are responsible for gas exchange across the alveolo-capillary barrier, and ATII cells that fulfill several functions, most notably production and release of surfactant (8).

In the intact lung, AFC constantly moves fluid from the alveolar space across the epithelial barrier into the interstitial space. Na^+ is actively absorbed on the apical side of alveolar epithelial cells, which is mediated by several sodium channels (**Figure 1**), most notably the amiloride-sensitive ENaC (9), the sodium glucose transporter (SGLT) (10, 11) and the sodium-coupled neutral amino acid transporter (SNAT) (12–14). On the basolateral surface, Na^+ extrusion to the interstitial space is driven through the Na^+-K^+-ATPase (8) and the Na^+/H^+ antiporter (15). For electroneutrality and osmotic balance, Cl^- and water follow the electrochemical gradient partly paracellularly and partly through aquaporins (specifically aquaporin 5) (16) and chloride channels, predominantly CFTR (17). Although it was thought that channels and transporter are primarily expressed in ATII cells, recent data demonstrated that ATI cells contain ENaC, CFTR, and the Na^+-K^+-ATPase suggesting a role for ATI cells in alveolar fluid transport (9, 18).

In response to lung injury and inflammation, the epithelial barrier becomes disrupted leading to increased influx of protein-rich fluid and formation of pulmonary edema (**Figure 1**). Furthermore, the physiological protection provided by active salt and water transport is attenuated, resulting in impaired AFC in patients with both cardiogenic (19) and permeability-type (3) lung edema. On top of that, AFC may reverse into active alveolar fluid secretion (AFS), thus promoting rather than resolving edema formation. Recently, work from our group identified basolateral NKCC1 and apical-expressed CFTR as critical for the reversal of an absorptive into a secretory alveolar epithelium by driving Cl^- secretion (20). Specifically, we could show that an acute increase in left atrial pressure decreases amiloride-sensitive Na^+ uptake across the alveolar epithelium and concomitantly stimulates Na^+ and Cl^- uptake via basolateral NKCC1 and Cl^- secretion into the alveolar space via apical CFTR, thus effectively reversing Na^+-driven AFC into Cl^- driven AFS. Importantly, inhibition of CFTR and NKCC1 improved AFC and attenuated edema formation. In line with this concept, previous studies have reported similar beneficial effects of NKCC inhibition on edema formation in different organs in that bumetanide reduced cerebral edema formation in response to ischemia (21) and furosemide improved fluid balance and reduced pulmonary edema in ARDS patients (22). Although these effects have traditionally been attributed to diuretic effects of non-specific NKCC inhibitors, improvement of respiratory function by furosemide in lung edema precedes the onset of diuresis (23, 24), suggesting alternative mechanisms such as NKCC/CFTR-mediated AFS in edema formation.

Pro-Inflammatory Cytokines in Lung Edema Formation

During early stages of injury, the lung is the site of acute inflammatory processes with excessive transepithelial neutrophil migration and continuous release and activation of pro-inflammatory mediators. Pro-inflammatory cytokines, produced by circulating

