# RECIPROCAL REGULATION OF LUNG INJURY AND RESOLUTION BY THE AKT-FOXO SIGNALING PATHWAY IN ACUTE RESPIRATORY DISTRESS SYNDROME

BY

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## ABSTRACT

Acute Respiratory Distress Syndrome (ARDS) is a fatal illness with high mortality rate of around 40% and is characterized by increased pulmonary vascular permeability resulting in inflammatory lung injury, loss of aerated lung tissue and severe hypoxia. Disruption of capillary endothelial- and alveolar epithelial-barrier marks the exudative phase of ARDS. Fate of an injured lung can either progress towards injury resolution or severe fibroproliferation leading to irreversible damage to the lungs. The objective of this project is to provide stage-specific therapeutic targets to prevent pulmonary edema in the exudative phase and enhance injury resolution in the fibrotic phase of ARDS. Using LPS induced lung injury model *in vivo* and LPS treatment on lung endothelial cells *in vitro*, we showed that LPS inhibits endothelial Akt and thus activates FoxO1/3a leading to the loss of tight-junction protein claudin5 expression and enhanced MMP3 expression/activity. Targeting FoxO1/3a and MMP3 protected from LPS-induced lung injury. Interestingly, we observed that lung MMP3 activity correlates with extent of lung injury. To further confirm the utility of MMP3 activity as a diagnostic marker for ARDS, MMP3 activity in human serum & plasma samples from ARDS patients were compared with controls. We found ~3-5-fold increase in MMP3 activity in ARDS patients. On the other end, regulatory T-cell (Treg)-mediated resolution of lung injury and prevention of fibroproliferation has been reported. However, molecular mechanisms to enhance quantity of Tregs after lung injury are yet to be explored. Here we show the importance

of inhibiting Akt, 48 hours after lung injury, to enhance FoxOs mediated increase in FoxP3 expressing Tregs and injury resolution. Together, these results indicate that endothelial Akt activity inhibition in the exudative stage is deleterious as it enhances pulmonary edema, while the T-cell Akt activity inhibition in fibrotic phase is beneficial as it enhances Treg-mediated resolution of lung injury. Overall, FoxOs and MMP3 could be potential therapeutic targets in high risk patients to prevent exudation and pulmonary edema while Akt could be an important target to enhance resolution during fibrotic phase of ARDS.

**INDEX WORDS:** ARDS; Akt; FoxO1/3a; MMP3; Claudin5; Regulatory T-cells; Inflammation; endothelium.

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## DEDICATION

I dedicate this thesis to my parents. My ever loving, caring and encouraging mother Indira Rani has supported me throughout my student life and continues to be my strength. My dad has always encouraged me and motivated me to achieve my goals. I would like to thank my parents for the immense support and this would not be possible if not for them. My brother and sister have also played an important role in this success and I would like to thank them for all their well wishes, support and prayers.

I love my family and this thesis is a tribute to them.

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## TABLE OF CONTENTS

# CHAPTER 1

# INTRODUCTION AND LITERATURE REVIEW

**1.1. Acute Respiratory Distress Syndrome (ARDS) / Acute Lung Injury (ALI) Definition and Diagnosis:** Acute respiratory distress syndrome and acute lung injury were first described in 1967 by Ashbaugh and colleagues. They described 12 patients with acute respiratory distress, cyanosis refractory to oxygen therapy, decreased lung compliance, and diffuse infiltrates evident on the chest radiograph along with hyaline membrane formation. Due to these findings previously thought to be specific for the respiratory distress syndrome of the newborn, this condition was defined as adult respiratory distress syndrome [1]. Since it also occurs in children, it was later changed to Acute Respiratory distress syndrome. In 1988, an expanded definition was proposed which used a four-point lung-injury scoring system that quantified the physiologic respiratory impairment and this was based on the level of positive end-expiratory pressure, the ratio of the partial pressure of arterial oxygen to the fraction of inspired oxygen, the static lung compliance, and the degree of infiltration evident on chest radiographs [2]. Although the lung-injury scoring system has been widely used to quantify the severity of lung injury in both clinical research and clinical trials, it cannot be used to predict the outcome during the first 24 to 72 hours after the onset of the acute respiratory distress syndrome and thus has limited clinical usefulness [3].

In 1994, the American–European Consensus Conference Committee (AECC) gave a standard definition for ARDS/ALI that was used in several clinical trials as well as observational clinical studies for over 15 years until the 2011 Berlin definition. AECC definition included:

1. Patients with bilateral infiltrates and less severe hypoxemia (as defined by a ratio of the partial pressure of arterial oxygen to the fraction of inspired oxygen [PaO2/FIO2] of 300 or less) are considered to have ALI,
2. and those with bilateral infiltrates and more severe hypoxemia (as defined by a ratio of 200 or less) are considered to have the ARDS [1, 4].

Since the AECC definition of ARDS in 1994, issues regarding the reliability and validity of this definition have emerged. Some of these include lack of explicit criteria for defining acute lung injury, sensitivity of PaO2/FIO2 to different ventilator settings, poor reliability of the chest radiograph criterion, and difficulties distinguishing hydrostatic edema. In order to address these issues, using a consensus process, a panel of experts convened in 2011 (an initiative of the European Society of Intensive Care Medicine endorsed by the American Thoracic Society and the Society of Critical Care Medicine) and developed the Berlin Definition.

**The Berlin Definition:** This definition proposed 3 mutually exclusive categories of ARDS based on degree of hypoxemia:

1. mild (200 mm Hg $< PaO_2/FiO_2 \leq$ 300 mm Hg),
2. moderate (100 mm Hg $< PaO_2/FiO_2 \leq$ 200 mm Hg), and
3. severe ($PaO_2/FiO_2 \leq$ 100 mm Hg)

And 4 ancillary variables for severe ARDS: radiographic severity, respiratory system compliance (≤ 40 mL/cm H2O), positive end expiratory pressure (≥ 10 cm H2O) and corrected expired volume per minute (≥10 L/min). The term acute lung injury as defined by the AECC was removed, due to the perception that clinicians were misusing this term to refer to a subset of patients with less severe hypoxemia rather than its intended use as an inclusive term for all patients with the syndrome. Berlin definition had better predictive validity for mortality, with an

area under the receiver operating curve of 0.577 (95% CI, 0.561-0.593) vs 0.536 (95% CI, 0.520-0.553; P<.001) [5].

**Diagnosis:** A lack of a specific biomarker that can assist in the ARDS diagnosis in clinic has been a major hindrance.

a. One of the first diagnostic efforts in ARDS is to identify potential pathogens responsible for disease. Initial assessment should include: blood cultures, urinary antigen testing for Legionella pneumophila and Streptococcus pneumoniae, serologic tests for Mycoplasma pneumoniae and Chlamydia pneumoniae, and microbial sampling of the lung, along with BAL analysis for the presence of pathogens.
b. Pulmonary CT scan showing morphological patterns such as consolidated regions, ground glass regions and normally aerated regions of lungs can clarify the underlying pathophysiology. While Pulmonary ARDS is characterized by similar amounts of consolidated and ground glass areas, extrapulmonary ARDS have a higher amount of ground glass areas. CT scans are also used to evaluate the extent of lung recruitability.
c. Ultrasonographic artifacts such as the presence of B-line artifact are pivotal findings in patients with respiratory failure. The finding of three or more B-lines in one intercostal space is considered abnormal and is referred to as a B-pattern. In comparison to cardiogenic edema, ARDS presents a non-homogeneous distribution of B-pattern, C (consolidative) pattern and pleural line abnormalities[6-8].

**1.2. Pathophysiology and Therapeutic Targets:** Alveolar epithelial-endothelial capillary barrier is crucial for physiological function of lungs and damage to this barrier symbolizes the pathological progression into exudative phase of ARD/ALI. Gaseous exchange occurs through this barrier which is composed of alveolar epithelium and underlying capillary endothelium fused by basal lamina [9]. The slightly thicker part of this blood-air barrier is also composed of extracellular matrix (ECM) between the epithelial and endothelial cells and is responsible for

liquid and solute exchange [10]. Endothelial injury and consequent enhanced vascular permeability ensuing influx of protein rich edema fluid into alveolar air spaces is a well-established pathological event occurring in the acute/exudative stage of ARDS [1]. Therefore, targeting disruption of capillary endothelial barrier could provide a potential therapy for ARDS.

Endothelial barrier regulates transport of fluids, inflammatory cells, proteins, etc. through paracellular and transcellular pathways. Paracellular pathway plays a prominent role in pathological inflammation and vascular leak [11, 12]. Endothelial paracellular barrier function is largely regulated by tight junctional proteins and claudin-5 is the predominant tight junction protein found in pulmonary microcirculation and the endothelium [13, 14]. Pulmonary endothelium expresses predominantly greater extents of claudin-5 than the alveolar epithelium. Decreased claudin-5 expression is associated with more severe lung injury because of enhanced vascular permeability and lungs of human patients with pneumonia exhibit reduced claudin-5 expression [14, 15]. Claudin-5 deficient mice also have disrupted blood-brain permeability indicating its importance in regulating vascular permeability [16]. Recent studies have indicated a potential interaction between endothelial tight junctions (TJ) and adherent junction (AJ) proteins in regulation of vascular permeability. In capillaries, TJs are more developed and AJs are more dominant in post-capillary venules [17]. Lung edema results from capillary leak and hence TJs play prominent role.

The critical importance of epithelial injury to both the development of and recovery from the disorder has become better recognized. The extent of alveolar epithelial injury is an important predictor of the outcome. The normal alveolar epithelium is composed of two types of cells which include type I that cover up to 90% of alveolar surface area and are easily injured, while the cuboidal type II cells make up the remaining 10% and these are more resistant to injury[18]. Type I cells form the barrier and the type II cells are involved in surfactant production, ion transport and proliferation and differentiation into type I cells after injury. Epithelial injury in

ARDS contributes to alveolar flooding, disruption of normal epithelial fluid transport impairing edema fluid clearance from alveolar space, decreased surfactant production leading to alveolar collapse and finally uncontrolled epithelial proliferation resulting in fibrosis [1, 19-21].

The second, or proliferative, phase of ARDS initiates repair processes that are essential for host survival. Reabsorption of alveolar edema and restoration of provisional matrix restoring alveolar architecture and function results from restoration of epithelial integrity. The final, or fibrotic, phase of ARDS does not occur in all patients but has been linked to prolonged mechanical ventilation and increased mortality [22].

### 1.3. Therapeutic Management

**Supportive Therapy:** The treatment for ARDS to date remains largely supportive. The priority of care is identifying and treatment of underlying causes, for example sepsis induced ARDS requires early resuscitation, appropriate antibiotic agents and source control. A 30-degree elevation of the head of the bed can help prevent nosocomial pneumonia. Supportive therapy in ARDS is primarily focused on limiting further lung injury and preventing lung edema while promoting edema resorption. This is achieved by a combination of lung protective ventilation along with conservative fluid therapy [22, 23].

*A. Non-Invasive Mechanical Ventilation*

- Reduces the work of breathing and improves gas exchange.
- Advantage of avoiding deep sedation and lowering the risk of nosocomial pneumonia.
- Disadvantages include high risk of failure and possible consequent risk of delaying tracheal intubation and invasive mechanical ventilation.
- Recently introduced high flow nasal cannulae (HFNCs) can deliver high $O_2$ flow through the nose along with sufficient heat and humidity thus improving oxygenation and $CO_2$

clearance while reducing the work of breathing. HFNCs have been shown to have lower intensive care mortality [23].

*B. Invasive Mechanical Ventilation:*

- Goal of mechanical ventilation is to provide sufficient gas exchange by enhancing arterial $O_2$ and removing $CO_2$.
- The effect of mechanical ventilation is twofold; first, it allows for titration of $FiO_2$ (fraction of inspired $O_2$), secondly, during inspiration, it provides sufficient positive pressure to prevent atelectrauma.

*Low Tidal volume ventilation:* Because of reduced volume of aerated lung in ARDS patients, even normal tidal volumes delivered with safe airway pressure for uninjured lungs, might cause regional overdistension (volutrauma), which further worsens injury and enhances inflammation. Clinical practice guidelines recommend mechanical ventilation with low tidal volume and airway pressure.

In the landmark ARDS network trial comparing high tidal volumes vs low tidal volumes, the use of lower tidal volume (tidal volumes are reduced from 6 ml per kilogram of predicted body weight to a minimum of 4 ml per kilogram if plateau airway pressures exceed 30 cm of water) is associated with reduction of 9 percentage points in mortality [24].

*PEEP (Positive end-expiratory pressure) selection:* While a PEEP of at least 5 cm of water is recommended, the optimum method for PEEP adjustment is unclear. Meta-analysis of three randomized trials suggests that mortality is reduced when the PEEP is kept relatively high, as compared with a strategy involving a higher PEEP (a mean initial PEEP of approximately 16 cm of water), in patients with moderate-to-severe ARDS[25]. A rational approach involves minimizing driving pressure (the difference between plateau airway pressure and PEEP) by adjusting PEEP or tidal volume [26, 27].

*Prone Positioning:* For moderate-to-severe ARDS (oxygenation ration = $PaO_2/Fi\ O_2$< 120 mm Hg) mechanical ventilation while the patient is in the prone position is associated with reduced mortality and is currently recommended. Prone positioning reduces ventilation induced injury through combined effect of uniform distribution of ventilation and less compression of the left lower lobe by the heart [28].

***Pharmacological Therapy***

- So far, no pharmacological therapy for ARDS has been shown to reduce either short-term or long-term mortality.
- Inhaled nitric oxide transiently improves oxygenation and may improve long term lung function in those who survive without any benefit on mortality. However, its benefit is limited as it is associated with kidney injury [29].
- Glucocorticoids may hasten radiographic improvement in pneumonia patients and improve oxygenation without any survival benefits. If started 14 days or more after ARDS, they are harmful [30].
- In severe ARDS patients, spontaneous breathing seems to be dangerous as it enhances patient-ventilator synchrony and enhances oxygen consumption due to respiratory muscle hyperactivity. Therefore, neuromuscular blocking agents seem to be beneficial for severe ARDS conditions [31].
- Intravenous delivery of mesenchymal stem cells that interact with injured tissue through the release of multiple soluble bioactive factors is one of the novel therapeutic approaches in early clinical development [32].

## 2. Role of Hypoxia in Lung Injury and Resolution

**2.1 Effect of hypoxia that result due to pathological conditions:** During the early stages of lung injury, hypoxia plays a major role in the progression of lung injury. Interestingly, in chronic

pulmonary pathological conditions, hypoxia enhances injury resolution. Studies indicate a protective and anti-inflammatory role of hypoxia inducible transcription factors such as HIF1A in lung protection during the early exudative phase of lung injury. Hypoxia inactivates Propyl Hydroxylases (PHDs) and stabilizes HIF1A [33-35]. During the acute stage of lung injury, inflammation, including enhanced neutrophil activity within the alveoli, leads to an increased alveolar edema and decreased alveolar gaseous exchange capacity. HIF stabilization has been shown to have anti-inflammatory role in conditions like intestinal inflammation. The protective role of HIF activators in the treatment of inflammatory bowel disease or ischemia and reperfusion injury of several organs has been shown in several studies [36-38]. Interestingly, Eckle T et al. showed the beneficial role of normoxic HIF1A stabilization in lung protection during ALI, where HIF-dependent control of alveolar-epithelial glucose metabolism function as an endogenous feedback loop to dampen lung inflammation. *In vivo* HIF1A increased glycolysis, lactate production and glucose flux rates in alveolar epithelium. Overall, this normoxic stabilization of HIF1A in alveolar epithelium increased glycolytic capacity and TCA flux thus optimizing mitochondrial respiration to enhance ATP production. This HIF dependent protection of mitochondrial function in ALI, not only enhanced ATP production but also concomitantly prevented ROS accumulation and lung inflammation [39]. Hence the role of hypoxia and subsequent HIF stabilization in suppressing inflammation might have a significant role in resolution of acute lung injury.

**2.2 Hypoxia and adenosine signaling in lung injury resolution:** Emigration of PMNs (polymorphonucleotide neutrophils) through the endothelial barrier in an injured lung creates a potential for vascular fluid leakage leading to edema formation and decreased oxygenation. The endothelial adaptations to hypoxia include enhanced extracellular adenosine production during limited oxygen availability. In response to hypoxia, endothelial cells have enhanced expression of surface ecto-nucleotidases, CD39 that converts ATP/ADP to AMP (ecto-apyrase), as well as

CD73 that is involved in phosphohydrolysis of AMP to adenosine thus forming the source for extracellular adenosine [40]. This enhanced extracellular adenosine can then signal through four different G-protein coupled adenosine receptors, all of which are present on vascular endothelia thus enhancing adenosine signaling that is implicated in tissue protection in different models of injury including lung injury. Several studies, notably couple of them from Sean P.colgan and colleagues have shown the role of extracellular adenosine and its signaling in attenuating hypoxia induced vascular leakage. They also showed that the source of ATP in hypoxic milieu are the PMNs (polymorphonucleotide neutrophils). Hypoxia induces the production of ATP by PMNs, however the exact mechanism by which ATP is produced still needs to be explored. This ATP is then phosphohydrolysed as mentioned above to produce extracellular adenosine [41]. Enhanced adenosine concentrations activate adenosine receptor, ($AdoRA_{2A}/A_{2B}$) on endothelial cells, which when activated increases intracellular cyclic AMP (cAMP) and activates protein kinase A (PKA) to induce resealing of the endothelial barrier. The resealing of endothelial barrier during PMN transmigration was obviated by inhibition of cAMP formation. This resealing effect is mediated by PKA-induced phosphorylation of vasodilator-stimulated phosphoprotein (VASP), a protein responsible for changes in the geometry of actin filaments and distribution of junctional proteins as a result affecting the characteristics of junctional proteins and increasing barrier function. Intriguingly, adenosine not only activates the endothelial $A_{2B}$ receptor, but also neutrophil $A_2$ adenosine receptor which has been shown to play an important role in limitation and termination of PMN mediated systemic inflammatory responses. Few others have also demonstrated that PMN $A_2$ adenosine receptor stimulation decreased leukocyte adherence and transmigration which might contribute to attenuated vascular leak associated with leukocyte accumulation [41-43]. Thus, hypoxia induced adenosine signaling in vascular endothelial cells and PMNs contributes to decreased vascular leak and inflammation, both of which are beneficial in inflammatory conditions such as ALI (Fig 1).

