



## Review Article

# The roles of sodium-potassium-chloride cotransporter isoform-1 in acute lung injury

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### ABSTRACT

Acute lung injury (ALI) is often characterized by severe lung inflammation and pulmonary edema with poor gas exchange and hypoxemia. Alveolar inflammation and water flooding are, in fact, notable features of ALI pathogenesis. The sodium-potassium-chloride co-transporter isoform 1 (NKCC1), localized at the basolateral surface of the lung epithelium, drives water transport via back transport of Na<sup>+</sup> and Cl<sup>-</sup> to the alveolar air space. NKCC1, therefore, is crucial in regulating alveolar fluid. Increased expression of NKCC1 results in increased alveolar fluid secretion and impaired alveolar fluid clearance. During ALI, the with no lysine kinase (WNK), oxidative stress responsive kinase 1 (OSR1), and STE20/SPS1-related proline/alanine-rich kinase (SPAK) pathways are activated, which upregulates NKCC1 expression. Proinflammatory cytokines also enhance the expression of NKCC1 via c-Jun N-terminal kinase-and p38-dependent pathways. NKCC1 activation also increases the expression of proinflammatory cytokines via cell rupture and activation of macrophages. Increased proinflammatory cytokines, in turn, recruit inflammatory cells to the site of injury and cause further lung damage. Animals with high expression of NKCC1 show more severe lung injury with presentations of more severe pulmonary edema and microvascular permeability, higher expression of proinflammatory cytokines, and greater neutrophilic infiltration. In contrast, animals with low expression of NKCC1 or those treated with NKCC1 inhibitors show less severe lung injury with milder levels of presentations of ALI. These reports collectively highlight a novel role of NKCC1 in ALI pathogenesis. Manipulation of NKCC1 expression levels could, therefore, represent novel modalities for effective ALI treatment.

**KEYWORDS:** *Acute lung injury, Lung inflammation, Pulmonary edema, Sodium-potassium-chloride co-transporter isoform 1*

### ACUTE LUNG INJURY

Acute lung injury (ALI) is often characterized by severe lung inflammation and pulmonary edema with poor pulmonary gas exchange and hypoxemia [1]. ALI usually has a great impact on morbidity and mortality rates in intensive care units (ICUs) [1]. Even with modern and progressive treatment strategies, treating patients with ALI remain challenging. Therefore, it is very important to gain more insights into the pathophysiology of ALI and develop possible modes of effective treatments [1].

The pathophysiological features of ALI include acute lung inflammation accompanied by pulmonary microvascular hyperpermeability and pulmonary edema [1]. Alveolar fluid flooding represents critical pathogenesis of ALI. Therefore, regulating the flow of alveolar fluid into or out of the alveolar air space is an important step in controlling ALI progression.

The sodium-potassium-chloride co-transporter (NKCC) is localized in alveolar epithelial cells and regulates the transport of alveolar fluids by coupling the transport of sodium (Na<sup>+</sup>), chloride (Cl<sup>-</sup>), and potassium (K<sup>+</sup>) [2]. Since NKCC plays a role in the regulation of alveolar fluid transport, it is expected to be important in ALI pathogenesis.

### Alveolar fluid clearance (AFC) and alveolar fluid secretion (AFS)

The alveolar fluid clearance (AFC) and alveolar fluid secretion (AFS) constitute important mechanisms of lung

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edema in ALI [3,4]. Although it was initially thought the transporters are primarily expressed in alveolar type II cells (ATII), it was recently demonstrated that alveolar type I cells (ATI) also contain these transporters, suggesting the role of ATI and ATII cells in alveolar fluid transport [3,4].