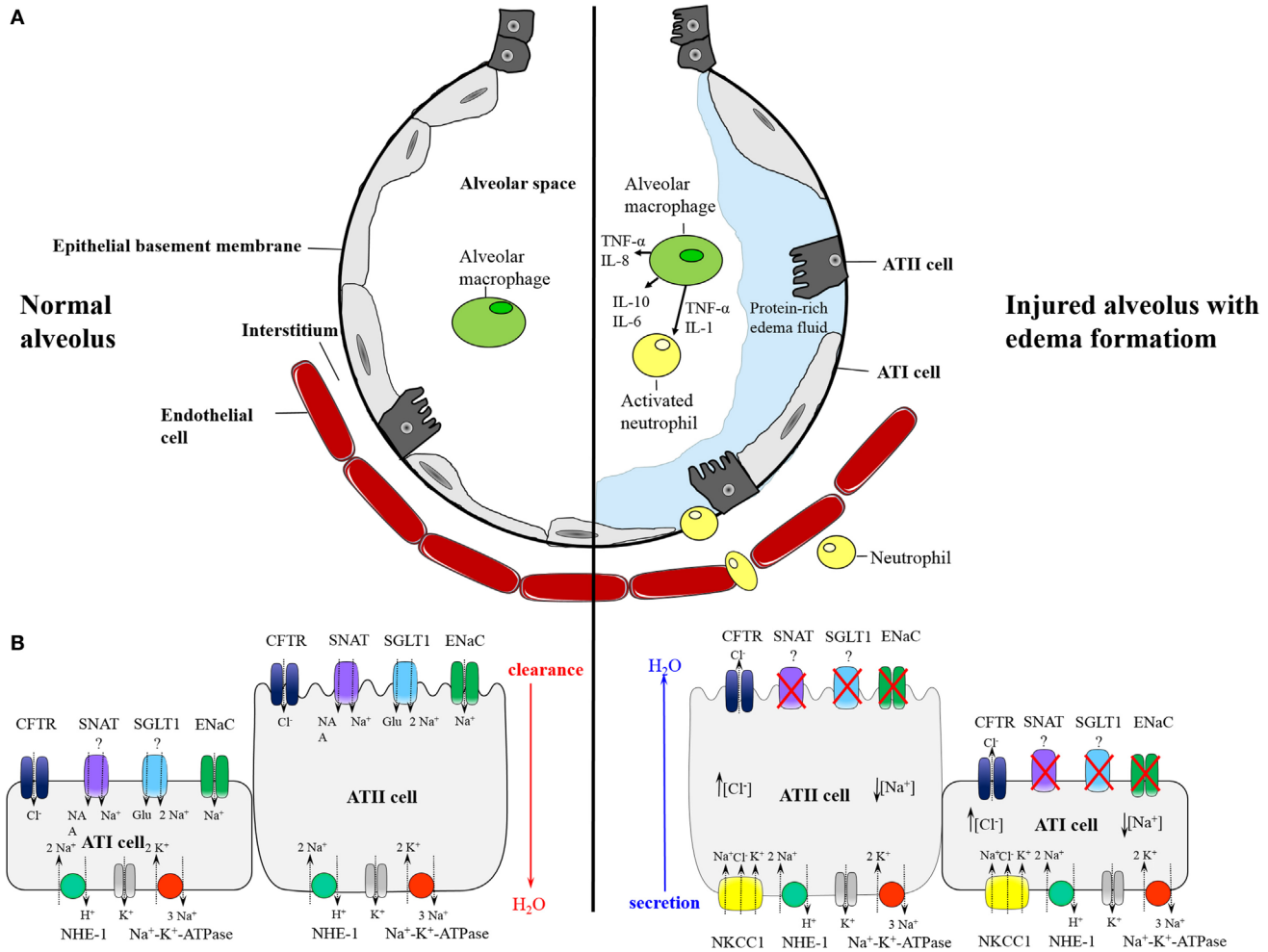

FIGURE 1 | **(A)** Schematic model of the normal alveolus (left) and the injured alveolus (right) with edema formation in inflammatory lung disease. In pulmonary inflammation, the epithelial and endothelial barrier become disrupted leading to influx of protein-rich edema fluid and migration of neutrophils from the vasculature into the alveolar space. In the air space, alveolar macrophages secrete proinflammatory cytokines that stimulate chemotaxis and activate neutrophils which in turn produce and release further cytokines. **(B)** Distribution of epithelial ion transporter and proposed mechanism of alveolar fluid clearance (AFC) (left) and secretion (right). In alveolar type II and presumably also type I cells, AFC is mediated through apical Na^+ entry by sodium channels like epithelial Na^+ channel (ENac), sodium-coupled amino acid transporter (SNATs), and sodium glucose transporter (SGLT). Basolateral extrusion is driven by Na^+-K^+-ATPase and sodium hydrogen exchanger (NHE). Water and Cl^- follow for electroneutrality. In pulmonary edema, ENaC and probably other sodium transporter are inhibited generating a gradient for Na^+ influx via basolateral NKCC1. Cl^- enters in cotransport with Na^+, and exits along an electrochemical gradient on the apical side through CFTR, resulting in Cl^--driven fluid secretion and formation of lung edema.

monocytes, alveolar macrophages, and neutrophils, promote recruitment and activation of additional immune cells and inflammatory molecules. A variety of cytokines and growth factors can be detected in BALF and edema fluid of ARDS patients including TNF-α, IL-1β, IL-8, and TGF-β1 (5, 6, 25, 26). These cytokines have been implicated to play a crucial role in the pathophysiology of pulmonary edema formation.