When adenosine signaling was inhibited in transgenic mice with targeted disruption of Cd73 that were subjected to hypoxia, fulminant vascular leakage, associated with severe edema and inflammation was seen. Recently, studies have shown three other mechanisms by which hypoxia enhances extracellular adenosine levels, including hypoxia-mediated repression of the equilibrative nucleoside transporters (ENT-1 and ENT-2) which are responsible for adenosine transport across the membrane into the cytoplasm; HIF-1α mediated inhibition of intracellular adenosine kinase that converts intracellular adenosine to AMP; and transcriptional induction of $AdoRA_{2B}$ receptor. These studies indicate the protective role of adenosine signaling during hypoxia especially in pulmonary tissue. On the other hand, chronically increased adenosine levels are detrimental as seen in pathological conditions like asthma and chronic obstructive pulmonary disease (COPD) and they also correlate with degree of inflammation in COPD. In order to regulate excessive adenosine signaling, chronic exposure to hypoxia eventually induces endothelial CD26 and extracellular adenosine deaminase (ADA). CD26 on endothelial surface acts as the ADA-complexing protein and localizes ADA accumulation on endothelial surface limiting extracellular adenosine accumulation during prolonged hypoxia [44-46].

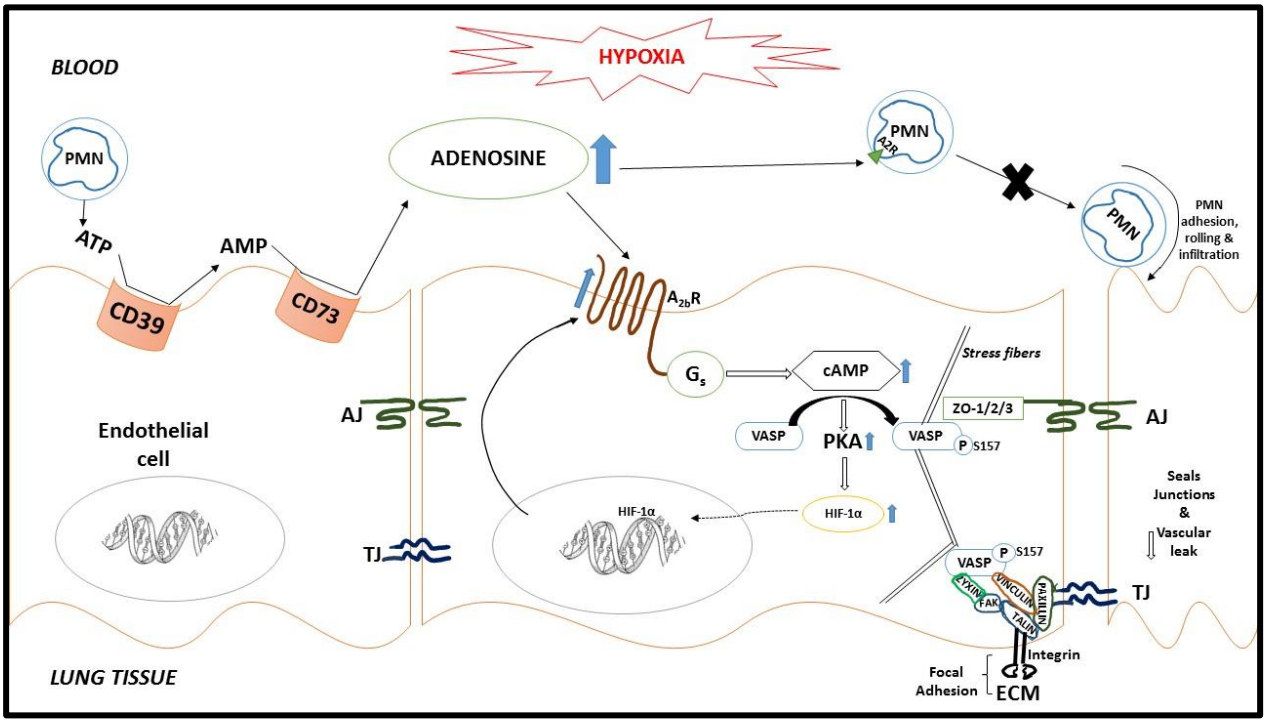


***Figure 1.1: Hypoxia and adenosine signaling in the lungs.*** *Hypoxia induced extracellular adenosine production acts through adenosine receptors on endothelial cells to enhance intracellular cAMP and PKA production. PKA catalyzes the phosphorylation of VASP, which integrates into stress fibers and help seal the endothelial barrier by enhancing expression of AJs, TJs and also focal adhesion. PKA also enhances HIF-1A expression, which translocate into nucleus and enhances adenosine receptor transcription. Extracellular adenosine also acts on A2-receptors on PMNs and prevents their adhesion, rolling and infiltration into lung tissue. Thus, hypoxia induced extracellular adenosine seals endothelial junctions, prevents PMN infiltration and protects lung tissue by preventing alveolar edema accumulation. PMN-Polymorphonuclear Neutrophils; ATP- Adenosine tri-phosphate; AMP-Adenosine mono-phosphate; $A_{2b}$R-Adenosine 2b receptor; cAMP-cyclic AMP; VASP- vasodilator-stimulated phosphoprotein; AJ-Adherent junction; TJ-Tight Junction; ECM-Extracellular matrix. (Adopted from: Sandeep Artham and Payaningal R. Somanath. "Stage specific effects of hypoxia on interstitial lung disease". Hypoxia by InTech Publishers [2017])*

### 2.3 Hypoxia and lung Inflammation

Uncontrolled inflammation is one of the major players in lung injury and suppression of inflammation is beneficial for injury resolution. Interestingly, as mentioned above, hypoxia induced HIF-1 mediated enhanced expression of adenosine $A_2$ receptor on different types of immune cells, along with enhanced extracellular adenosine levels which activate these receptors, are responsible for anti-inflammatory and tissue protecting effects of hypoxia. This anti-inflammatory effect is attributed to elevated intracellular cAMP levels via activation of adenylyl cyclase. Even pharmacological immunosuppressive molecules such as catecholamines, neuropeptides, histamine, and prostaglandins are known to have their effects through elevation of cAMP levels. Therefore, this extracellular adenosine serves to report excessive collateral immune damage and prevents further damage by suppressing activated

immune cells. Adenosine triggers high-affinity $A_{2A}$ adenosine receptors on activated immune cells resulting in enhanced intracellular cAMP levels to suppress these immune cells. Few studies also show that hypoxia inhibits adenosine kinase, an enzyme responsible for re-phosphorylation of adenosine to AMP, in order to maximize the anti-inflammatory effect.

## 3. Mechanisms of Pulmonary endothelial barrier disruption in ARDS: What do we know so far?

### 3.1 Pulmonary endothelial barrier permeability

The coefficient of vascular permeability ($K_{fc}$) across the endothelium in isolated perfused lung preparations under basal conditions in experimental animals showed a gradation of permeability across the microvascular bed, with the microvascular site constituting approximately 42%, the arterial region approximately 19%, and the venous area approximately 37% indicating that arterial system was more restrictive [47]. However, the endothelial cells of pulmonary microvessels form a monolayer that is approximately 4 times less permeable to albumin compared with the confluent monolayer of cells from venous or arterial regions. The transendothelial electrical resistance (TER) was also found to be higher in microvascular endothelial cells compared to cells isolated from larger arteries. Studies have shown that the constitution of interendothelial junctions proteins, ECM and cytoskeletal proteins may contribute to this variation in endothelial permeability. The interendothelial junction proteins include adherens junctions (AJs), tight junctions (TJs) and gap junctions (connexins). [48-51].

The genesis and progression of several pathological conditions that involve impairment of endothelial barrier function include pulmonary edema, ischemic stroke, neurodegenerative disorder, angioedema, sepsis and cancer. Among the barrier-forming adhesive structures, the most important are the AJ, gap junctions (GJ), and TJ. AJs mediate endothelial cell-cell contact in all types of blood vessels and is composed mainly of VE-cadherin. VE-cadherin binds to actin

cytoskeleton via the cadherin-catenin binding ( b-catenin, p120 catenin and a-catenin) [52]. In comparison to AJs, TJs are more predominant in small arterioles and localized at the outermost part of interendothelial junctions but are also found to be intermingled with AJs [53, 54].

### 3.2 Disruption of Actomyosin Contractility in ALI

The balance between actomyosin contractile forces and adhesive EC-EC (Endothelial cell) and EC-matrix tethering forces regulate endothelial barrier. Dysregulation of this balance results in paracellular gaps and an increase in permeability.

a. Myosin Light Chain Kinase (MLCK) activation in lung injury: The actomyosin cytoskeletal forces are regulated by the motor activity controlled by phosphorylation of myosin light chain (MLC) at Thr18 and Ser19. MLCK phosphorylates MLC and regulates tension at endothelial barrier junctions. Increased actomyosin contractility drives endothelial barrier dysfunction and vascular leakage [17]. While LPS activates MLCK in ECs and causes EC contraction, LPS induced endothelial hyper-permeability between MYLK1 (responsible for MLCK) KO and WT mice was the same. This finding suggests that MLCK might not be a major player in LPS induced hyper-permeability [55]. However, other studies indirectly imply the correlation between MLCK activation in lung injury with enhanced edema. LPS activates Ang2 and enhances Ang2-Tie2 signaling. Ang2 induced signaling activates MLCK and enhances permeability. Excessive circulating Ang2 has been linked to vascular leak in humans [56, 57]. Therefore, the direct effect of endothelial MLCK activation in lung injury induced edema needs further confirmation.
b. Role of RhoA and ROCK in lung edema after lung injury: RhoA, Rac and Cdc42 comprise the Rho family of small GTPases which are key regulators of endothelial cytoskeletal dynamics and thus effect several vascular processes including endothelial permeability, angiogenesis, cell proliferation, contraction, differentiation and apoptosis[58]. While Rac1 and Cdc42 mediate the assembly, stabilization and maturation of AJs, RhoA mainly

contributes to AJ destabilization and enhanced endothelial permeability. Upon activation of RhoA, it translocases to the plasma membrane where it mediates downstream effectors such as ROCK. ROCK phosphorylates and inhibits MLCP (MLC phosphatase) which is responsible for deactivating MLC. Thus, ROCK activation enhances actomyosin contraction and endothelial barrier dysfunction [17].

The involvement of ROCK in LPS induced pulmonary inflammation as measured by lung wet-dry ratio is prominent as the ROCK inhibitor Y-27632 prevents LPS induced pulmonary edema [59]. Other RhoA signaling downstream components like $pp60^{Src}$ [60], a member of src family kinase (SFK), Protein Kinase C δ [61] as well as upstream components like thrombin [62] have been shown to be activated by LPS and induce endothelial hyper-permeability.

### 3.3 LPS induced calcium influx in Endothelial Barrier Disruption

LPS through TLR4 binding induces calcium influx across the endothelial membrane by activating membrane calcium channels. The transient receptor potential channel (TRPC) superfamily is the largest known cation channel family contributing to calcium entry in the lung endothelium [63]. LPS induced increase in diacylglycerol (DAG, a second messenger in ECs) directly activates TRPC6 in a TLR-4 dependent manner. TRPC6 induced calcium entry enhances intracellular calcium levels, which then activate MLCK resulting in EC actomyosin contractility and lung vascular hyper-permeability [64]. Another sub-family of TRPC, TRPV4 (sub-family vanilloid) activation has been suggested to play a major role in pulmonary vascular collapse and lung edema contributing to acute lung injury. Calcium-activated potassium channels (Kca3.1) are crucial downstream molecular components of TRPV4 that are responsible for TRPV4-induced pulmonary arterial relaxation, fluid extravasation and pulmonary circulatory collapse [65].

### 3.4 LPS induced Apoptosis of Endothelial cells in ALI

Endothelial apoptosis is a crucial factor for pulmonary barrier disruption. Studies have shown that LPS induced endothelial apoptosis causes pulmonary edema. Interestingly, Vascular endothelial growth factor (VEGF), a vascular permeability enhancing agent was shown to attenuate LPS induced extravascular albumin leakage and edema formation by significantly reducing the number of apoptotic endothelial cells as well as attenuating active caspase-3 levels. The beneficial effects of VEGF were attributed to its anti-apoptotic effect on endothelial cells. [56, 66]. LPS induced pulmonary cell apoptosis was also reduced when treated with the anti-inflammatory resolving D1 (RvD1). Mechanistically, RvD1 upregulated the expression of HO-1, which protected from the reduced ZO-1 and occludin expression, thus preventing barrier disruption and apoptosis [67]. The transcription factor responsible for LPS induced initiation of the apoptotic cascade was found to be p38 mitogen-activated protein kinase (MAPK) and its direct downstream effector mitogen activated protein kinase 2 (MK2) [68].

### 3.5 LPS induced tyrosine kinase phosphorylation in ALI

Src family tyrosine protein kinase (SFK) and Focal adhesion kinase (FAK) have been extensively studied in the pulmonary endothelium. Tumor necrosis factor (TNF) α-associated factor (TRAF) 6 and TLR4 interaction leads to SFK activation and loss of barrier integrity in human lung microvascular endothelial cells (HLMVECs). LPS activates SFK and induces tyrosine phosphorylation of VE-cadherin. VE-cadherin internalization and subsequent barrier disruption follows. Studies indicate that 4 members of SFK family, c-Src, Fyn, Yes and Lyn are expressed in the HLMVECs. While silencing c-Src, Fyn and Yes decreased LPS induced VE-cadherin phosphorylation and barrier disruption, this wasn't the case with Lyn [69, 70]. SFKs also effect permeability through regulating focal adhesions. It was found that, unlike other SFKs, Lyn interacts with FAK, phosphorylates FAK and strengthens endothelial junctions [69].

### 3.6 Other factors

LPS acts through TLRs to initiate innate immunity and induce adaptive immune responses by recognizing distinct pathogen-associated molecular patterns. This involves signaling through MyD88 and/or TICAM1/TRIF dependent pathways to activate NF-κB and MAPK pathways, leading to production of inflammatory cytokines. While inhibition of MAPK and NF-κB inflammatory cascade is beneficial to the integrity of the endothelial barrier, studies indicate that vascular ECs do not express the TRIF-dependent gene subset [71]. This questions the involvement of TRIF-mediated signaling in regulation of pulmonary ECs. Further studies are required to confirm this. LPS induced inflammation involves neutrophil migration into the alveoli. Neutrophils are a major source of ROS (reactive oxygen species), which are also implicated in endothelial barrier disruption [72]. (Fig 2)

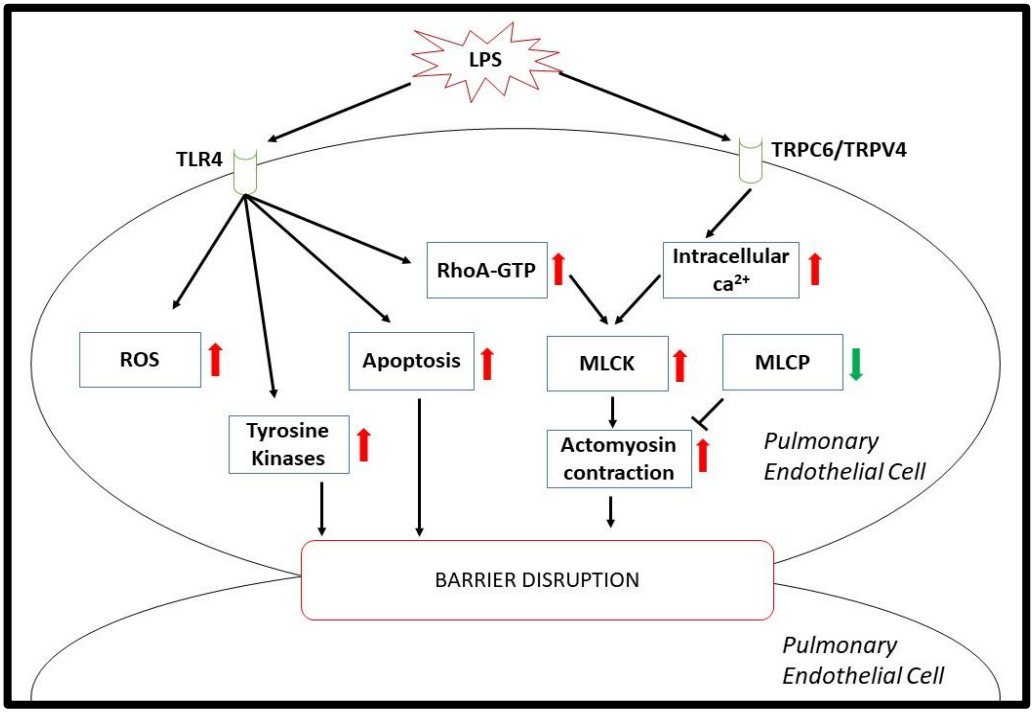



***Figure 1.2: Schematic representation of mechanisms involved in endothelial barrier disruption during LPS induced lung injury.*** *ROS: Reactive oxygen species; MLCK: myosin light chain kinase; MLCP: myosin light chain phosphatase; LPS: Lipopolysaccharide; TLR4: Toll-like receptor 4; TRPC6: transient receptor potential channel; TRPV: TRP channel subfamily V (vanilloid) type 4;* ⬆ *: increased activity;* ⬇ *: decreased activity.*