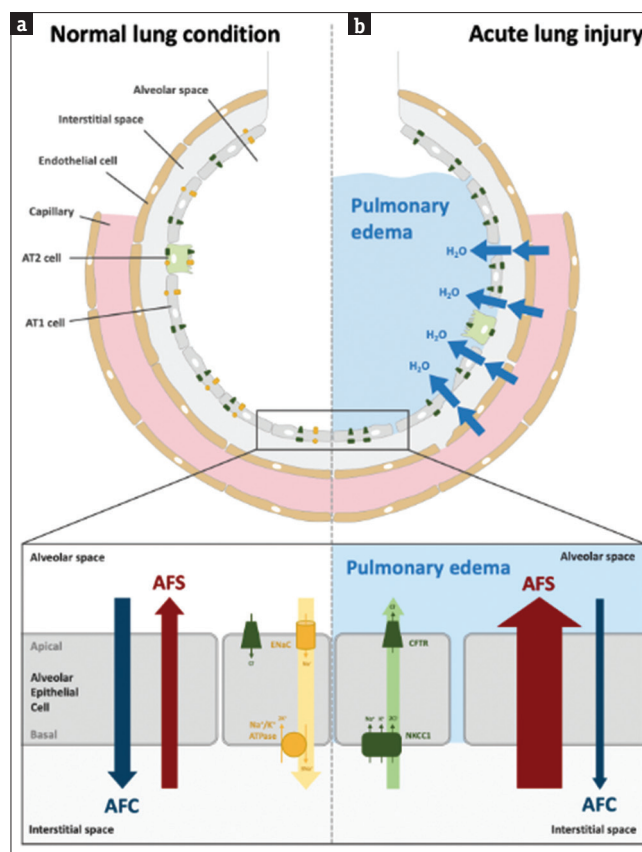
AFC is the ability to remove excess water from the alveolar space. This system of ion-driven alveolar fluid reabsorption is the primary mechanism of AFC [3]. AFC depends on an intact epithelial lining that transports ions to create an osmotic gradient for fluid absorption. AFC is an active mechanism driven by epithelial  $\text{Na}^+$  absorption by the apical side through the epithelial sodium channel (ENaC) and across the basolateral side via the sodium/potassium ATPase pump (Na-K ATPase) [3].  $\text{Cl}^-$  and water are considered to follow for electroneutrality and osmotic balance.  $\text{Cl}^-$  is transported via the cystic fibrosis transmembrane conductance regulator (CFTR) channel [3]. This vectorial ion transport creates an osmotic gradient that drives the clearance of fluid [3].

In contrast to AFC, AFS is the secretion of fluid into the alveolar space [5]. The process of AFS is triggered by the active flux of  $\text{Na}^+$  and  $\text{Cl}^-$  gradients, that drive ions and fluid secretion from the lung interstitium and pulmonary circulation into the airspaces [5]. AFS is driven by transepithelial  $\text{Cl}^-$  transport [4]. The basolateral expressed NKCC1 and the apical CFTR involve in the pathogenesis of pulmonary edema in ALI [4]. The direction of  $\text{Cl}^-$  flux by CFTR may reverse depending on electrochemical gradients, thus turning absorptive into a secretory epithelium [4]. Therefore, AFS is driven by a transepithelial  $\text{Cl}^-$  transport that is facilitated by basolateral uptake of  $\text{Cl}^-$  with  $\text{Na}^+$  by NKCC1 and apical  $\text{Cl}^-$  extrusion by CFTR [4].

### Regulation of AFC and AFS during normal lung condition and acute lung injury

The regulation of AFC and AFS is shown in Figure 1. In the normal lung condition, ion transporters regulate AFC and AFS, maintain fluid balance and optimal gas exchange [2]. AFC constantly moves fluid from the alveolar space across the epithelial barrier into the interstitial space and pulmonary circulation. The force drives ions and water from the alveolar into the interstitial space by ENaC and Na-K ATPase [6].

During ALI, dysregulation of ion transporters leads to impaired AFC or increased AFS, which results in abnormal accumulation of fluid in the alveolar spaces and subsequent pulmonary edema. Apical CFTR and basolateral NKCC1 generate an electrochemical gradient for  $\text{Na}^+$  influx, thereby driving fluid into the alveolar space that promotes the formation of lung edema [4]. Previous studies have shown that mice with higher NKCC1 expression exhibited impaired AFC and more severe water accumulation in the lungs after ischemia-reperfusion lung injury [5]. Conversely, mice with lower expression levels of NKCC1 showed relatively normal AFC and less accumulation of water in the lungs [5]. Because NKCC1 is important in ALI, we are here to review the roles of NKCC1 in ALI.