A critical involvement of TNF-α in edema formation has been documented in a series of experimental and clinical studies, which have previously been reviewed in detail (27). In brief, TNF-α reduces the expression of ENaC mRNA in alveolar epithelial cells and thereby decreases amiloride-sensitive sodium uptake (28). However, there is also evidence for a protective effect of TNF-α in pulmonary edema formation, as demonstrated by Borjesson and colleagues who identified in a rat model of intestinal ischemia-reperfusion, a TNF-α-dependent stimulation of AFC in the early phase of injury (29). Similarly, a TNF-dependent and amiloride-sensitive increase in alveolar fluid resorption was detected in a rat model of *Pseudomonas aeruginosa* pneumonia (30).

Inhibition of growth factor TGF-β1 protects wild-type mice from pulmonary edema in a bleomycin-induced lung injury model (31). An increased TGF-β1 activity in distal airways has been shown to promote edema by reducing alveolar epithelial sodium uptake and AFC. This effect of TGF-β1 is considered to be dependent on activation of the MAPK-ERK1/2 pathway resulting in decreased expression of ENaC mRNA (32). A similar effect

has been described for IL-1β, which was shown to reduce ENaC expression through p38–MAPK-dependent inhibition of ENaC promoter activity (33). In contrast, an *in vitro* study reported an IL-1β-mediated increase in epithelial repair induced by edema fluid (34).

The chemotactic mediator IL-8 promotes edema formation by blocking AFC (35). Accordingly, inhibition of IL-8 significantly diminishes edema caused by smoke inhalation, acid aspiration, or ischemia-reperfusion injury (36–38).

Overall, there is evidence that cytokines are important regulators of active ion transport and AFC. However, exact regulation of ion channels by inflammatory cytokines may be a complex phenomenon with functional effects depending on temporal and spatial profiles, interdependence between various cytokines, and the presence (*in vivo* situation) or absence (*in vitro* assays) of immune cells. Detailed dissection of these scenarios poses a considerable challenge in terms of both resources and appropriate assays, yet would provide an invaluable platform for a better understanding of the complex crosstalk between inflammation and ion channel activity in a wide range of pulmonary and systemic inflammatory diseases.

CFTR AND NKCC1 IN INFLAMMATORY LUNG DISEASE AND PULMONARY EDEMA

Na^+-K^+-Cl^- Cotransporter

The Na-K-Cl cotransporter (NKCC) mediates active electroneutral uptake of one Na^+ and K^+ with 2 Cl^- molecules along an inwardly directed electrochemical gradient for Na^+ and Cl^-. Of the two known isoforms, NKCC1 and NKCC2, NKCC1 is found on the basolateral side on epithelial and endothelial cells in several organs, including the alveolar epithelium. In contrast, apically expressed NKCC2 is only present in the kidney epithelium (39). Both isoforms are sensitive to loop diuretics like bumetanide and furosemide, which inhibit ion translocation (40).

To maintain cell shape and integrity during active salt and water secretion, activation of NKCC1 is strictly regulated. Activity of NKCC1 can be induced through hyperosmotic stress (41), low intracellular Na^+ level, increase in intracellular cAMP, or changes in cell shape, and depends on direct phosphorylation by Ste20-related proline/alanine-rich kinase (SPAK) and oxidative stress responsive kinases (OSR1) (42).

Cystic Fibrosis Transmembrane Conductance Regulator (CFTR)

CFTR, which has been identified as the mutated gene in cystic fibrosis patients (43), is considered an atypical ATP-binding cassette (ABC) transporter which is activated by phosphorylation and ATP hydrolysis (44). It permits bidirectional transport of Cl^- anion depending on the electrochemical gradient. CFTR is expressed on apical membranes of epithelial cells in distal airways and alveolar epithelium, where it mediates Cl^- transport to maintain alveolar fluid homeostasis (45). CFTR expression and activation depends on intracellular cAMP or cGMP, which activate PKA and cGKII (46) leading to upregulation of CFTR expression and phosphorylation (47, 48).

Expression of NKCC1 and CFTR in Inflammatory Lung Diseases

NKCC1 and CFTR are both involved in a variety of biological processes ranging from ion transport to regulation of macrophage activation and modulation of cytokine production (49–52). Of relevance for this review, NKCC1 and CFTR have also been implicated in pulmonary inflammatory processes.

NKCC1 is upregulated in response to Gram-negative bacterial toxins like lipopolysaccharide (LPS) in the lung and kidney (53). Whether this enhanced NKCC1 gene expression is, however, mediated directly by LPS binding to its receptor inducing intracellular signaling or via released inflammatory cytokines like TNF-α after LPS stimulation remains to be elucidated.