## 4. Role of Endothelial Akt pathway in regulation of endothelial barrier function

### 4.1 Akt structure and isoforms

Akt (also known as protein kinase B or PKB), was originally identified by Stephen Staal in 1987 as the likely transforming gene component, v-Akt, of the Akt8 provirus [73]. Akt is an oncogene present in mammalian genome. It exists as three isoforms, Akt1/PKBα, Akt2/PKBβ, and Akt3/PKBγ which encode proteins containing a pleckstrin homology (PH) domain in the amino terminus, a central kinase domain, and a carboxy terminal regulatory domain [74]. All 3 isoforms of Akt gene are widely expressed in various mammalian tissues, however, Akt1 is most abundantly expressed in brain, heart and lung, whereas Akt2 is predominantly expressed in skeletal muscle and embryonic brown fat and Akt3 is predominant in brain kidney and embryonic heart [75-77]. In unstimulated cells, Akt protein exists in the cytoplasm. The two regulatory phosphorylation sites of Akt are threonine at 308 and serine at 473 which remain un-phosphorylated in unstimulated cells. Upon growth factor stimulation, the PH domain binds to the lipid products of phosphoionositide3 kinase (PI3K), and Akt is recruited to the plasma membrane. At the membrane, upstream kinases known as 3-phosphoinositide-dependent protein kinase 1 (PDK1) and PDK2 sequentially phosphorylate Akt at T308 and S473 respectively, fully activating the kinase [78]. (Figure 3)

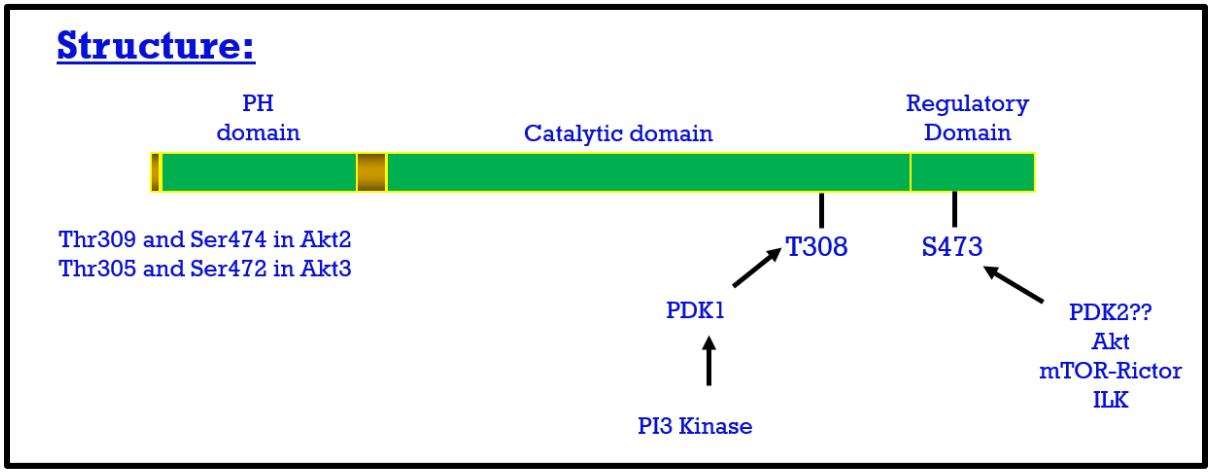



***Figure 1.3:*** *The mammalian Akt gene structure [79].*

### 4.2 Akt1 as a major player in endothelial barrier function

The majority of studies that investigate VEGF-induced endothelial barrier breakdown report SFKs induced VE-cadherin tyrosine phosphorylation at various sites as a potential mechanism involved in the vascular leakage [80, 81]. However, recent studies also showed the importance of serine threonine kinase, Akt, in VEGF induced vascular permeability as an equally important mechanism in regulating vascular permeability. In endothelial cells, Akt1 is the predominant isoform of Akt. Our previous studies have shown that Akt1 null endothelial cells have reduced transendothelial barrier resistance *in vitro* and EC Akt1 KO mice have a leaky blood vessel phenotype *in vivo*. VEGF induced angiogenesis was enhanced in Akt1 null mice because of the enhanced vascular permeability in these mice. Only prolonged as opposed to short term Akt1 expression corrected excessive vascular permeability *in vivo* and *in vitro* indicating the importance of Akt1 in maintaining vascular permeability, which of course is governed by endothelial barrier function [82]. Mechanistically, we showed previously that Akt1 is directly responsible for the expression of thrombospondins (TSPs). TSPs (including TSP-1, TSP-2) regulate several endothelial functions including, angiogenesis, apoptosis and permeability. TSP-2-deficient mice exhibit a leaky vascular phenotype indicating the importance of TSP-2 in maintaining vascular permeability [83]. Hence, decreased TSP-2 in Akt1 null endothelial cells may render this vasculature leaky [82]. More recently, we showed that TNFα induced stress response in endothelial cells that prepared these cells for barrier breakdown and inflammatory extravasation, involved sustained expression of TSP-1 through activation of Akt and p38 MAP kinase signaling in endothelial cells. This scenario again indicates the importance of Akt signaling in the regulation of TSP-1 expression by TNFα [84].

Over the past two decades, studies investigating the importance of Akt in endothelial barrier regulation have been reported, but several unanswered questions regarding the role of Akt in endothelial barrier function still continue to perplex the scientific community. Some of

these questions include 1. How VEGF and Ang-1 both activate Akt1, but exert opposing effects on endothelial barrier? 2. What is the short-term vs long-term effect of Akt1 activity on endothelial barrier function? What mechanisms downstream of Akt1 regulate the differential effects of opposing growth factors on endothelial barrier function? We tried to address some of these questions in our recent study, where we showed the importance of Akt1-FoxO-mediated tight junction protein turnover as a molecular mechanism downstream of Akt1 that protects the endothelial barrier in the long-term following endothelial barrier disrupting/protecting stimuli from growth factors such as VEGF and Ang-1 respectively [85]. While we showed in this study that the acute/short-term VEGF induced barrier breakdown was dependent on src and independent of Akt1, the recovery of barrier function after VEGF insult (or barrier enhancing effect with Ang1) was dependent on Akt1 signaling and independent of src signaling [85]. Interestingly, we showed, for the first time, a potential cross talk between these two signaling pathways that have been implicated in vascular permeability for over 2 decades now. Although inhibition of Akt did not result in any significant change in the levels of Tyr416 Src phosphorylation in the short-term (0–6 hr), it resulted in increased levels of activating Tyr416 Src phosphorylation in the long-term (12–24 hr). The increase in Src Tyr416 phosphorylation was accompanied by a decrease in Akt Ser473 phosphorylation at 12 and 24 hr following TCBN treatment, indicating that long-term inactivation of Akt enhances Src activity, thus contributing to the long-term endothelial-barrier disruption following Akt inhibition. Although our data indicate a reciprocal regulation of Akt and Src pathways in long-term endothelial barrier function, the underlying molecular mechanisms regulating this cross-talk need further extensive analysis [86]. Thus, our studies indicate a vital role of Akt1 in regulating endothelial barrier function. Since, the exudative phase of ARDS involves loss of endothelial barrier as the initial trigger of inflammation and injury, investigating the role of Akt1 in the initial exudative phase could provide a potential therapeutic target to treat this deadly condition in the early stages.

## 5. MMPs in ARDS

Since ECM in the blood-air barrier regulates exchange of liquid and solute, disruption of ECM contributes to vascular leak. Matrix metalloproteinases (MMPs) are a diverse family of ECM proteinases that have recently been implicated in destructive pulmonary pathologies [10]. Studies have shown the importance of stromelysin-1 (MMP3) in regulating lung injury with the help of genetic knockout (KO) mice lacking stromelysin-1. Lack of stromelysin-1 ameliorated immunoglobulin G induced lung injury [87, 88]. Clinical studies have associated an increase in stromelysin-1 levels with more severe ARDS accompanied by increase in distal organ failure and mortality [89, 90]. While it has been shown that the presence of stromelysin-1 is associated with worsened lung injury, the mechanisms regulating the role of stromelysin-1 in inflammation and migration of neutrophils from circulation into alveoli has not been well explored. Moreover, MMPs can be present in latent form and the net MMP activity depends on the balance between active protease concentration and the presence of endogenous inhibitors [10]. Therefore, for stromelysin-1 to have an active role in regulating the pathology of ALI/ARDS, demonstration of its increased activity is more relevant than just its presence. We studied specifically stromelysin-1 activity associated with its expression in this project.

## 6. Role of FOXP3$^+$ Tregs in Lung injury resolution

Regulatory T cells (Tregs) are a suppressive subset of CD4$^+$ T lymphocytes that function to antagonize immune responses. The suppressive function of Tregs is directed by a transcription factor known as FOXP3 [91]. Humans lacking functional FOXP3 develop a severe autoimmune disease early in infancy known as IPEX (immunodysregulation, polyendocrinopathy and enteropathy, X-linked syndrome) [92]. Natural Tregs and inducible Tregs have been implicated in several pathological conditions including cancers and inflammatory diseases. Treg infiltration in ovarian cancer is correlated with poor survival of patients. Elevated proportions of Tregs among tumor-infiltrating lymphocytes have been also

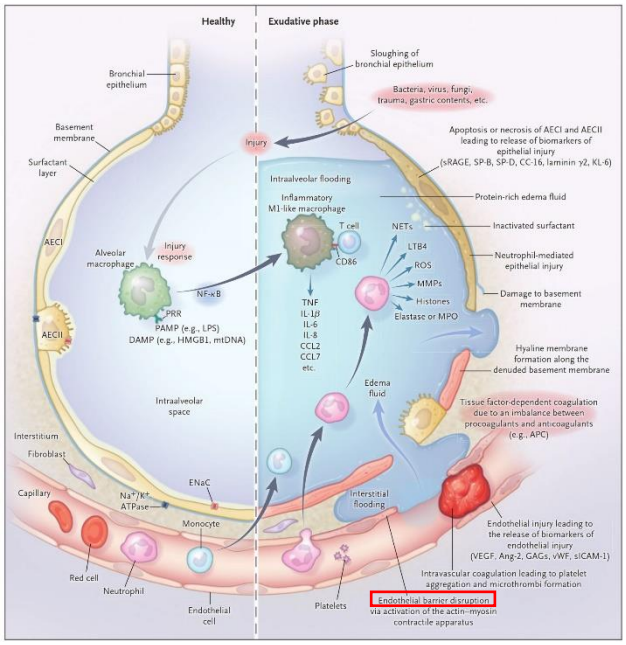
described in many other types of cancers [93]. While Tregs are associated with deleterious effects in progression of cancers, Tregs play a positive role in recovery from inflammatory conditions. Reduction in the number or functional impairment of Tregs have been reported in numerous human allergic, inflammatory or autoimmune disease [93, 94].

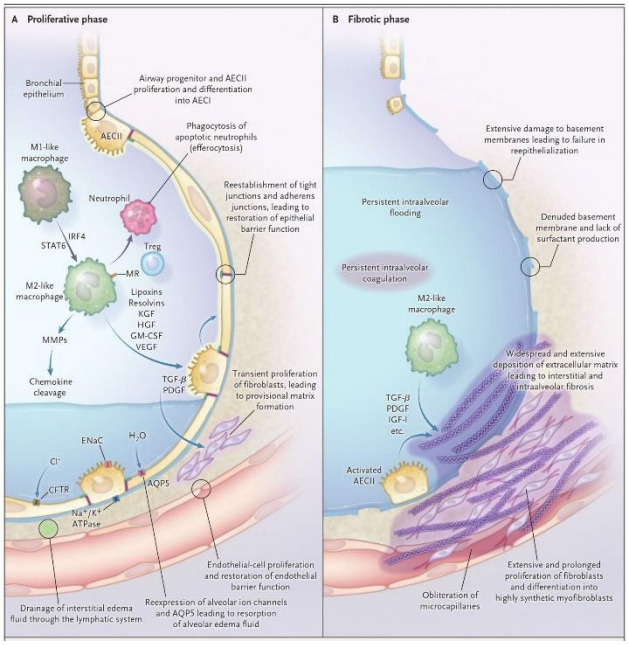
Injury resolution in ALI requires a series of integrated steps including transitions in the balance of pro- and anti-inflammatory cytokines, as well as clearance of neutrophils from sites of inflammation. Recent studies have shown the importance of Tregs in promoting resolution of LPS induced lung injury [95-97]. Enhancing the differentiation or expression of the suppressive Tregs in lung injury is a potential and attractive therapy. However, molecular mechanisms that regulate the differentiation and expression of Tregs is not well understood.

The lipid phosphatase PTEN (phosphatase and tensin homolog) has recently been identified as an important signaling pathway in Tregs. Tregs specific depletion of PTEN causes Treg instability, leading to progressive conversion into proinflammatory effector cells and eventual lupus-like autoimmunity. Thus, Tregs in tumor microenvironment have been shown to be reliant on PTEN (a negative regulator of Akt) pathway to regulate their suppressive effects[98]. Hence targeting Akt expression in the resolution stage of ALI could potentially enhance Treg mediated resolution of ALI.

## OBJECTIVE AND CENTRAL HYPOTHESIS

The *objective* of my proposal is to study the "*Reciprocal regulation of lung injury and resolution by Akt-FoxO signaling in the pathogenesis of ARDS*.". I hypothesize that Akt1-mediated modulation of MMP3 and Claudin-5 expressions in the exudative phase and $T_{reg}$ activation in the resolution phase lead to its dual role in ARDS.




**Stage specific role of Akt-FoxO signaling in ARDS:**

While Akt inhibition (downstream FoxO1/3a activation) deleteriously enhances pulmonary edema and lung injury in the initial **exudative phase**,

*Obtained from:* Thompson, B.T., R.C. Chambers, and K.D. Liu, *Acute Respiratory Distress Syndrome.* N Engl J Med, 2017. **377**(6): p. 562-572.

in the later **fibroproliferative phase**, Akt inhibition (downstream FoxO1/3a activation) beneficially enhances regulatory T-cells mediated resolution of lung injury

## SPECIFIC AIMS

I tested my overall hypothesis by pursuing the following two specific aims:

**Aim 1:** Endothelial stromelysin1 (MMP3) synthesis by Akt1-FoxO pathway is crucial in the pathogenesis of acute respiratory distress syndrome

**Aim 2:** Akt inhibition promotes resolution of LPS induced lung injury through $CD4^+/Foxp3^+/CD103^+/CTLA4^+$ activated (effector) Tregs

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# CHAPTER 2

## ENDOTHELIAL STROMELYSIN1 REGULATION BY THE FORKHEAD BOX-O TRANSCRIPTION FACTORS IS CRUCIAL IN THE EXUDATIVE PHASE OF ACUTE LUNG INJURY[1]

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## ABSTRACT

Enhanced vascular permeability is associated with inflammation and edema in alveoli during the exudative phase of acute respiratory distress syndrome (ARDS). Mechanisms leading to the endothelial contribution on the early exudative stage of ARDS are not precise. We hypothesized that modulation of endothelial stromelysin1 expression and activity by Akt1-forkhead box-O transcription factors 1/3a (FoxO1/3a) pathway could play a significant role in regulating pulmonary edema during the initial stages of acute lung injury (ALI). We utilized lipopolysaccharide (LPS)-induced mouse ALI model in vivo and endothelial barrier resistance measurements in vitro to determine the specific role of the endothelial Akt1-FoxO1/3a-stromelysin1 pathway in ALI. LPS treatment of human pulmonary endothelial cells resulted in increased stromelysin1 and reduced tight junction claudin5 involving FoxO1/3a, associated with decreased trans-endothelial barrier resistance as determined by electric cell-substrate impedance sensing technology. In vivo, LPS-induced lung edema was significantly higher in endothelial Akt1 knockdown (EC-Akt1–/–) compared to wild-type mice, which was reversed upon treatment with FoxO inhibitor (AS1842856), stromelysin1 inhibitor (UK356618) or with shRNA-mediated FoxO1/3a depletion in the mouse lungs. Overall, our study provides the hope that targeting FoxO and styromelysin1 could be beneficial in the treatment of ALI.



Keywords: stromelysin1; MMP3; Akt1; FoxO; claudin5; lung injury; ARDS

## 1. INTRODUCTION

Tight regulation of alveolar epithelial and capillary endothelial barrier integrity is crucial for the gaseous exchange and other physiological function of lungs [1]. Any damage to the alveolar capillary unit symbolizes the pathological progression into an exudative phase of acute respiratory distress syndrome (ARDS) and acute lung injury (ALI; a previous name for mild ARDS) [2, 3]. The slightly thicker part of this blood-air barrier is also composed of the extracellular matrix (ECM) between the epithelial and endothelial cells, in addition to the fused basal lamina, which is responsible for liquid and solute exchange [4]. Endothelial injury and consequent vascular permeability ensuing influx of protein rich fluid into the alveolar air spaces is a well-established pathological event occurring in the acute/exudative stage of ARDS [5]. Therefore, targeting disruption of capillary endothelial barrier could provide a potential therapy for ARDS.

Among the paracellular and transcellular pathways that regulate the transport of fluids, inflammatory cells, and proteins, etc. across the endothelial barrier, the former plays a prominent role in the pathological inflammation and edema in the lungs [6, 7]. Endothelial paracellular barrier function is largely regulated by adherens junction (AJ) proteins such as VE-cadherin and tight junction (TJ) proteins such as claudins [8, 9]. Pulmonary endothelium expresses predominantly higher levels of claudin5 than the alveolar epithelium and a decrease in claudin5 expression is associated with aberrant vascular permeability and severe lung injury in patients with pneumonia [9, 10]. *Claudin5*$^{-/-}$ mice have disrupted blood-brain permeability indicating its importance in maintaining blood-tissue barrier integrity [11]. Recent studies have indicated a potential interaction between endothelial TJ and AJ proteins in the regulation of vascular permeability. Whereas TJs are more developed in capillaries, AJs are dominant in post-capillary venules [12]. Lung edema results from the capillary leak, and hence TJs play a prominent role in the maintenance of alveolar-capillary integrity.

Since ECM in the alveolar-capillary unit regulates the selective exchange of liquid and solute, disruption of ECM significantly contributes to vascular leak [13]. Matrix metalloproteinases (MMPs) are a diverse family of ECM proteinases that have recently been implicated in destructive pulmonary pathologies [4]. Clinical studies have demonstrated an increase in MMPs in general with more severe ARDS accompanied by an increase in distal organ failure and mortality [14, 15]. Gene knockout studies have shown the importance of stromelysin1 (MMP3) in acute inflammatory tissue injury [16, 17]. While it has been shown that the presence of stromelysin1 is associated with worsened lung injury, specific involvement of stromelysin1 in mediating lung injury, mechanisms regulating its expression and activity, its utility as a screening marker in biological fluids and its therapeutic potential etc. have not yet been explored. Furthermore, in order for stromelysin1 to have an active role in regulating the pathology of ALI/ARDS, demonstration of its increased activity is more relevant than just its presence in latent form, which often is the case.