**Figure 1:** Ion transporters regulate alveolar fluid transport. (a) In the normal lung condition, AFC constantly moves fluid from the alveolar space across the epithelial barrier into the interstitial space and keeps optimal gas exchange. Apical ENaC and basolateral Na-K ATPase drive ion and water from the alveoli into the interstitial space (b) during ALI, dysregulation of ion transporters leads to impaired AFC or increased AFS, which results in abnormal accumulation of fluid in the alveolar spaces. Apical CFTR and basolateral NKCC1 generate an electrochemical gradient for  $\text{Na}^+$  influx, thereby driving fluid into the alveolar space that promotes the formation of lung edema. AFC: Alveolar fluid clearance; AFS: Alveolar fluid secretion; ALI: Acute lung injury; CFTR: Cystic fibrosis transmembrane conductance regulator; ENaC: Epithelial sodium channel; Na-K ATPase: Sodium/potassium ATPase pump; NKCC1, sodium-potassium-chloride co-transporter isoform

### FUNCTION OF SODIUM-POTASSIUM-CHLORIDE CO-TRANSPORTER ISOFORM 1

NKCC belongs to the superfamily of 12 membrane-spanning cation-chloride cotransporters [7]. It mediates the electroneutral transport of ions and water across the cell membrane [7]. Two NKCC isoforms have been identified. NKCC1 is present in epithelial and nonepithelial cells in a wide variety of organs. On the other hand, the occurrence of NKCC isoform 2 is restricted to the ascending limbs of kidneys. Therefore, lung tissues only have NKCC1 [7].

NKCC1 is located at the basolateral surface of the lung epithelium. NKCC1 transports ions in the stoichiometric ratio of 1  $\text{Na}^+$ :1  $\text{K}^+$ :2  $\text{Cl}^-$  in an electrically neutral manner and is accompanied by water transport that mediates the flow of these ions into cells [7]. NKCC1, therefore, regulates ions and water influx into the alveolar space and is important in the regulation of alveolar fluid in ALI.

## SODIUM-POTASSIUM-CHLORIDE CO-TRANSPORTER ISOFORM 1 IN EPITHELIAL CELL INTEGRITY

The alveolar epithelium forms an interface with the external environment and maintains a continuous surface for optimum gas exchange [8]. Pulmonary vascular permeability is mainly determined by the epithelial and endothelial barriers [9]. The intactness of these barriers prevents the leakage of protein-rich fluid from capillaries into the alveolar spaces [10].

Epithelial and endothelial barriers and pulmonary microvascular permeability are important determinants in ALI pathophysiology. ALI often involves epithelial basement membrane destruction, epithelial cell necrosis, and damage to epithelial and endothelial barriers [8]. The destruction of epithelial and endothelial barriers leads to pulmonary microvascular hyperpermeability with protein and fluid retention in the alveolar space [8]. In addition, the destruction of epithelial and endothelial barriers further results in increased neutrophilic migration into lung tissues and leads to lung damage [11].

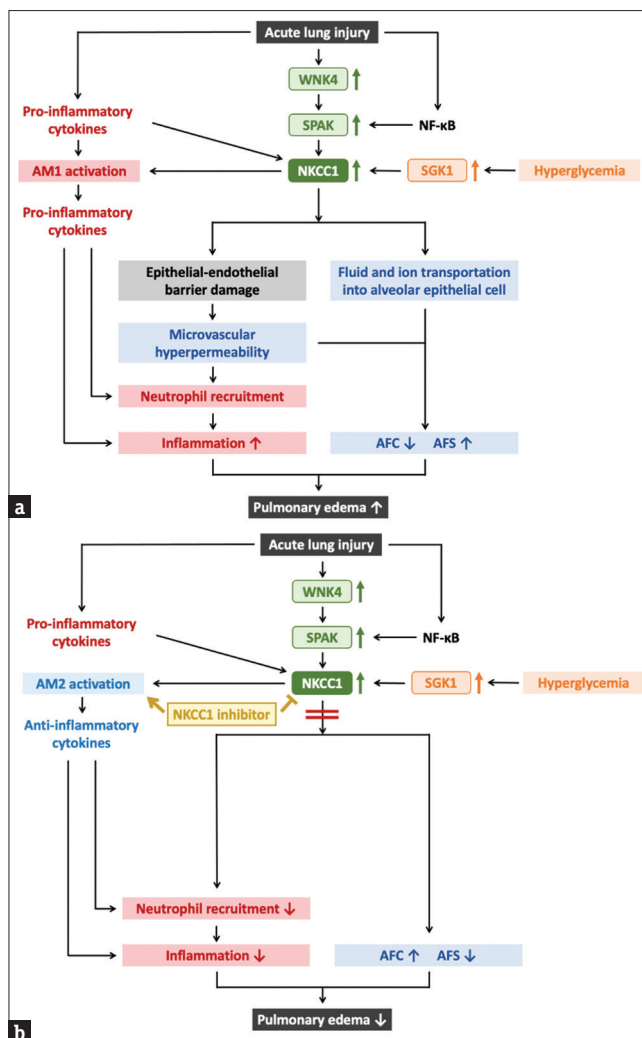
NKCC1 is believed to regulate the endothelial and epithelial barriers and therefore, influence pulmonary microvascular permeability [12]. NKCC1 regulates the volume of alveolar epithelial and endothelial cells [12]. The activation of NKCC1 causes swelling of epithelial and endothelial cells, which may damage intercellular gaps [12]. This process damages the integrity of endothelial and epithelial barriers and results in pulmonary microvascular hyperpermeability and pulmonary edema.