Nguyen and colleagues (54) proposed a role for NKCC1 in inflammatory processes in response to *Klebsiella pneumoniae* infection. Mice lacking NKCC1 were protected from bacteremia and lethal sepsis after infection and showed decreased vascular permeability. The number of migrated neutrophils in the air space was increased leading to a reduced number of *K. pneumoniae* in the lung of NKCC1-deficient mice. A potential mechanism that may explain the involvement of NKCC1 in edema formation and neutrophil transmigration was proposed by Matthay and Su (55), who speculated that expression of NKCC1 in endothelial and epithelial cells might be upregulated by inflammatory molecules in response to bacterial infections; however, this hypothesis still awaits functional validation, and regulatory pathways involved remain to be clarified. Along similar lines, a study by Andrade and colleagues reported upregulated NKCC1 expression along with downregulated ENaC in response to *Leptospirosis* infection, a model of sepsis leading to edema formation and ARDS (56). The authors proposed a regulation of transporter expression via JNK and NF-κB pathways during leptospirosis-induced pulmonary edema, yet exact signaling cascades remain unclear.

CFTR is considered an important modulator of inflammatory responses in the lung. Absence of a functional CFTR leads to chronic pulmonary inflammation, as seen in cystic fibrosis patients (57). In a murine model of cerebral and uterine *Chlamydia trachomatis* infection, CFTR mRNA and protein were shown to be upregulated causing increased tissue fluid accumulation and edema formation (58). The authors hypothesized that increased CFTR expression and abnormal fluid accumulation upon *C. trachomatis* infection may depend on increased cytokine release. In pulmonary epithelial cells, CFTR functions as receptor for *P. aeruginosa* internalization leading to CFTR translocation into lipid rafts (59) and NF-κB mediated expression of IL-1β (60). However, the role of CFTR in lung injury and edema formation may be more complex. In a CF mouse model using CFTR-deficient animals, Bruscia and colleagues (61) demonstrated an enhanced pulmonary inflammatory response with elevated cytokine levels in response to chronic LPS exposure. Similarly, Su and coworkers (51) reported aggravated inflammatory cytokine release and edema formation following LPS challenge in mice bearing the human functional CFTR mutation, F508del-CF,

or in mice treated with a pharmacological CFTR inhibitor. Importantly, subsequent chimeric experiments in wild-type mice reconstituted with F508del neutrophils or bone marrow, respectively, revealed that pro-inflammatory, pro-edematous effect of functional CF inhibition was attributable to the lack of CFTR on immune cells, specifically neutrophils, rather than epithelial or other parenchymal cells (51). Hence, CFTR may promote both pro-and anti-edematous effects in inflammatory lung disease depending on its site of expression.

Taken together, functional concepts involving NKCC1 and CFTR in fluid transport and edema formation (**Figure 1A**) in combination with data demonstrating differential regulation of NKCC1 and CFTR in lung injury that coincides with edema formation point toward a critical role for CFTR and NKCC1 in infection-induced pulmonary edema. While molecular mechanisms underlying the regulation of NKCC1 and CFTR by infectious pathogens remain to be elucidated, it is tempting to speculate on a critical role for inflammatory cytokines as putative mediators in the regulation of these channels. As discussed in the following section, multifunctional cytokines like TNF-α, IL-1β, and IL-8 are particularly attractive candidates as key regulators of NKCC1 and CFTR.

Regulation of NKCC1 and CFTR by Cytokines

Various signaling pathways have been suggested to play a role in NKCC1 activation and expression including WNK, MAP kinase/ERK, p38, and JNK pathways (62–64). Notably, these pathways are also known to be stimulated by cytokines and growth factors like TNF-α and TGF-β, which activate intracellular p38 and JNK pathways (65, 66). In pulmonary inflammation, cytokines may bind to receptors on alveolar epithelial cells and induce intracellular pathways resulting in activation of NKCC1, yet, direct evidence for such an effect in the intact, inflamed lung is presently outstanding. Consistent with this notion, however, NKCC1 mRNA and protein levels were found to be selectively upregulated by TNF-α and IL-1β in endothelial and epithelial cells (53, 67). Conversely, inhibition of TNF-α and IL-1β by hypertonicity was found to be beneficial in cerebral edema, and the functional role of TNF-α and IL-1β in the regulation of NKCC1 expression was validated *in vitro*, leading the authors to propose that TNF-α and IL-1β may directly upregulate NKCC1 expression via JNK- and p38-dependent pathways (67).