The molecular mechanisms responsible for disruption of junctional proteins in the capillary endothelium and ECM of the alveolar-capillary unit during ARDS could provide a suitable therapeutic target. Our study addresses the gap in knowledge in targeting pulmonary endothelial barrier disruption due to loss of TJs and ECM observed during the exudative stage. We previously reported that the Akt1-FoxO pathway is integral in the maintenance of endothelial-barrier function and prevents aberrant vascular leakage [18]. However, the stage-specific, differential effects of the highly complex Akt pathway deem it unfit for therapeutic development for ARDS, a disease with very limited therapeutic options. In the current study, we investigated the role of the Akt1-FoxO pathway in the regulation of claudin5 and stromelysin1 expression and activity in ECs, mouse lungs and BALF in an LPS-induced lung injury model. Further, we tested whether FoxO and stromelysin1 are druggable targets for ARDS therapy. Our results demonstrate that the pulmonary endothelial Akt1-FoxO-stromelysin1 signaling is

altered in LPS-induced lung injury. We also provide the first evidence of a 5-fold increase in stromelysin1 activity in the blood samples collected from ARDS patients compared to the healthy controls, indicating the utility of stromelysin1 activity assay as the potential diagnostic marker for the early screening of ARDS. Further, our preclinical results provide reasonable optimism on the use of pharmacological inhibitors of FoxO and stromelysin1 for treating ARDS patients.

## 2. MATERIALS AND METHODS

### 2.1 Cell culture and preparation of ShAkt1 stable cell lines

Human dermal (telomerase-immortalized) microvascular endothelial cells (HMEC) (CRL-4025; ATCC, Manassas, VA, USA) were maintained in Endothelial Cell Basal Medium-2 with a Growth Medium-2 Bullet Kit (Lonza; Walkersville, MD, USA). All cultures were maintained in a humidified 5 % CO2 incubator at 37 °C, and routinely passaged when 80–90 % confluent. Stable ShControl, ShAkt1 (ACGCT-TAACCTTTCCGCTG) HMEC cells were generated using SMART vector 2.0 lentivirus particles (109 pfu) (Thermo Scientific, Waltham, MA, USA). Lentivirus particles were mixed in 1 ml Hyclone SFM4Transfx-293 (Fisher, Hanover Park, IL, USA) and added along with 1 µl Polybrene (10 mg/ml, American bioanalytical, Natick, MA, USA). Three days later, transfection efficiency was tested through Turbo-GFP expression and subjected to 4 µg/ml puromycin (Life Technologies, Grand Island, NY, USA) selection until all cells expressed GFP. Primary Human pulmonary artery endothelial cells (HPAECs) (Part # 0055; Lifeline cell technology) were used as a model to study the in vitro effects of LPS (100ng/ml).

### 2.2 Measurement of endothelial-barrier resistance

Endothelial-barrier integrity (measured as electrical resistance of the endothelial monolayer) was determined using ECIS equipment (Applied Biophysics, Troy, NY, USA) as described previously[18, 19]. Endothelial-barrier resistance was measured at multiple frequency modes.

Endotheial cells were grown till a monolayer in full EBM-2 media and then treated with LPS, TCBN and AS1842856.

### 2.3 Immunofluorescence staining

Immunofluorescent staining of HMEC monolayers was performed using the chamber slides. Cells were then washed twice with PBS, fixed using 2 % paraformaldehyde for 30 min, permeabilized with 0.1 % Triton X-100 for 15 min, and blocked with 2 % BSA in sterile PBS. Cell monolayers were then incubated with antibodies against Claudin-5 (1:100, Rabbit antibody, Cell Signaling, Danvers, MA, USA) at 4°C overnight. Immunofluorescence was revealed using AlexaFlour secondary antibodies (1:2000 dilution of goat anti rabbit 488 and goat anti-mouse 594) obtained from Life Technologies, Grand Island, NY, USA. Cells on chamber slides were mounted on to a glass slide using DAPI containing mounting medium (Vector Laboratories). Samples were observed under a confocal microscope equipped with argon and helium/neon lasers (LSM510, Zeiss, Germany). Controls were performed by omitting either one or both primary antibodies. All controls gave negative results with no detectable non-specific labeling.

### 2.4 Western blot analysis

Western blot analysis was performed as described previously[18, 20, 21]. Antibodies used include Akt1, P-Akt (s473), P-Akt (T308), MMP3/9, P-FoxO1/3a and FoxO3a from Cell Signaling, anti-b-actin and claudin-5 from Sigma, St. Louis, MO, USA and Zo-1, from Abcam, Cambridge, MA, USA.

### 2.5 Experimental protocol for LPS induced acute lung injury model

Both male and female mice of equal quantity were randomly divided into 4 groups and each group contained 8-10 mice (equal number of males and females). The groups were as follows: 1. Control group (saline injury), 2. LPS group (LPS injury), 3. LPS injury with lung FoxO1/3a inhibition (These animals were intra-tracheally injected with $10^7$ particles of LentiShFoxO1/3a 5 days prior to LPS injury to inhibit FoxO1/3a activity locally in the lung tissue) and 4. LPS injury

with stromelysin-1 inhibition (i.p.UK-356618 15mg/kg). LPS (5 mg/kg) was administered intra-tracheally to induce acute lung injury, and the control group was injected with the same dose of saline. The doses of these drugs were on the basis of previous studies and our preliminary experiments [22]. All the mice in each group were euthanized and sacrified after 48 hrs. of LPS injury. The BAL, lung tissue specimens were collected at the time of sacrifice. Lower lobes of lungs were snap frozen for further molecular analysis and the lobe of left lung was used for sectioning and staining, while the upper lobes of right lung was used for lung W/D ratio.

**2.6 Lung wet/dry weight ratio**

The wet/dry (W/D) ratio was assayed in the upper lobe of right lung to assess lung edema. The upper lobes of the right lung were excised and the wet weights were determined. Then, the lungs were placed on sterile nonenzyme paper. After incubation at 80°C for 96 hours to remove all moisture, the dry weights were measured and the W/D ratios were calculated.

**2.7 Lung injury scoring**

Lung injury was scored based on predefined criteria [23] by 3 blinded reviewer. Average of the three reviewers score was considered for analysis. Briefly, all lung fields at ×20 magnification were examined for each sample. Assessment of histological lung injury was performed by grading as follows [scoring from 1 to 5, with 1 being the best (normal lung) and 5 being the worst (severe most lung injury)]: 1, normal; 2, focal (<50% lung section) interstitial congestion and inflammatory cell infiltration; 3, diffuse (>50% lung section) interstitial congestion and inflammatory cell infiltration; 4, focal (<50% lung section) consolidation (Combining into a solid mass without the alveoli structure) and inflammatory cell infiltration and 5, diffuse (>50% lung section) consolidation and inflammatory cell infiltration.

### 2.8 Lung injury area measurement

Lung injured area was measured using NIH software image J. Briefly, particle size was set, image was converted to black and white. The injured area measured as the black area was normalized to the uninjured area and compared between the groups.

### 2.9 BAL analysis

At the time of sacrifice, animals were anasthesized using i.p. ketamine/xylazine (100 mg/kg & 10 mg/kg respectively). When deep anesthesia was attained, the abdominal content and thorax were exposed through an abdominal midline incision. A tracheostomy tube was subsequently inserted and secured in the trachea, the diaphragm cut and the chest wall opened via a midline incision to expose the lungs. Lungs were then lavaged with 3 × 1 mL aliquots of saline, and each aliquot was instilled and withdrawn three times. The total volume of lavage fluid collected from each mouse was recorded. Lung lavage fluid was immediately centrifuged at 380 g for 10 minutes at 4°C to isolate the cell pellet; the supernatant was collected and aliquots were frozen at −80°C for subsequent analyses.

### 2.10 Stromelysin-1 activity assay

The enzymatic activity of MMP3 was determined using a fluorescence resonance energy transfer peptide and immunocapture assay as previously described elsewhere with minor modifications. Briefly, 50-µg total protein of macrovascular or brain homogenates were incubated at 4°C for 2 hours with rabbit polyclonal anti-MMP3 antibody (cat no. sc-6839-R; Santa Cruz Biotechnology, Dallas, TX). A/G agarose beads were then added and allowed to incubate overnight at 4°C. The beads were then washed and samples were transferred to black 96 well plate and 100 µL of 2 mmol/L 5-FAM/QXL 520 fluorescence resonance energy transfer peptide (cat no. 60580-01; AnaSpec, San Jose, CA) in assay buffer were added per well. Plates were incubated for 8 hours at 37°C, then relative fluorescence units were read and monitored at

excitation/emission wavelengths of 485 of 528 nm in a Synergy HT multimode microplate fluorescence reader (BioTek, Winooski, VT) running Gen5 data analysis software.

### 2.11 MPO activity assay

MPO activity assay was performed using Mouse MPO ELISA kit from Hycult biotech according to manufacturers protocol.

### 2.12 Ingenuity pathway analysis

Ingenuity Pathway Analysis (IPA, Qiagen Bioinformatics) is a system that transforms a list of genes into a set of relevant networks associated with pathology based on extensive records maintained in the Ingenuity Pathways Knowledge Base[24]. Highly interconnected networks are predicted to represent significant biological function[25]. IPA was used to connect 132 genome wide association study (GWAS)- implicated ARDS genes[26, 27] along with Akt-FoxO-MMP3 pathway. Only those genes that were directly affected by pathway of interest and ARDS are shown.

### 2.13 Statistical Analysis

All the data are presented as mean ± SD and were calculated from multiple independent experiments performed in triplicates. The ‘n’ value for each figure implies the multiple independent experiments we performed. All the data were analyzed by parametric testing using the Student's unpaired t-test or one-way ANOVA, followed by the post-hoc test using the GraphPad Prism 6.01 software. Data with $P<0.05$ were considered significant.

## 3. RESULTS

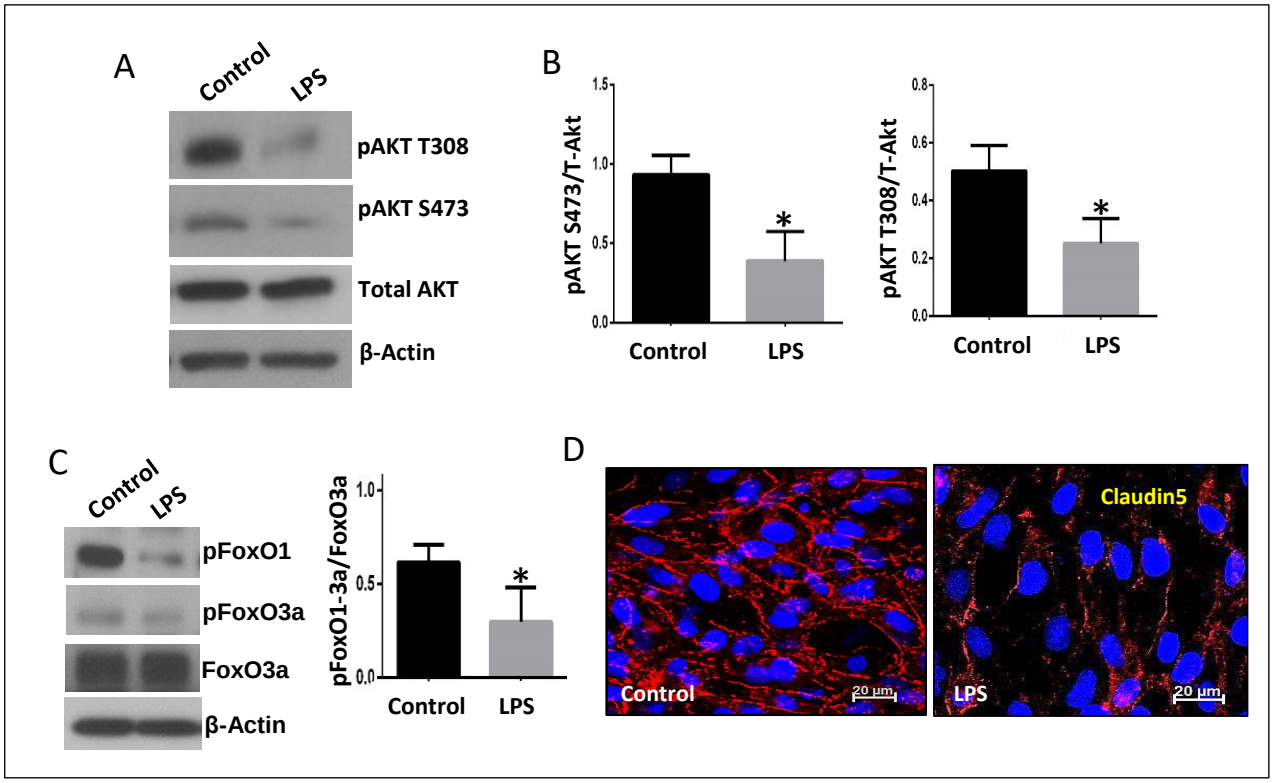
### 3.1 LPS treatment inhibits Akt1 activity and disrupts endothelial barrier integrity in primary human lung endothelial cells.

We have recently identified the integral role of Akt1 activity in regulating endothelial barrier function *in vitro* and vascular permeability *in vivo* through tight junction (TJ) protein turnover [18]. Hence, we sought to test our hypothesis that LPS mediated lung injury alters endothelial Akt1 activity leading to disruption of vascular barrier and enhanced vascular leakage induced lung edema. To explore the effect of LPS on Akt1 activity in endothelial cells, Human Pulmonary Artery Endothelial cells (HPAECs) were treated with 100ng/ml LPS or equal volume of PBS for 12 Hrs. Phosphorylation changes in endothelial Akt were observed at both serine 473 and threonine 308 sites of Akt at different time points (Supplement. Fig 1). LPS significantly decreased Akt phosphorylation in endothelial cells after 12 hrs. of treatment (Fig. 1A-B). Next, we studied the effect of LPS on endothelial barrier integrity in vitro. We used ECIS assay to measure real-time transendothelial barrier resistance in a monolayer of HPAECs. Single dose LPS treatment (100ng/ml) and Akt inhibitor triciribine (10μM) disrupt endothelial barrier integrity gauged in terms of decreased resistance in HPAEC monolayer treated with LPS/triciribine compared to PBS (Fig. 1G).

### 3.2 LPS disrupts endothelial barrier integrity through impaired TJ protein expression because of Akt inhibition and FoxO1/3a activation.

Tight Junction (TJ) proteins in endothelial cells regulate barrier integrity and vascular permeability. Claudin-5 is one of the most prominent TJ protein expressed in endothelial cells[8]. Since LPS disrupts endothelial barrier integrity, we explored its effect on claudin-5 expression in endothelial cells. To mimic the microvascular nature of capillary endothelial cells that surround the alveoli forming alveolar epithelial and endothelial barrier in lung, we used