A previous study showed that pulmonary microvascular permeability and alveolar protein leakage were more prominent in mice with higher NKCC1 expression in ALI conditions [13]. Mice with lower NKCC1 expression or those treated with NKCC1 inhibitors displayed lower pulmonary microvascular permeability and alveolar protein leakage in ischemia-reperfusion lung injury [13]. Another study suggested that lipopolysaccharides (LPS) activate NKCC1 in the pulmonary epithelium and endothelium, resulting in the destruction of the epithelial and endothelial barriers [2]. Hsieh *et al.* recently revealed that ALI conditions are characterized by higher NKCC1 expression levels and loss of integrity of alveolar epithelial cells in pulmonary air emboli-induced lung injury [14]. These authors also demonstrated pulmonary microvascular hyperpermeability, pulmonary edema, alveolar protein leakage, and neutrophilic infiltration in rats with high NKCC1 levels [14].

## SODIUM-POTASSIUM-CHLORIDE CO-TRANSPORTER ISOFORM 1 IN INFLAMMATORY RESPONSES

### Sodium-potassium-chloride co-transporter isoform 1 and cytokines

The mechanism of NKCC1 regulating inflammation is shown in Figure 2. Proinflammatory cytokines are important in the pathogenesis of ALI [15]. Here, we discuss the influence of NKCC1 on the expression of proinflammatory cytokines.



**Figure 2:** NKCC1 regulates inflammatory responses. (a) During ALI, NKCC1 is activated by the WNK4-SPAK kinase pathway and proinflammatory cytokines. In hyperglycemic conditions, SGK1 is activated, which triggers the expression of NKCC1. Activation of NKCC1 results in alveolar fluid flooding and causes inflammatory cell swelling, cell rupture, and release of proinflammatory cytokines. Activation of NKCC1 also increases cell volume of macrophages and increases proinflammatory cytokine production. The proinflammatory cytokine further recruit neutrophils into the alveoli and cause damage of lung tissues. (b) Administration of a NKCC1 inhibitor can inhibit both WNK4-SPAK-NKCC1 and SGK1-NKCC1 pathways and attenuate ALI by decreasing pulmonary edema, proinflammatory cytokine expression, and infiltration by inflammatory cells. AFC: Alveolar fluid clearance; AFS: Alveolar fluid secretion; ALI: Acute lung injury; NKCC1, sodium-potassium-chloride co-transporter isoform 1; OSR1: Oxidative stress responsive-1; SGK1: Serum-glucocorticoid kinase 1; SPAK: STE20/SPS1-related proline/alanine rich kinase; WNK: With-no-lysine kinase

NKCC1 is known to regulate the flow of  $\text{Cl}^-$  and water into cells and thereby influence intracellular fluid retention, cell volume, and cell shape [16]. A previous study revealed that activation of NKCC1 leads to dysregulation of cellular fluid transport and causes inflammatory cell swelling [17,18]. Cell swelling leads to cellular membrane rupture and the release of inflammatory mediators and proinflammatory cytokines [19]. Therefore, increased NKCC1 expression results in cell swelling, rupture, and subsequent release of pro-inflammatory cytokines.