For CFTR, the interplay between inflammatory cytokines and channel expression/activity is even more complex. As such, IL-8 has been shown to indirectly regulate activity and biosynthesis of CFTR through inhibition of the β_2-adrenergic receptor (AR) pathway (35) and subsequent phosphorylation of CFTR via cAMP-mediated PKA activation, which is considered to be essential for AFC (68). Downregulation of β2-AR in ATII cells by IL-8 blocked fluid transport across the alveolar epithelium via inhibition of CFTR phosphorylation and expression (35). Likewise, TGF-β has been proposed to diminish cAMP-driven chloride transport in colonic epithelia via inhibition of CFTR mRNA expression and protein synthesis (69). In 2007, Lee and colleagues (7) investigated the effect of edema fluid on transporter expression in alveolar epithelial cells and showed that cytokine-containing edema fluid decreases expression and activation of CFTR leading to decreased AFC. However, incubation with individual cytokines alone did not alter CFTR expression, suggesting a complex regulatory mechanism.

Other studies, reported an upregulation of CFTR mRNA and protein by IL-1β (70–72). The NF-κB-pathway has been identified to be involved in the IL-1β-dependent increase in CFTR expression (70). In agreement with this effect, CTFR-dependent AFS has been shown to be stimulated by IL-1β and TNF-α in airway submucosal glands via cAMP-dependent activation of PKA (73).

Contradictions in CFTR regulatory processes and its involvement in fluid transport and edema formation may result from differences in expressed transport system in various cell types, and such heterogeneity may also be present in the pulmonary epithelium. Recently, it has been proposed that the distal airways might be comprised of secreting areas, located in the contraluminal regions of the pleats, and absorbing areas in the folds (74). Secretion and absorption of fluid is considered to occur simultaneously and independently maintaining the required level of airway surface liquid. Regulation of transporter expression may also vary in these areas. NKCC1 has been found to be abundantly expressed in the pleats of distal airways and less in the folds (75). No change in CFTR expression was detected in different areas (75), which is not surprising assuming CFTR is involved in fluid secretion and absorption. In lung injury, impaired alveolar fluid transport might be triggered by cytokine-mediated differential expression of ion channels including NKCC1 and CFTR that may putatively result in an increased expression and/or activation of NKCC1 and CFTR in secretory epithelia and an inhibition of channels in absorptive areas (**Figure 2**). While largely speculative at this stage, this hypothetical concept of a spatially (and potentially temporally) differential regulation of ion channels involved in fluid absorption and secretion by inflammatory cytokines highlights the need for expanded research in this fascinating field. An in-depth understanding of changes in ion and fluid flux and their regulation may provide for optimized targets for edema resolution in acute inflammatory lung disease.

CONCLUSION

Acute respiratory distress syndrome with edema formation is a serious complication in critically ill patients. Resolution of edema needs strategies to restore epithelial barrier function and improve AFC. Formation of pulmonary edema in inflammatory lung diseases is caused by the loss of endothelial and epithelial barrier and impaired fluid and ion transport across the alveolar epithelium. Pro-inflammatory cytokines like TNF-α, TGF-β1, and IL-1β, which are released and activated in the early phase of lung inflammation, may regulate expression and activity of ion channels involved in fluid transport including NKCC1 and CFTR. Regulation of NKCC1 and CFTR is, however, complex, with discrepant results potentially depending on time profile, cell type, and co-stimulation by different cytokines resulting in a distinct multi-dimensional response that favors AFS while impairing AFC. Manipulation of expression and activity of NKCC1 and