Human Microvascular Endothelial Cells (HMECs) to examine the effect of LPS on claudin-5 expression. LPS treatment significantly decreased claudin-5 expression in HMECs at 24 Hrs. (Fig. 1D-E). This was also confirmed in primary HPAECs (Fig. 1F). Our previous study has shown that Akt1 protects endothelial barrier by inhibiting foxO-mediated TJ protein turnover[18]. To further investigate the signaling involved in LPS mediated claudin-5 suppression, we observed Akt-FoxO signaling in HPAECs after LPS treatment. As anticipated, LPS significantly repressed phosphorylation of both FoxO1 and FoxO3a in HPAECs (Fig. 1C). Moreover, to further confirm the involvement of Akt-FoxO signaling in LPS induced claudin-5 suppression, we used FoxO1 inhibitor AS1842856 (dose 10μM) along with LPS treatment in HPAECs. Prior treatment (4 Hrs.) with AS1842856 rescued LPS induced claudin-5 suppression in HPAECs conforming the role of FoxOs in suppression of claudin-5 expression (Fig. 1F). Real time transendothelial barrier resistance measure using ECIS showed a significant recovery in barrier resistance when FoxO inhibitor AS1842856 was used along with LPS treatment. This established the involvement of Akt-FoxO signaling in LPS induced disruption of endothelial barrier resistance (Fig 1G).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LPS
pAKT T308
pAKT S473
Total AKT
β-Actin
B
pAKT S473/T-Akt
1.5
1.0
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Control
LPS
pAKT T308/T-Akt
0.8
0.6
0.4
0.2
0.0
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Control
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LPS
pFoxO1
pFoxO3a
FoxO3a
β-Actin
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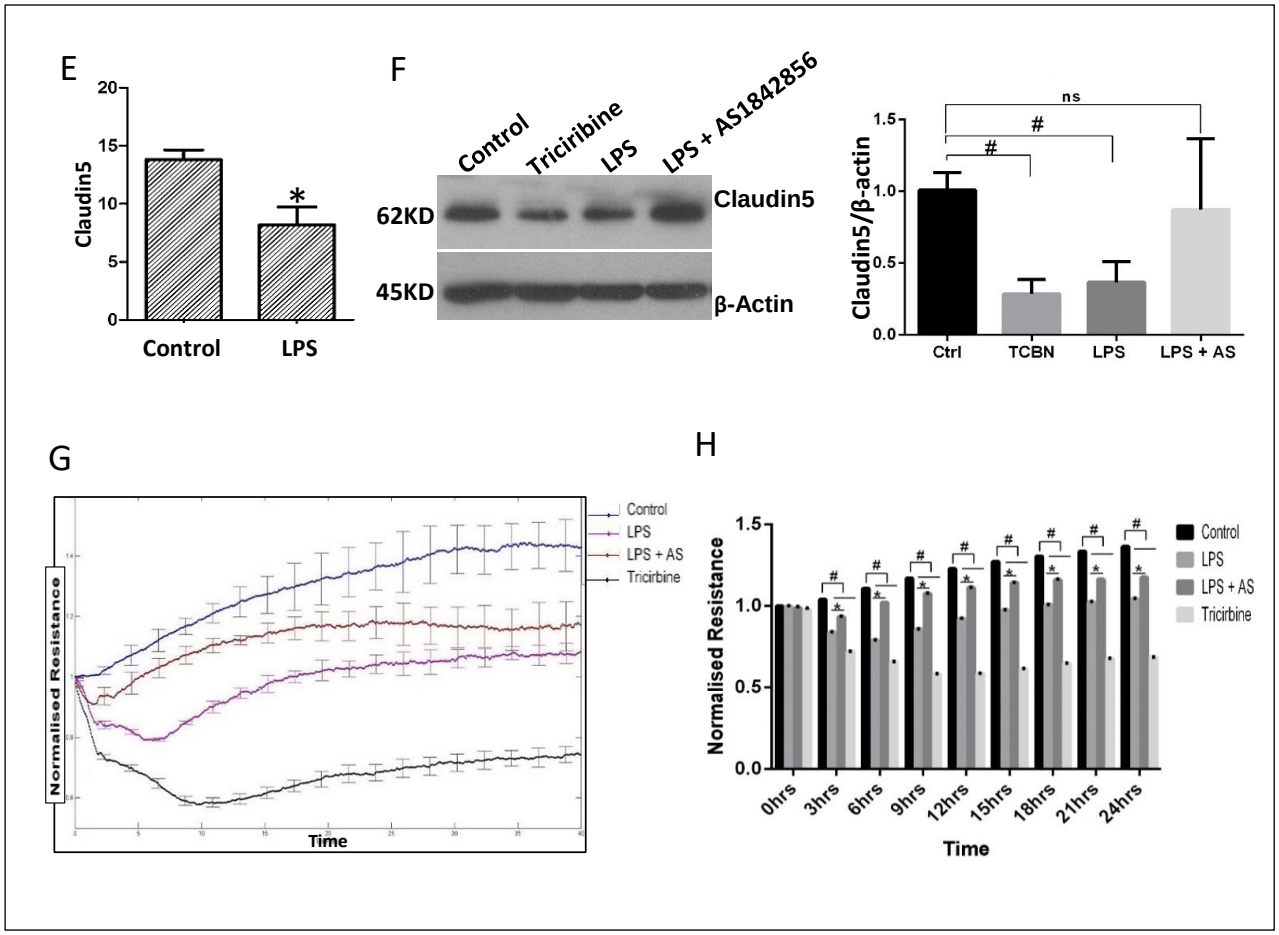
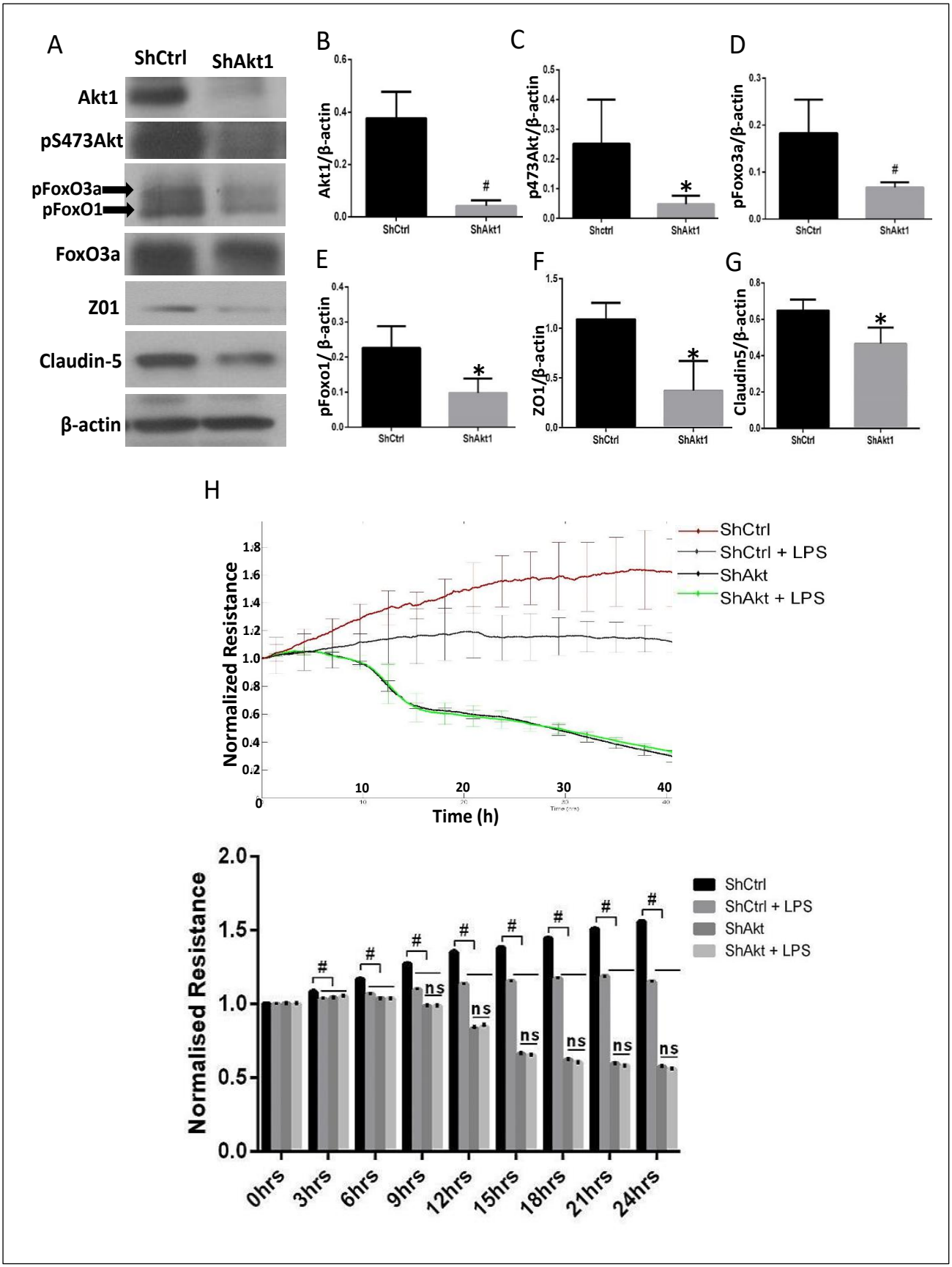



**Figure 2.1:** *LPS treatment disrupts EC-barrier integrity via the FoxO1/3a mediated suppression of claudin5 in primary HPAECs.* ***(A-B)*** *Representative Western blot images and the densitometry analysis indicating the inhibitory effect of LPS treatment (100 ng/ml) on Akt phosphorylation in primary HPAECs after 12 hrs (n=3).* ***(C)*** *Representative Western blot images and the densitometry analysis showing the effect of LPS on FoxO1/3a phosphorylation in HPAECs after 12 hrs treatment (n=3).* ***(D-E)*** *Representative images and bar graph showing percentage of claudin5 positive area in the HMEC monolayers probed with Claudin5 antibodies in the absence and presence of LPS treatment for 12 hrs.* ***(F)*** *Representative Western blot images and the densitometry analysis of HPAECs indicating the effect of Akt inhibitor triciribine (10 μM), LPS and with LPS in combination with FoxO inhibitor AS1842856 (10 μM) for 24hrs on claudin5 expression (n=5).* ***(G-H)*** *Graphs showing the real-time changes in resistance by the HPAEC monolayers upon treatment with LPS, triciribine, and LPS in combination with FoxO*

*inhibitor AS1842856 (10 μM; pretreated) compared to DMSO control as recorded using the ECIS assay (n=3). HPAEC: Human Pulmonary Artery Endothelial Cells; HMEC: Human Microvascular Endothelial cells. * (p<0.05); # (p<0.01); ns = non-significant.*

**3.3 Silencing Akt1 in HMECs mimics the effect of LPS treatment on HMECs.**

We previously showed that Akt1 is the predominant isoform of Akt in endothelial cells [28]. In order to determine if Akt1 suppression in ECs will have a similar effect of LPS treatment, we silenced Akt1 in HMECs using lentivirus-based ShRNAs followed by generation of a stable ShAkt1 HMEC cell line through antibiotic selection. ShAkt1 HMECs had ~80% reduction in their Akt1 expression and total Akt phosphorylation (Figure 2A-C) and significantly reduced FoxO1/3a phosphorylation (Figure 2D-E) as compared to the ShControl (ShCtrl) HMECs. This was accompanied by reduced expression of TJ proteins ZO-1 and claudin5 (Figure 2F-G), an effect similar to what was observed with LPS treatment of HMECs and HPAECs (Figure 1). ShAkt1 HMECs compared to ShCtrl HMECs exhibited a significant reduction in the normalized transendothelial barrier resistance in ECIS (Figure 2H). Interestingly, whereas LPS treatment decreased resistance in ShCtrl HMECs, it did not have any further effect on already resistance compromised ShAkt1 HMECs compared to PBS treatment (Figure 2H). These results indicated that LPS, through Akt1 inhibition and FoxO1/3a activation suppresses claudin5 expression in ECs thus disrupting endothelial barrier resistance.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Akt1
pS473Akt
pFoxO3a
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FoxO3a
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Claudin-5
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C
p473Akt/β-actin
D
pFoxo3a/β-actin
E
pFoxo1/ β-actin
F
ZO1/β-actin
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Claudin5/β-actin
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ShAkt
ShAkt + LPS
Normalised Resistance
0hrs
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6hrs
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12hrs
15hrs
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21hrs
24hrs

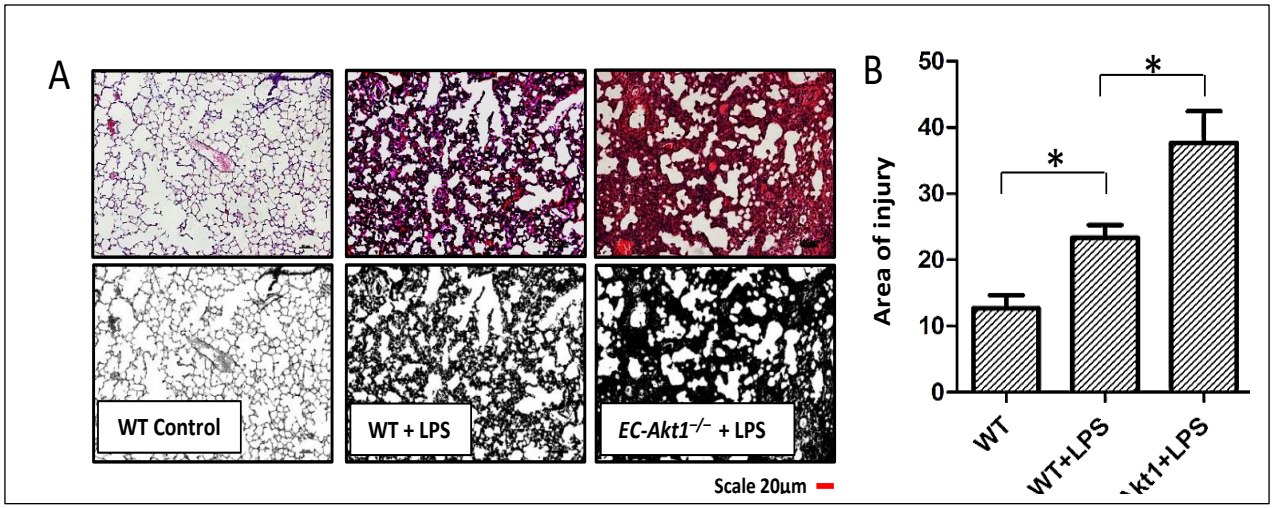
***Figure 2.2:*** *Silencing Akt1 in HMECs mimics the effect of LPS treatment on HMECs.*

***(A)*** *Representative Western blot images showing changes in the expression of expression of Akt1, pS473Akt, pFoxO1/3a and tight junction proteins ZO-1 and claudin5 in ShAkt1 compared to ShCtrl HMECs.* ***(B-G)*** *Bar graphs showing quantification of the band densitometry indicating significant reduction in the expression levels of Akt1, pS473Akt, pFoxO1/3a and tight junction proteins ZO-1 and claudin5 in ShAkt1 compared to ShCtrl HMECs normalized to the loading control, β-actin (n=5).* ***(H)*** *Graphs showing the real-time changes in resistance by the ShControl and ShAkt1 HMEC monolayers in the presence and absence of LPS treatment compared to PBS control as recorded using the ECIS assay (n=4). * (p<0.05); # (p<0.01); ns = non-significant.*

### 3.4 *Endothelial Akt1 loss exacerbates LPS-induced acute lung injury in EC-Akt1$^{-/-}$ mice via FoxO1/3a activation*

To further investigate the involvement of Akt1-FoxO1/3a pathway in LPS-induced lung injury *in vivo*, we utilized a previously characterized tamoxifen-inducible VE-cadherin promoter driven endothelial specific Akt1 knock down (*EC-Akt1$^{-/-}$*) mice. Intra-tracheal (i.t.) instillation of LPS (5mg/kg) resulted in lung injury in both WT and *EC-Akt1$^{-/-}$* mice, where injury was exacerbated in *EC-Akt1$^{-/-}$* mice compared to WT as confirmed by the blinded analysis of the H&E stained mouse lung sections (Figure 3A-B). Lung wet/dry weight ratio used as a measure of lung edema was significantly higher in LPS injured *EC-Akt1$^{-/-}$* mouse lungs compared to the LPS injured WT mouse lungs (Figure 3D). Similarly, vascular leakage was significantly enhanced in LPS injured *EC-Akt1$^{-/-}$* mouse lungs compared to the LPS injured WT mouse lungs even in an Evans blue dye extravasation assay in the mouse lungs, thus confirming enhanced pulmonary injury and edema in LPS injured *EC-Akt1$^{-/-}$* mouse lungs compared to the LPS injured WT mouse lungs (Figure 3C&E). Next, we investigated the effect of FoxO1/3a inhibition *in vivo* on LPS induced lung injury in WT and *EC-Akt1$^{-/-}$* mouse lungs. Lung specific knockdown of FoxO1/3a achieved

by intratracheal instillation of LentiShFoxO1/3a ($10^7$ virus particles) 5 days prior to LPS administration (supplementary figure 2B-D) and systemic inhibition of FoxO achieved by treatment with FoxO inhibitor AS1842856 (30mg/kg), both blunted the increased lung wet/dry weight ratio and Evans blue extravasation in *EC-Akt1*$^{-/-}$ mouse lungs compared to untreated controls (Figure 3C&E). Thus, both lung specific FoxO1/3a knockdown, as well as pharmacologic FoxO inhibition, were found to be inhibiting LPS induced ALI. Genome wide association studies (GWAS)-implicated ARDS genes interaction with FoxO1/3a in humans, mice and rats further confirms the importance of FoxO1/3a in regulation of ARDS affected genes and pathology. (Figure 3F).



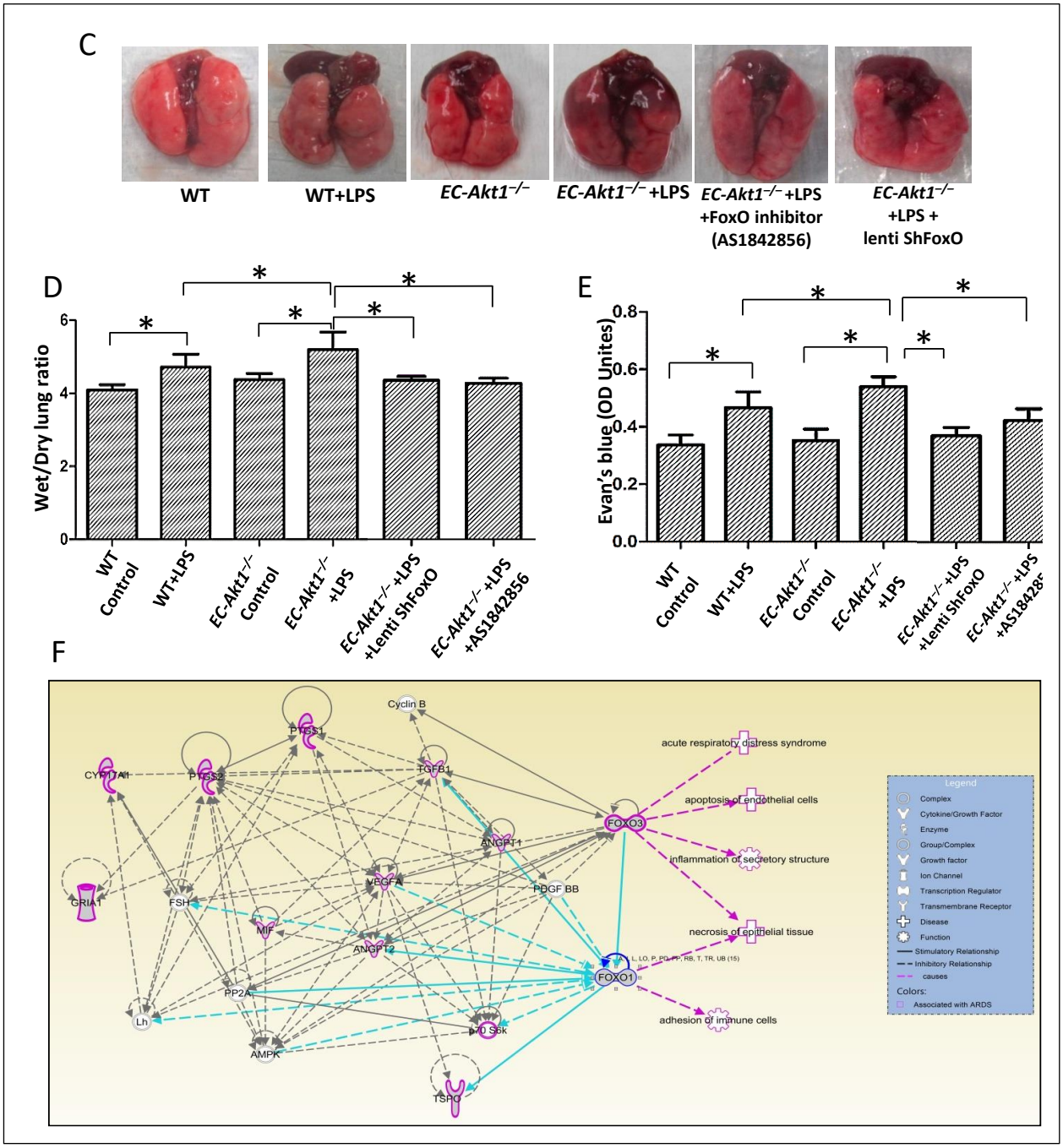


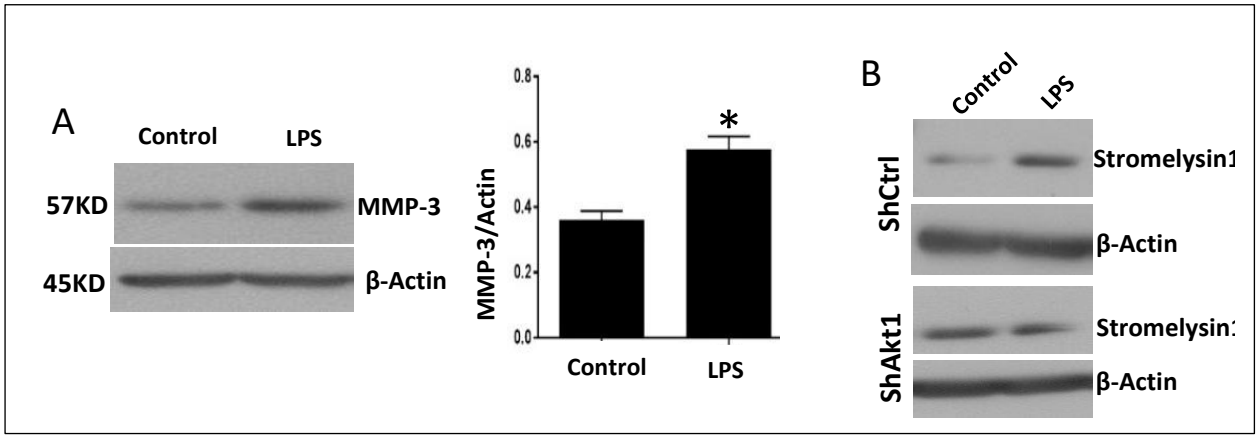

***Figure 2.3:*** *<u>Endothelial Akt1 loss exacerbates LPS-induced acute lung injury (ALI) in VE-Cad-CreAkt1 mice which is partly rescued by FoxO1/3a inhibition</u>.* ***(A-B)*** *H&E staining of mice lung sections showed enhanced LPS induced lung injury in Cre-Akt1 mice compared to WT C57BL/6 mice.* ***(C)*** *Representative lung images showing enhanced lung vascular leak in Cre-Akt1 mice which was rescued by FoxO1/3a inhibition.* ***(D)*** *Lung wet/dry ratio as a measure of lung edema further confirmed that the edema was greater in LPS injured Cre-Akt1 mice and this was partly*