### **Sodium-potassium-chloride co-transporter isoform 1 and alveolar macrophages**

Alveolar macrophages (AMs) are one of the major types of inflammatory cells involved in ALI [20]. There are two kinds of AMs, namely proinflammatory/cytotoxic macrophages (M1) and anti-inflammatory/wound repair macrophages (M2) [20]. AM1 produces and secretes proinflammatory cytokines, including interleukin-1 $\beta$  (IL-1 $\beta$ ), IL-6, IL-8, and tumor necrosis factor- $\alpha$  [20]. These cytokines recruit neutrophils and monocytes into the lungs and cause further damage to the lungs. On the other hand, AM2 secretes anti-inflammatory cytokines, including IL-4, IL-10, and IL-13, which accelerate the reversal of inflammation [20]. There is a balance between AM1 and AM2 under normal circumstances. In ALI conditions, AM1 is preferentially activated and triggers the inflammatory response, which leads to further lung damage [20].

Wu *et al.* recently investigated the influence of NKCC1 on the balance of AM1 and AM2 [21]. They revealed that the upregulation of NKCC1 expression leads to the activation of AM1 and proinflammatory cytokines and suppression of AM2. Treatment with a NKCC1 inhibitor reduced AM1 and proinflammatory cytokine expression and increased AM2 levels. Recently, Hung *et al.* also reported the expression of NKCC1 on AMs [16]. The authors showed that LPS-induced activation of NKCC1 resulted in increased cell volume [16]. This increase in cell volume of AM1 further activated AM1 and resulted in increased production of proinflammatory cytokines. The NKCC1 inhibitor decreased the cell volume of AMs and decreased the production of proinflammatory cytokines [16]. This study provided novel evidence that NKCC1 plays a role as an amplifier of LPS-induced macrophage functions.

Taken together, NKCC1 plays a critical role in accelerating the inflammation cascade. NKCC1 increases proinflammatory cytokines by two mechanisms. The first mode involves the activation of NKCC1, which leads to cell swelling, rupture, and release of proinflammatory cytokines; whereas the other is associated with increasing cell volume, which activates AMs and increases the production of proinflammatory cytokines.

### **Sodium-potassium-chloride co-transporter isoform 1 and neutrophils**

The influx of neutrophils into the lungs is an important inflammatory response in ALI [22]. Nuclear factor  $\kappa$  B (NF- $\kappa$ B) activation leads to increased expression of proinflammatory cytokines. The chemotactic effect of proinflammatory cytokines, in turn, induces neutrophil migration into the alveoli. In addition, neutrophils degranulate and release toxic products, cytotoxic enzymes, and free radicals into the lungs [22]. The accumulation of neutrophils in lung tissues, therefore, aggravates lung injury.

Previously, NKCC1 was proposed to be involved in neutrophil transmigration [18]. Many previous studies have demonstrated that animals (rats and mice) with higher expression of NKCC1 exhibited greater neutrophilic infiltration in lung tissues during ALI [13,21]. The degree of neutrophil sequestration was less severe in animals with lower NKCC1 levels or could be markedly attenuated by treatment with an

NKCC1 inhibitor [13,21]. However, the direct relationship between NKCC1 and neutrophils remains unknown.

### **REGULATION OF SODIUM-POTASSIUM-CHLORIDE CO-TRANSPORTER ISOFORM 1**

Various signaling pathways, as discussed below, have been known to regulate NKCC1 activation and expression.

#### **Regulation of sodium-potassium-chloride co-transporter isoform 1 by hyperosmotic stress**

NKCC1 is important to maintain cell shape and integrity during active salt and water secretion [2]. Therefore, activation of NKCC1 is strictly regulated by hyperosmotic stress, low intracellular Na<sup>+</sup> level, increase in intracellular cAMP, and changes in cell shape [2]. Liedtke and Cole also suggested that hyperosmotic stress activates NKCC1 and this activation is regulated by PKC-delta and extracellular-signal-regulated kinase (ERK) [23]. The activation of NKCC1 drives water and salt into the cell. Therefore, NKCC1 has important function in maintaining cellular homeostatic fluid status [2,23].