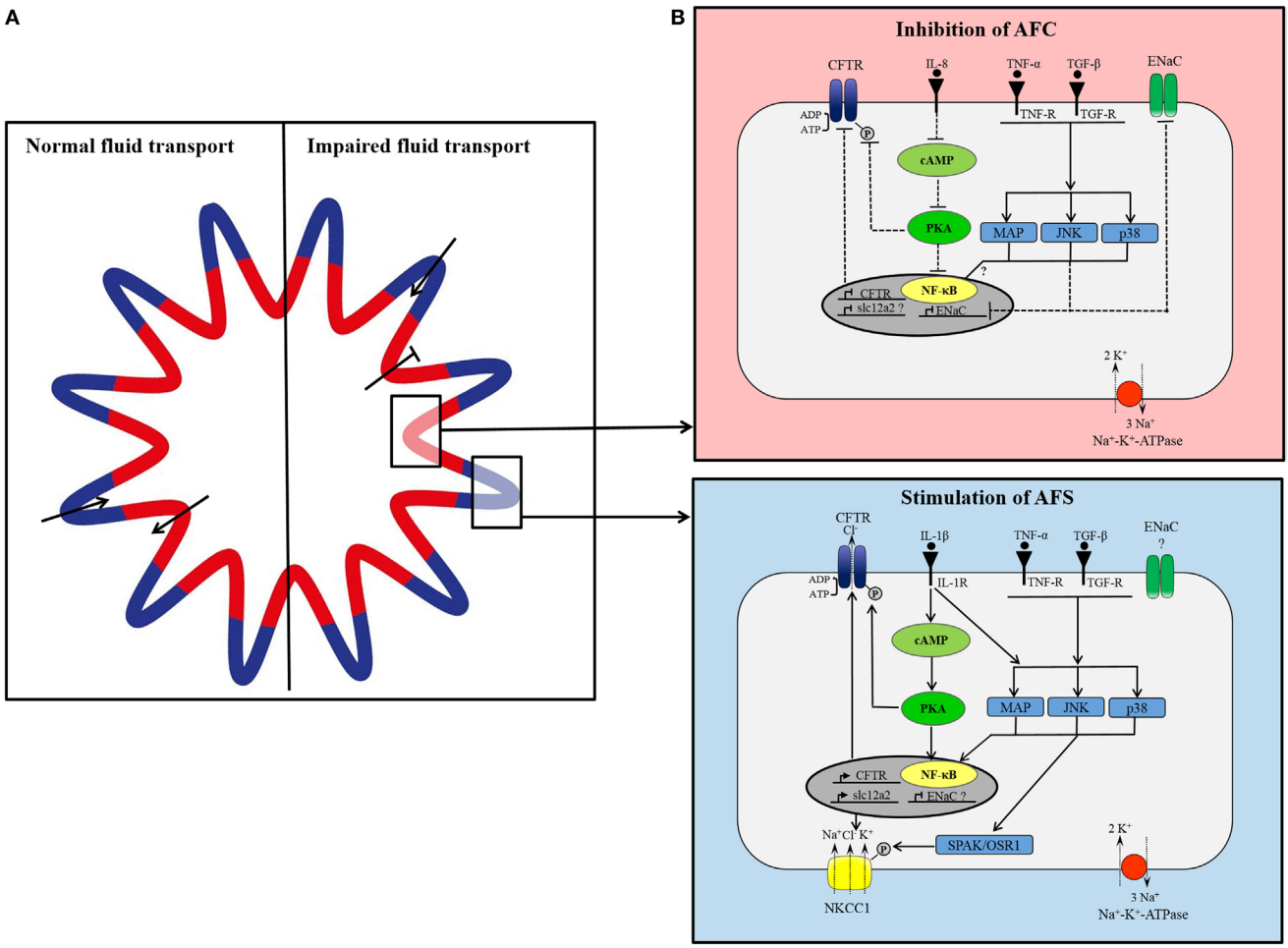

FIGURE 2 | **(A)** Proposed model of organization of small airways. In lung epithelium, independent groups of cells simultaneously secrete and absorb to maintain fluid homeostasis. Under normal conditions, cells located in the pleats secrete fluid (blue) and cells located around the folds concurrently absorb secreted fluid (red). In lung injury, fluid transport in absorptive areas may be blocked while fluid secretion stays intact or increases. **(B)** Simplified, hypothetical concept of differential cytokine-dependent regulation of ion transporter in absorptive (red) and secretory (blue) areas in inflammatory lung disease. In apsorptive areas, AFC is impaired presumably through cytokine-mediated inhibition of CFTR and epithelial Na^+ channel (ENaC). Proinflammatory cytokines [tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), interleukin1β (IL-1β)] bind to their receptor inducing intracellular signaling cascades of MAP-kinases, JNK, p38, which prevent expression of ENaC. IL-8 blocks CFTR expression and activation by inhibition of cAMP/PKA pathway. In secretory areas, fluid secretion is stimulated by cytokines. Receptor binding of IL-1β, TNF-α, and TGF-β induces intracellular signaling pathways leading to activation and expression of CFTR and NKCC1.

CFTR might serve as therapeutic target in inflammatory lung diseases with edema formation.

AUTHOR CONTRIBUTIONS

Both authors, SW and WK, meet the following four criteria of authorship: 1. Substantial contributions to the conception or design of the work. 2. Drafting the work and revising it critically for important intellectual content. 3. Final approval of the version to be published; 4. Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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