*rescued with pulmonary FoxO1/3a inhibition using LentiShFoxO1/3a or FoxO1 inhibitor (AS1842856) i.p. 30mg/kg.* ***(E)*** *Evans blue extravasation further confirmed enhanced lung vascular leak in Cre-Akt1 mice and this was partly rescued with pulmonary FoxO1/3a inhibition using LentiShFoxO1/3a or FoxO1 inhibitor (AS1842856) i.p. 30mg/kg. (n=5).* ***(F)*** *FoxO1/3a regulates ARDS genes and pathology in humans, mice and rats as identified from genome wide association studies using ingenuity pathway analysis (IPA). * (p<0.05); # (p<0.01).*

### 3.5 *Treatment with LPS or Akt1 knock down in endothelial cells, both increases stromelysin1 expression and activity involving FoxO1/3a*

Since the involvement of MMPs in the disruption of Alveolar epithelial-endothelial barrier in lung injury is prominent [4], we determined whether LPS treatment or Akt1 knock down in endothelial cells will lead to increased stromelysin1 (MMP3) expression, which has the potential to break down the TJ proteins such as claudins and ZO-1 with its proteolytic activity in addition to its ability to digest the ECM [29-31]. We observed that LPS treatment for 24hrs on HPAECs significantly increased stromelysin1 expression (Figure 4A). To further investigate the involvement of endothelial Akt1 signaling in LPS-induced stromelysin1 expression, both ShCtrl and ShAkt1 HMECs were treated with LPS or PBS. Loss of Akt1 activity by itself was sufficient to increase stromelysin1 expression in ShAkt1 HMECs compared to ShCtrl HMECs. Interestingly, while LPS significantly enhanced stromelysin1 expression in ShCtrl HMECs, it did not have any additive effect in ShAkt HMECs (Figure 4B-C). To confirm that increased stromelysin1 expression correlates with its activity, we performed stromelysin1 activity assay in HMEC lysates. In order to further investigate the signaling downstream of Akt1 that is involved in the LPS-induced stromelysin1 expression, we used inhibitors of FoxO and β-catenin, two major downstream signaling pathways regulated by Akt1 in ECs. HMECs treated with LPS alone or in combination with inhibitors of FoxO (AS1842856 10μM) or β-catenin (10 μM of ICG001 or IWR1) signaling, and stromelysin1 expression was assessed. While LPS treatment

of HMECs enhanced stromelysin1 expression, prior treatment with FoxO inhibitor, but not β-catenin inhibitors reversed LPS-induced stromelysin1 expression (Figure 4D-E). Silencing Akt1 significantly resulted in increased stromelysin1 activity in HMEC lysates (Figure 4F). Suppressive effect of FoxO inhibitor on LPS-induced stromelysin1 expression was also observed in its reduced activity in LPS and AS1842856 treated HMEC lysates compared to LPS only treated controls (Figure 4G). The effect of LPS on stromelysin1 activity and its suppression by FoxO inhibitor was also confirmed in HPAECs (Supplemental Figure 2A). Interestingly, saline instilled *EC-Akt1*$^{-/-}$ mouse lung lysates exhibited higher stromelysin1 expression compared to *WT* lungs, which was further elevated upon LPS treatment (Figure 4H). These results indicate the role of Akt1-FoxO1/3a mediated stromelysin1 expression and activity in ECs on LPS-induced vascular injury.



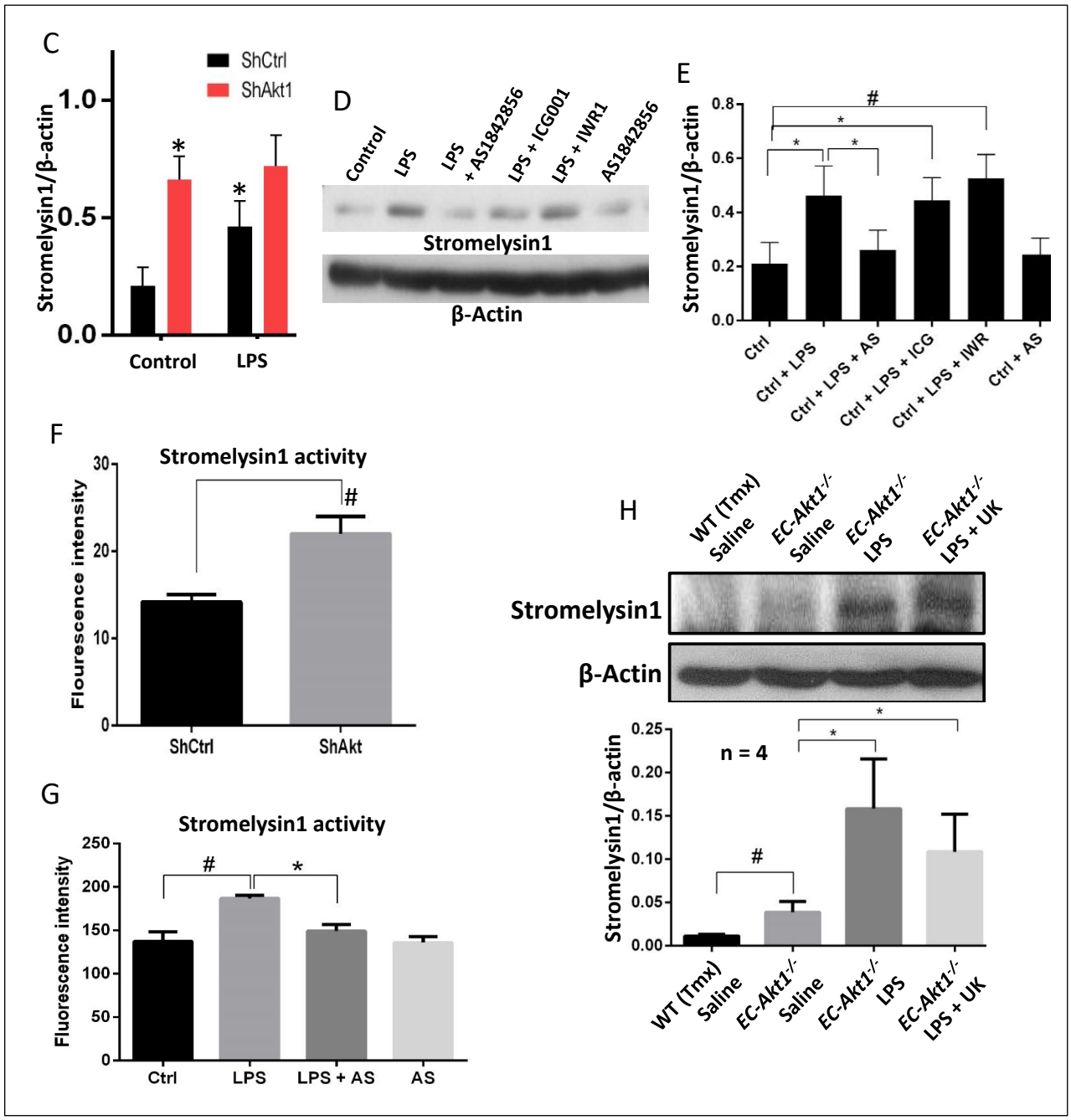



**Figure 2.4:** *Treatment of primary HPAECs with LPS induces increased expression and activity of stromelysin1 via Akt1-FoxO1/3a signaling.* ***(A)*** *Western blot images and densitometry analysis of HPAEC lysates after treatment with 100ng/ml LPS showing enhanced stromelysin1expression after 24 hrs (n=4).* ***(B-C)*** *Western blot images and densitometry analysis of ShControl and ShAkt1 HMEC lysates showing enhanced stromelysin1 expression upon treatment with LPS in ShControl, but not in ShAkt1 cells and that expression levels of*

*Stromelysin1 is already high in ShAkt1 HMECs compared to ShControl cells (n=5).* ***(D-E)*** *Western blot images and densitometry analysis of HMEC lysates showing increased expression of stromelysin1 with LPS treatment and a significant reversal of stromelysin1 expression upon co-treatment with FoxO1/3a inhibitor (10 μM AS1842856), but not with β-Catenin inhibitors (10 μM each of ICG001 and IWR-1) (n=4).* ***(F)*** *Bar graph showing increased activity of stromelysin1 in ShAkt1 HMEC lysates compared to ShControl (n=5).* ***(G)*** *Bar graph showing Stromelysin1 activities in LPS treated HMEC lysates compared to PBS treated controls and its reversal upon co-treatment with FoxO1/3a inhibitor AS1842856 (n=3).* ***(H)*** *Western blot images and densitometry analysis of WT and EC-Akt1$^{-/-}$ mouse lung lysates probed for stromelysin1. Bar graph shows a significant increase in stromelysin1 expression in EC-Akt1$^{-/-}$ mouse lung lysates compared to WT, which is further elevated upon LPS injury (n=6). * (p<0.05); # (p<0.01).*

### 3.6 *Both FoxO1/3a and stromelysin1 are druggable targets in LPS-induced lung injury in mice*.

Next, we sought to investigate whether targeting Akt1-FoxO1/3a signaling would be beneficial to inhibit LPS-induced lung injury *in vivo*. In order to do this, 8-10-weeks old C57BL/6 mice were divided into four groups of i.t. Saline (sham) injury with i.p. DMSO (control), LPS injury with i.p.DMSO, LPS injury with FoxO1/3a inhibition locally in the lung (i.t. Lenti-ShFoxO1/3a) and LPS injury with stromelysin1 inhibition using i.p. UK-356618 (15 mg/kg). H&E staining of mice lung tissue 48hrs after injury exhibited enhanced inflammatory cell infiltration, interstitial congestion and lung consolidation with LPS injury (Figure 5A-B). To a significant extent, local FoxO1/3a inhibition, as well as systemic stromelysin1 inhibition, reduced LPS-induced lung injury (Figure 5A-B). Lung wet/dry weight ratio depicted a significant decrease in lung edema with FoxO1/3a and stromelysin1 inhibition (Figure 5C). Examination of mice BALF revealed reduced LPS-induced protein and cell counts upon FoxO1/3a or stromelysin1 inhibition (Figure 5D-E).

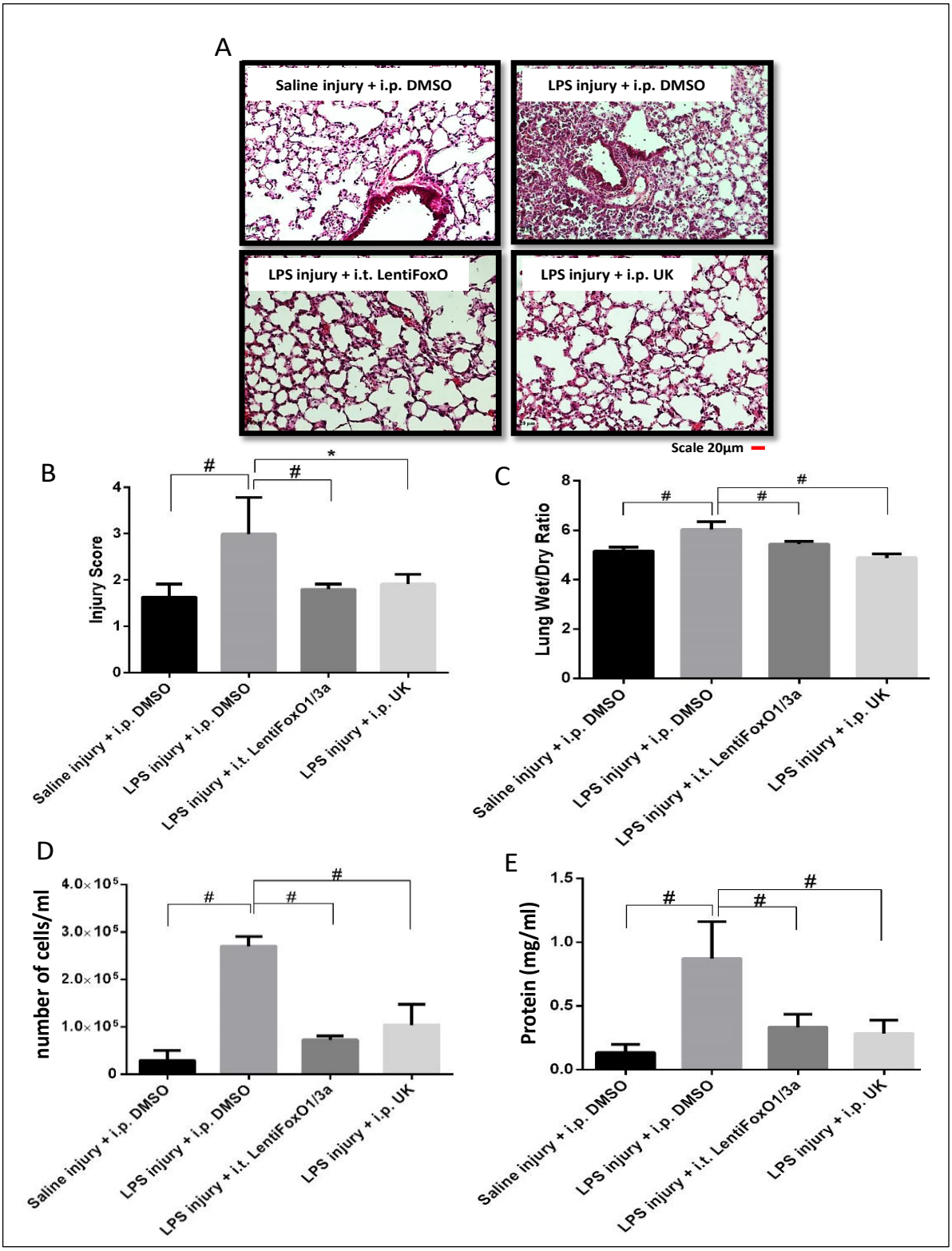


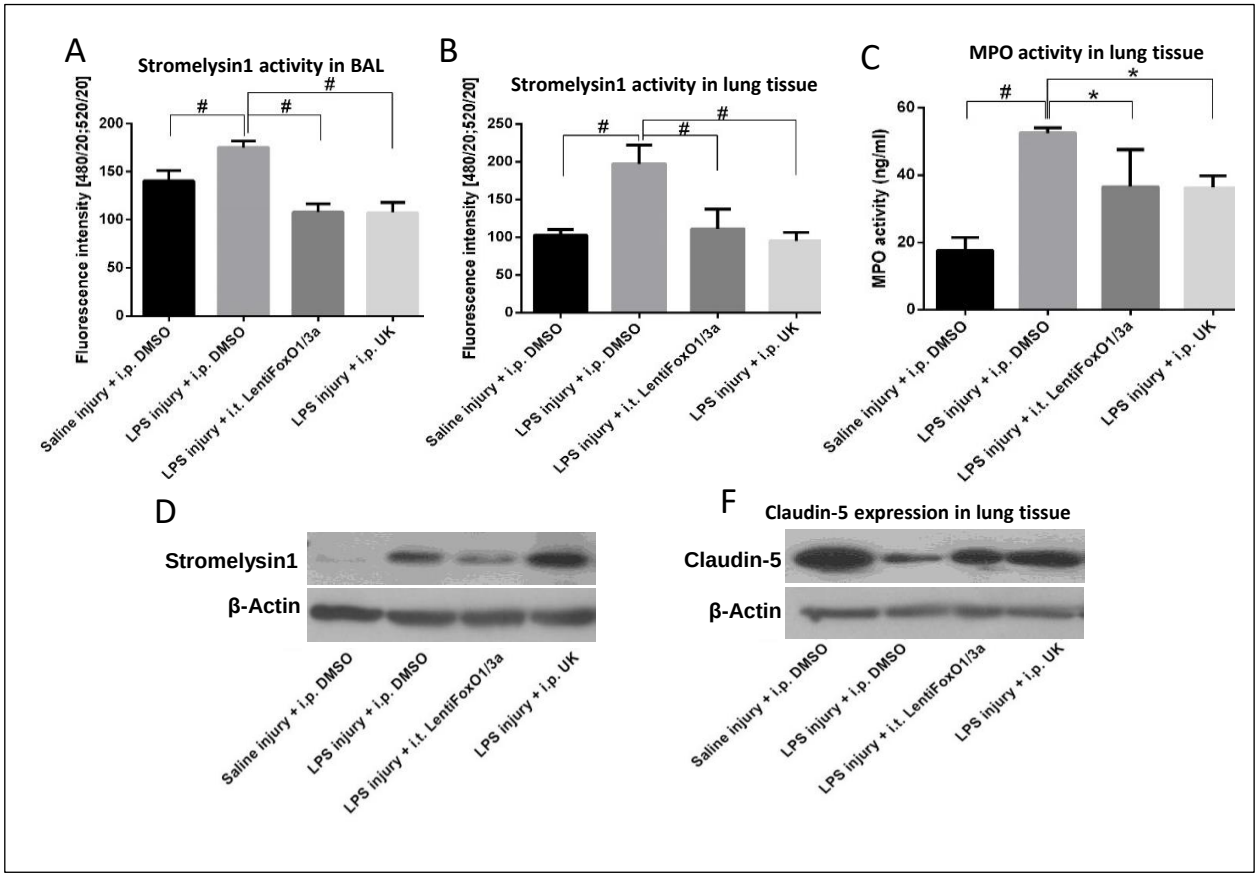

***Figure 2.5:*** *<u>Inhibition of FoxO1/3a or stromelysin1 blunts LPS-induced Acute Lung Injury in Mice</u>.* ***(A)*** *Representative H&E staining (20X) of mouse lung sections showing LPS (5mg/kg) induced lung injury that is significantly inhibited upon inhibition of FoxO1/3a (Lentiviral*