#### **Regulation of sodium-potassium-chloride co-transporter isoform 1 by WNK pathway**

The activity of NKCC1 is regulated by signaling cascades of with-no-lysine kinase (WNK), oxidative stress-responsive kinase-1 (OSR1), and STE20/SPS1-related proline/alanine-rich kinase (SPAK) [7]. SPAK and OSR1 are downstream substrates of WNK4 kinases, and the activation of WNK4 is known to trigger the OSR1/SPAK phosphorylation cascade [7]. In addition, SPAK and OSR1 are upstream regulators of NKCC1, which activate NKCC1 by phosphorylation at key conserved threonine residues within the NH<sub>2</sub>-terminal domain [7]. Therefore, the WNK-SPAK/OSR1 kinase pathway is important in the regulation of NKCC1. Lan *et al.* showed that WNK4 knockin (WNK4<sup>D561A/+</sup>) mice had higher NKCC1 expression and more pulmonary microvascular permeability, lung edema, and inflammation in ALI [13]. They also showed that SPAK knockout (SPAK<sup>-/-</sup>) mice exhibited lower NKCC1 expression and had less severe lung inflammation, pulmonary microvascular permeability, and pulmonary edema.

#### **Regulation of sodium-potassium-chloride co-transporter isoform 1 by cytokines**

Previously, we have reported the regulation of proinflammatory cytokine expression by NKCC1 [13]. However, pro-inflammatory cytokines have also been reported to regulate the transport of ions and AFC [2]. Previously, the mechanisms underlying cytokine-mediated regulation of ion transport and AFC was unknown. Recently, the c-Jun N-terminal kinase (JNK), p38, and mitogen-activated protein kinases/ERK pathways, known to regulate NKCC1 activity [2], are be activated by proinflammatory cytokines [2]. Therefore, proinflammatory cytokines could regulate the expression of NKCC1 by activation of these pathways.

At the early stages of lung injury, AMs and neutrophils produce proinflammatory cytokines [2]. These cytokines bind

to receptors, upregulate JNK-and p38-dependent pathways, and activate the expression of NKCC1 in alveolar epithelial cells [2]. NF- $\kappa$ B also plays an important role in SPAK regulation [24]. NF- $\kappa$ B activates SPAK via an NF- $\kappa$ B binding site in the 5'-flanking region of the SPAK gene [24]. During ALI, the NF- $\kappa$ B-SPAK pathway is activated and leads to activation of SPAK and NKCC1 [24].

### Regulation of sodium-potassium-chloride co-transporter isoform 1 by serum/glucocorticoid-inducible kinase 1 pathway during acute hyperglycemia

Acute hyperglycemia often occurs in severe sepsis and other systemic inflammatory response syndromes and is associated with exacerbating lung injury [25]. Hyperglycemia is known to activate the pathway of serum/glucocorticoid-inducible kinase 1 (SGK1) [26]. The expression of SGK1 further augments activation of NKCC1 during acute hyperglycemia [21].

Wu *et al.* studied acute hyperglycemia and NKCC1 in ALI. They revealed that rats with acute hyperglycemia exhibited higher NKCC1 expression and more severe ALI, accompanied by hypoxemia, pulmonary edema, pulmonary hypertension, alveolar protein leakage, pro-inflammatory cytokine levels and neutrophils infiltration [21]. They found that during ALI, the WNK4-SPAK-NKCC1 pathway was activated, while acute hyperglycemia also activated the SGK1-NKCC1 pathway [21]. Therefore, WNK4-SPAK-NKCC1 and SGK1-NKCC1 were concurrently activated when acute hyperglycemia is combined with ALI. Activation of these two pathways lead to higher expression of NKCC1, and lung injury was, therefore, more severe. The administration of NKCC1 inhibitors inhibited both the WNK4-SPAK-NKCC1 and SGK1-NKCC1 pathways, thus reduced the severity of acute hyperglycemia combined with ALI [21].

### SODIUM-POTASSIUM-CHLORIDE CO-TRANSPORTER ISOFORM 1 IN DIFFERENT TYPES OF ACUTE LUNG INJURY

There are many causes of ALI such as sepsis, ischemia-reperfusion injury, ventilation-induced injury, and pulmonary air emboli. Sepsis-related lung injury due to infection is common in ICUs and contributes highly to patient mortality and morbidity [1]. Some invasive iatrogenic procedures may cause air embolism in the lungs and when the air emboli exceed the absorption capacity of the lungs, it causes pulmonary air emboli-induced ALI [14]. Ischemia-reperfusion-induced ALI occurs when the lungs are exposed to periods of ischemia and reperfusion [13]. Clinically, ischemia-reperfusion-induced ALI occurs in cardiopulmonary bypass, lung transplantation, and other surgical procedures [27].

There are some studies on NKCC1 in different kinds of ALI. In a study of air emboli-induced ALI, NKCC1 expression was significantly increased in rats with air embolism, along with an increase in pulmonary edema, neutrophilic infiltration, hyperpermeability, and expression of proinflammatory cytokines [28]. Similar findings were noted in hyperoxia [29], ischemia-reperfusion [13], and ventilator-induced [21] ALI. In

all these studies, treatment with an NKCC1 inhibitor decreased the expression of NKCC1 and decreased pulmonary edema, hyperpermeability, neutrophilic infiltration, and expression of proinflammatory cytokines [13,21,28]. Hung *et al.* studied the role of NKCC1 in AMs in a cellular model of sepsis and found that NKCC1 was activated by LPS, which led to increased cell volume of AMs and increased proinflammatory cytokine expression [16]. These authors also suggested that NKCC1 inhibitors could reduce the cell volume of AMs and decrease the levels of proinflammatory cytokines.

### CLINICAL IMPLICATIONS

ALI has a great impact in the mortality and morbidity rates associated with ICUs [1]. Alveolar inflammation and fluid flooding are important in ALI pathogenesis [2]. NKCC1 regulates alveolar inflammation and fluid transportation, and NKCC1 activation results in lung inflammation and pulmonary edema during ALI [5]. Therefore, manipulating the expression and biological activity of NKCC1 may be a way to treat ALI [13].

Conventionally, NKCC1 inhibitors are used as diuretics due to their effect on the loop of Henle in the kidneys. In this review, we suggest that NKCC1 can modulate alveolar fluid and inflammation in the lungs. In a clinical scenario, it is difficult to differentiate whether NKCC1 impacts the kidneys (as diuretics) or lungs. However, Lan *et al.* investigated the influence of NKCC1 in a model of isolated perfused lung, which excludes the effect of NKCC1 in the kidneys [13]. This study clearly highlighted the effect of NKCC1 inhibitors in the lungs and establish the suitability of NKCC1 inhibitors in attenuating lung injury by the reduction of alveolar water and inflammation. These results, therefore, provide an impetus to address NKCC1 inhibitors in ALI treatment in a clinical setting.

### Limitations

There are some limitations to the studies discussed here. First, most of these investigations were performed in animals or cells. Studies on NKCC1 in human beings are quite limited. Clinical studies on the roles of NKCC1 and the effects of NKCC1 inhibitors in humans are, therefore, warranted. Secondly, most of these experiments were conducted in early ALI (a few hours within ALI induction). The influence of NKCC1 on long-term outcomes, such as post-ALI fibrosis, should also be confirmed.

### CONCLUSIONS

ALI is characterized by pulmonary edema, pulmonary microvascular hyperpermeability, lung inflammation, and expression of proinflammatory cytokines. AFS and AFC are important in the formation of pulmonary edema during ALI. NKCC1 regulates  $\text{Cl}^-$ ,  $\text{Na}^+$ , and  $\text{K}^+$  flux with concurrent water transport in alveolar epithelial cells. NKCC1, therefore, is crucial in regulating alveolar fluid. In addition, it regulates intracellular volume and activation of AMs, thereby modulating proinflammatory cytokine production. Activation of NKCC1 causes increased production of proinflammatory cytokines, which further recruit inflammatory cells (such as

neutrophils) into the alveoli and cause further damage to the lungs. Recent studies have identified the novel contributions of NKCC1 in the pathogenesis of ALI. Manipulation of NKCC1 manipulation may, therefore, emerge as a new direction for the treatment of ALI.

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### Conflicts of interest

There are no conflicts of interest.

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