*expression of ShFoxO1/3a) or stromelysin1 (15 mg/kg of UK-356618).* ***(B-C)*** *Bar graphs showing blinded analysis of lung injury score (by 3 different reviewers), and lung wet/dry weight ratio analysis depicting a significant reduction in lung injury and lung edema with FoxO1/3a or stromelysin1 inhibition, respectively (n=6).* ***(D-E)*** *Bar graph showing elevated BALF total cell counts and BALF total protein in LPS-injured mouse lungs that is reversed upon inhibition of FoxO1/3a or stromelysin1 (n=6). * (p<0.05); # (p<0.01). BALF: Bronchoalveolar lavage fluid.*

### 3.7 *Changes in tissue and BALF stromelysin1 activity is a reliable diagnostic and prognostic marker for lung injury*

Since stromelysin1 is a secreted enzyme and its activity can be measured in a laboratory set up, we determined whether changes in stromelysin1 activity can be utilized as a screening and/or prognostic marker in lung injury. Intriguingly, FoxO1/3a inhibition reduced stromelysin1 activity in the mouse lungs and airways confirming an *in vivo* role of FoxO1/3a contribution in stromelysin1 expression and activity (Figure 6A-B). Neutrophil infiltration and activity is a marker of lung injury in the exudative phase [4, 29]. Neutrophil activity was also significantly decreased with FoxO1/3a and stromelysin1 inhibition (Figure 6C). Next, we assessed stromelysin1 expression in the whole mouse lung lysates. Whereas LPS-induced injury enhanced stromelysin1 expression in mouse lungs, prior inhibition of FoxO1/3a activity in the lungs significantly prevented LPS-induced stromelysin1 expression in the mouse lung tissue lysates (Figure 6D&E). Increased mouse lung stromelysin1 expression and activity with LPS-induced injury and its reversal with FoxO inhibition once again confirmed the contribution of FoxO1/3a signaling in the regulation of stromelysin1 expression. Whereas stromelysin1 inhibitor UK356618 did not alter stromelysin1 expression in mouse lungs, it significantly inhibited its activity (Figure 6B). *In vitro* results indicated that LPS-treatment resulted in the reduced expression of claudin5 leading to enhanced vascular permeability. Assessment of claudin5 in mouse lungs in injured mice further confirmed these results from *in vitro* studies, where LPS-

induced decrease in claudin5 expression in the mouse lungs was partially rescued with FoxO1/3a or stromelysin1 inhibition (Figure 6F&G). Sromelysin1 interaction with GWAS-implicated ARDS genes in humans, mice and rats indicates its importance in regulation of ARDS affected genes and pathology. (Figure 6H).



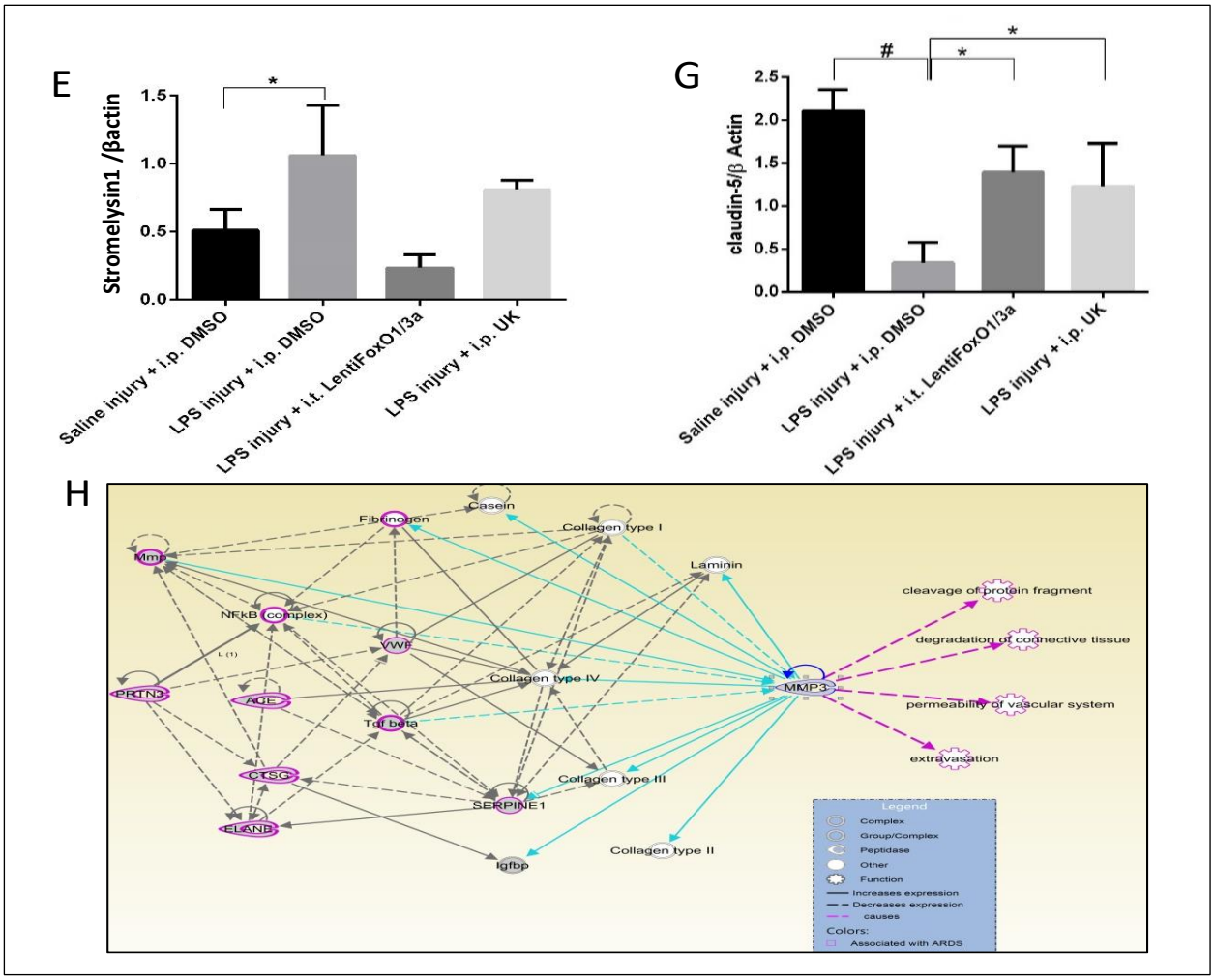



***Figure 2.6:*** *<u>Pharmacological inhibition of FoxO1/3a or stromelysin1 has beneficial effects on LPS- induced lung injury and edema.</u>* ***(A)*** *Bar graph showing increased stromelysin1 activity in mouse BALF upon LPS injury and its significant inhibition upon FoxO1/3a knock down or co-treatment with inhibitor of stromelysin1 (n=6).* ***(B)*** *Bar graph showing increased stromelysin1 activity in mouse lung tissues upon LPS injury and its significant inhibition upon FoxO1/3a knock down or co-treatment with inhibitor of stromelysin1 (n=6).* ***(C)*** *Bar graph showing significantly increased myeloperoxidase (MPO) activity in mouse lungs after LPS injury and a significant inhibition with FoxO1/3a and stromelysin1 inhibition (n=6).* ***(D-E)*** *Representative Western blot images and band densitometry analysis showing increased stromelysin1 expression in lung tissue lysates with LPS injury and its significant reversal upon FoxO1/3a knock down or co-*

*treatment with inhibitor of stromelysin1 (n=6).* ***(F-G)*** *Representative Western blot images and band densitometry analysis showing decreased claudin5 expression in lung tissue lysates with LPS injury and its significant reversal upon FoxO1/3a knock down or co-treatment with inhibitor of stromelysin1 (n=6). * (p<0.05); # (p<0.01). MPO: Myeloperoxidase).* ***(H)*** *Stromelysin1 (MMP3) regulates ARDS genes and pathology in humans, mice and rats as identified from genome wide association studies using ingenuity pathway analysis.*

### 3.8 *Stromelysin1 inhibition significantly rescued LPS-induced lung injury in EC-Akt1$^{-/-}$ mice*.

Since we observed a significant decrease in LPS-induced lung injury with FoxO1/3a inhibition in *EC-Akt1$^{-/-}$* mice, we determined if we would also see reduced lung injury in *EC-Akt1$^{-/-}$* mice with stromelysin1 inhibition. Stromelysin1 inhibition using 15 mg/kg of UK356618 significantly reduced lung injury score (Figure 7A-B), decreased lung edema (Figure 7C), decreased cell count and total protein in BALF in *EC-Akt1$^{-/-}$* mice compared to PBS-treated controls (Fig. 7D & 7E), suggesting that stromelysin1 inhibition is also effective to treat lung injury in *EC-Akt1$^{-/-}$* mice.

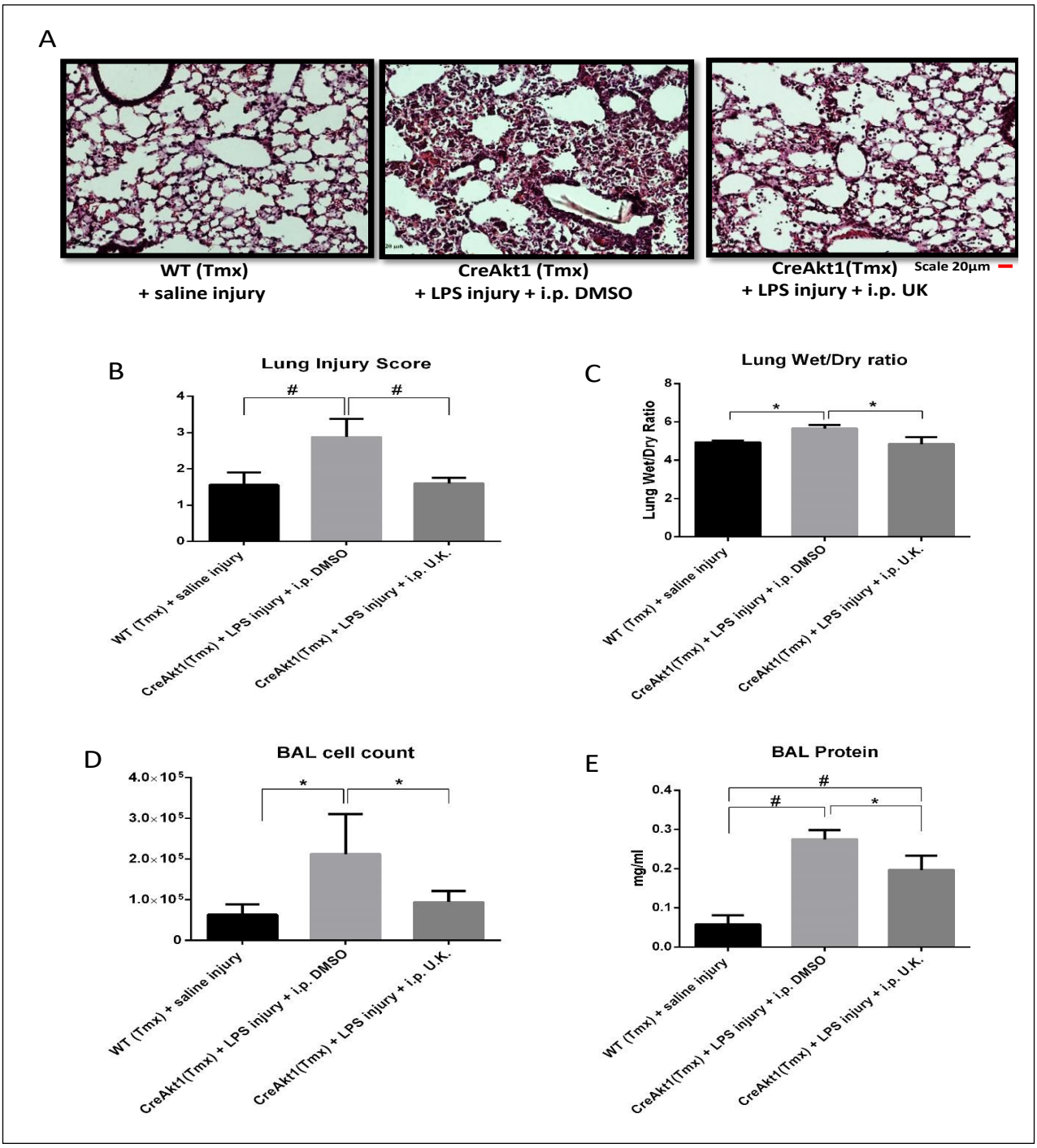



***Figure 2.7:*** *Augmented LPS-induced lung injury and edema in EC-Akt1$^{-/-}$ mouse lungs is rescued by co-treatment with stromelysin1 inhibitor.* ***(A)*** *Representative H&E images of (20X) of EC-Akt1$^{-/-}$ mouse lungs showing significantly increased LPS-induced lung injury compared to WT mouse lungs, and that co-treatment with stromelysin1 inhibitor (15 mg/kg UK-356618) reverses this effect.* ***(B-C)*** *Bar graphs showing lung injury score (blindly scored by 3 different*

*reviewers) and the lung wet/dry weight ratio in EC-Akt1$^{-/-}$ mice, respectively, depicting a significant reduction in lung injury score and edema with stromelysin1 inhibition (n=6).* ***(D-E)*** *Bar graph showing total cell count and total protein content in mouse BALF indicating significantly higher cell count and protein content in EC-Akt1$^{-/-}$ mice compared to WT mice, and its significant reversal with stromelysin1 inhibition (n=6). * (p<0.05); # (p<0.01).*

### 3.9 *Human serum and plasma samples from ARDS patients exhibit significantly higher levels of stromelysin1 activity compared to healthy subjects*.

In order to confirm that stromelysin1 can serve as an important diagnostic and prognostic tool for lung injury patients, we determined the activity levels of stromelysin1 in plasma and serum samples collected from ARDS patients compared to healthy smoking and non-smoking subjects. Human subject characteristics are shown in Supplemental Tables 1 and 2. Comparison of stromelysin1 activity in blood samples from healthy human subjects with ARDS patients showed a 3-6-fold increase in stromelysin1 activity in ARDS subjects (Figure 8A-B). Analysis of different components of blood (serum and plasma) stromelysin1 activity showed no difference between these components (Figure 8C). Smoking is an important confounding factor in the etiology of lung injury. Analysis of blood stromelysin1 activity in healthy smokers versus healthy non-smokers showed no difference in the basal stromelysin1 activity in the blood of healthy subjects (Figure 8D). Interaction of Akt-FoxO-stromelysin1 pathway with GWAS-implicated ARDS genes along with their functions depicts the impact that this pathway bears in pathology of ARDS (Figure 8E).

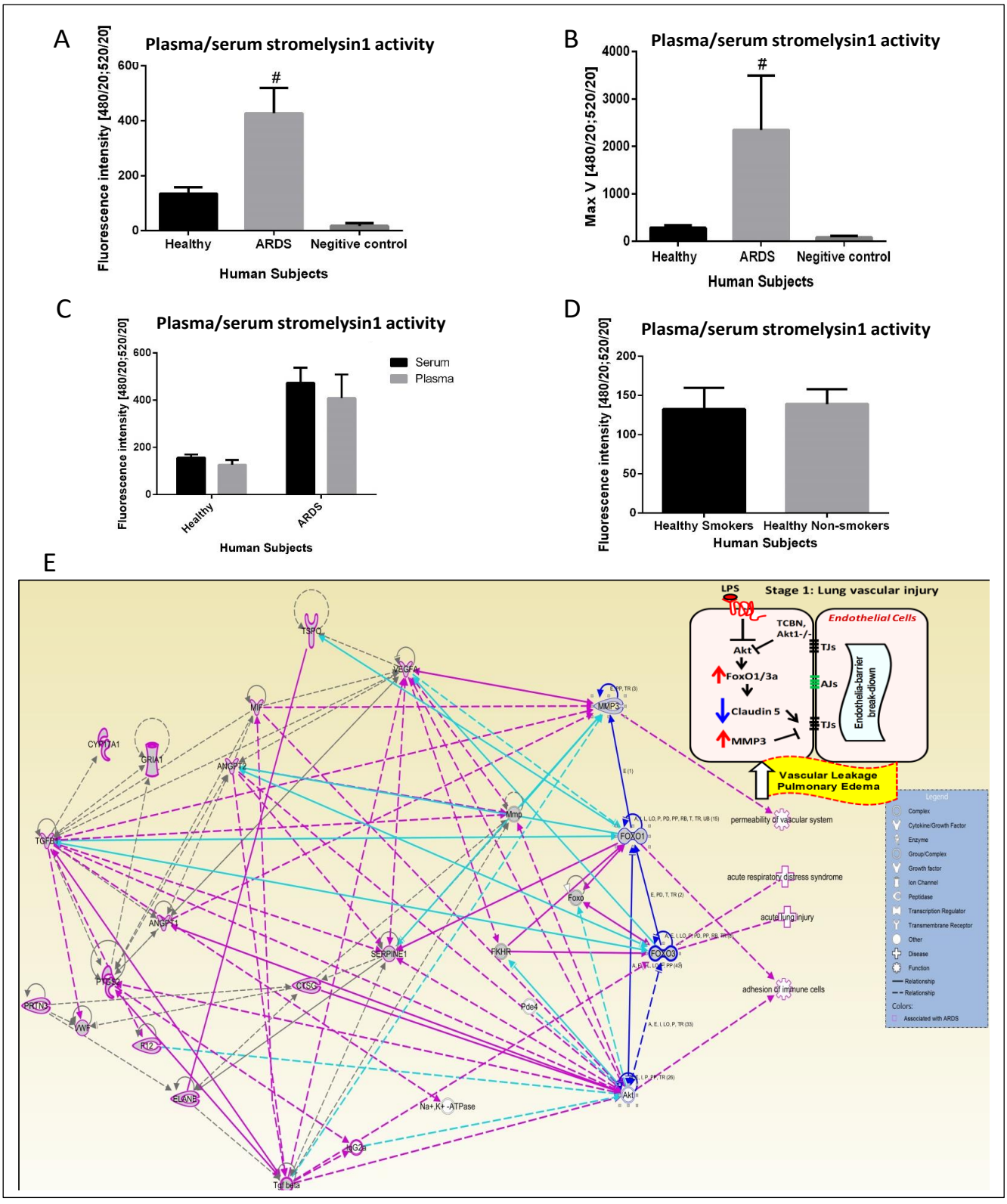



***Figure 2.8:*** *Human serum and plasma samples collected from ARDS patients showed a significant increase in stromelysin1 activity.* ***(A-B)*** *Human Plasma and serum samples from*

*healthy subjects and ARDS patients were analyzed for stromelysin1 activity. Bar graphs indicating a >3 fold increase in stromelysin1 activity in ARDS patient plasma/serum samples compared to healthy subjects. Max V values showed a ~6-fold increase in stromelysin1 activity in ARDS subjects over 15 Hrs. of analysis (n=10).* ***(C)*** *Bar graphs indicating both plasma and serum samples had similar stromelysin1 activity (n=3-7).* ***(D)*** *Bar graphs indicating no significant difference in stromelysin1 activities between the plasma/serum samples collected from healthy smokers and nonsmokers (n=4-6)).* ***(E)*** *Akt-FoxO1/3a-Stromelysin1 pathway regulates ARDS genes and pathology in humans, mice and rats as identified from genome wide association studies using ingenuity pathway analysis, along with cartoon showing the working hypothesis that suppression of Akt1 activity by LPS in pulmonary endothelial cells results in increased FoxO1/3a activation, in turn, leading to increased stromelysin1 expression/activity and reduced expression of tight junction proteins, particularly claudin5. * ($p<0.05$); # ($p<0.01$).*

**Supplementary Figures and Tables:**

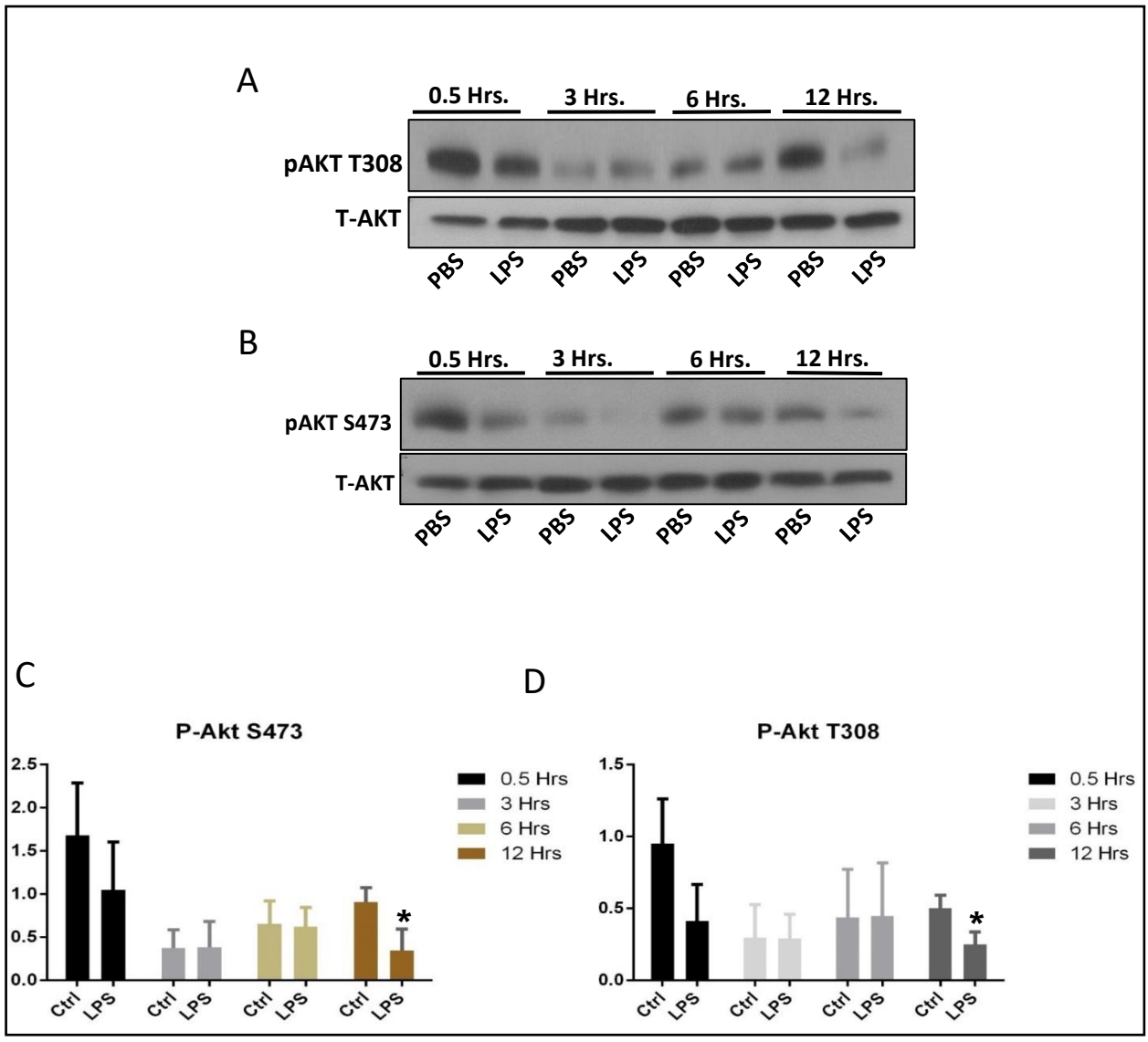



***Figure S1: (A-B)*** *Western blot representatives and* ***(C-D)*** *quantification data showing phosphorylation changes with LPS 100ng/ml or PBS treatment on HPAECs at various time points. LPS treatment was compared to PBS control at each time point. LPS treatment significantly inhibited Akt phosphorylation only at 12 hrs. although there is trend in decrease as early as 30 mins. * ($p<0.05$); # ($p<0.01$).*

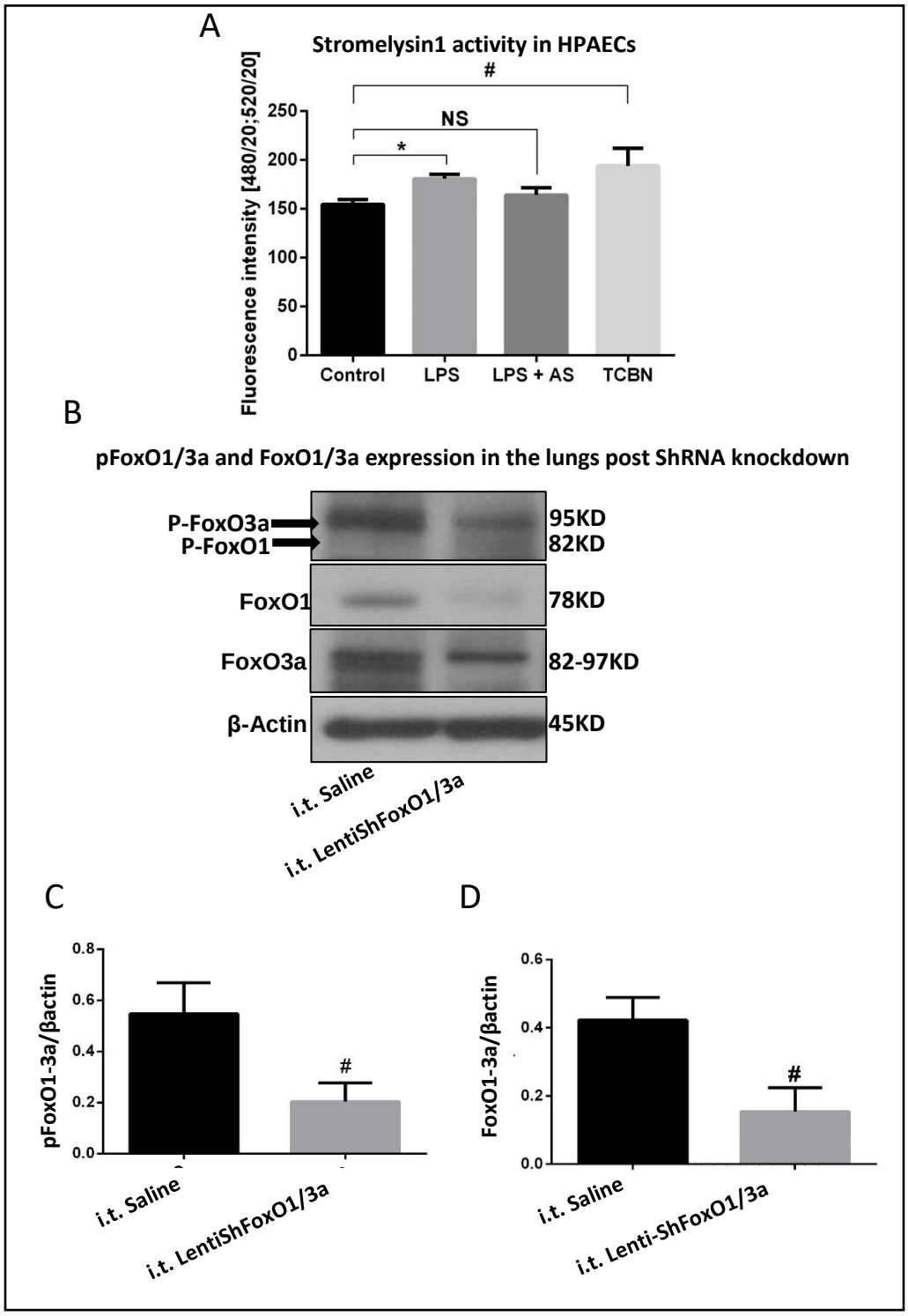



***Figure S2: (A)*** *stromelysin-1 activity assay on HPAECs treated with triciribine (TCBN), LPS and LPS with AS1842856.* ***(B-D)*** *Western blot and quantification data showing mice lung P-FoxO1/3a and total FoxO1/3a expression changes with LentiShFoxO1/3a. Greater than 60% reduction in lung FoxO1/3a activity was achieved with lentivirus particles (107 particles) (n=6). * (p<0.05); # (p<0.01).*

| # | Sample Name | Sample Date | Gender | Age | Ethnicity | Smoke | Matrix |
|---|---|---|---|---|---|---|---|
| 1 | R313816 | 4/3/2017 | Male | 26 | Black | Yes | Lithium Heparin |
| 2 | R313824 | 4/3/2017 | Male | 21 | Black | Yes | Lithium Heparin |
| 3 | N124401 | 4/6/2017 | Male | 40 | Black | No | Lithium Heparin |
| 4 | R314046 | 4/6/2017 | Male | 36 | Black | No | Lithium Heparin |
| 5 | R314059 | 4/6/2017 | Female | 35 | Black | No | Lithium Heparin |
| 6 | R313897 | 4/4/2017 | Male | 33 | Black | Yes | Na Heparin |
| 7 | R313904 | 4/4/2017 | Male | 33 | Black | Yes | Na Heparin |
| 8 | DLS17-025697 | 11/23/2016 | Male | 37 | Black | Yes | Serum |
| 9 | DLS17-025776 | 11/29/2016 | Male | 44 | Black | Yes | Serum |
| 10 | DLS17-025695 | 11/29/2016 | Male | 63 | Black | No | Serum |

**(A) Patient Characteristics (Healthy Subjects):**

| # | Sample Name | Sample Date | Gender | Age | Ethnicity | Matrix |
|---|---|---|---|---|---|---|
| 1 | DLS14-32067 | 6/14/2016 | M | 33 | Black | Na Heparin |
| 2 | DLS15-18601 | 3/6/2016 | F | 38 | White | Lithium Heparin |
| 3 | DLS16-69301 | 11/16/2016 | M | 46 | Black | Lithium Heparin |
| 4 | DLS15-18166 | 1/22/2016 | M | 27 | White | Lithium Heparin |
| 5 | DLS15-18537 | 3/2/2016 | M | 20 | White | Lithium Heparin |
| 6 | DLS14-31892 | 6/10/2016 | M | 73 | White | Serum |
| 7 | DLS16-31851 | 8/4/2016 | M | 37 | White | Serum |
| 8 | DLS16-32224 | 9/11/2016 | M | 44 | White | Serum |
| 9 | DLS15-18413 | 2/29/2016 | F | 43 | White | Lithium Heparin |
| 10 | DLS15-17563 | 11/28/2015 | M | 41 | White | Na Heparin |

**(B) Patient Characteristics - ARDS Subjects (International Classification of the Disease-10 code J80):**

***Supplemental Table 1: (A)*** *Table showing patient characteristics of age matched healthy subjects used as controls.* ***(B)*** *Table showing patient characteristics of ARDS subjects (whose samples were used in study) classified under international disease-10 code J80 (ARDS).*

## 4. DISCUSSION

The endothelial barrier plays an integral role in mediating a selective transport of biological agents, fluids and gases across the alveolar-capillary unit [32]. Compromising blood-air barrier integrity leads to lung injury and edema that occur in the initial exudative phase of ARDS [2, 32, 33]. The mechanisms leading to the disruption of capillary barrier and a translational approach to prevent this is still an area to be vastly explored. In this study, we investigated the Akt1-FoxO-stromelysin1 pathway as a potential mechanism mediating aberrant vascular permeability, lung edema and inflammation similar to the exudative phase of ARDS in a LPS-induced lung injury model, and determined whether FoxO and stromelysin1 are druggable targets for ARDS therapy. We also determined whether stromelysin1 expression and activity in lung tissues and biological fluids can be developed into a diagnostic and prognostic marker for ARDS.

The current study first identifies the similarities between pharmacological or genetic suppression of Akt1-FoxO pathway and LPS stimulation on endothelial injury and TJ protein claudin5 and ZO1 expression. Subsequently, we showed that LPS treatment results in the inhibition of Akt and activation of FoxO1/3a in human pulmonary artery and microvascular ECs leading to reduced claudin5 expression and impaired EC-barrier resistance. Both the reduced resistance of the EC monolayer and a decrease in claudin5 expression by LPS was reversed upon genetic ablation or pharmacological inhibition of FoxO1/3a. Both Akt1 knock down and treatment with LPS in ECs resulted in significantly increased expression and activity of

stromelysin1, which once again was blunted by co-treatment with panFoxO inhibitor or ShRNA-mediated knock down of FoxO1/3a. Interestingly, no additive effect of LPS stimulation in Akt1 deficient ECs on stromelysin1 expression was observed *in vitro* indicating that Akt1 suppression downstream of LPS stimulation in ECs is responsible for stromelysin1 expression. *In vivo*, LPS-induced lung injury score, edema and stromelysin1 expression and/or activity were significantly higher in the lungs and BALF collected from *EC-Akt1*$^{-/-}$ mice. Intriguingly, saline instilled *EC-Akt1*$^{-/-}$ mouse lungs expressed significantly higher levels of stromelysin1 compared to saline instilled *WT* mice demonstrating an EC-specific expression of stromelysin1 in the lungs contributing to lung vascular injury. Inhibition of FoxO or stromelysin1 were effective in treating lung injury in both *WT* and *EC-Akt1*$^{-/-}$ mice confirming that the changes in Akt and FoxO activities, and changes in claudin5 and stromelysin1 expression/activity leading to lung injury follow the same pathway. Interestingly, plasma and serum samples collected from ARDS patients exhibited ~3 to 5-fold increase in stromelysin1 activity compared to healthy smoking or non-smoking subjects, occur where a significant difference between smoking and non-smoking subjects on stromelysin1 activity was not observed. Together, we present a novel role for pulmonary ECs in stromelysin1 expression in response to Akt1-FoxO1/3a pathway contributing to the initial exudative phase of lung edema and injury induced by LPS administration.

Using the cellular and skin vascular permeability experimental models, we have recently shown the importance of Akt1-FoxO1/3a signaling in regulating VEGF and Ang1-induced long-term EC barrier protection *in vitro* and the prevention of aberrant vascular permeability *in vivo* [18]. In a pathological condition like ARDS, the effect of LPS on the EC barrier and the mechanism underlying its effects leading to lung edema and injury may also depend on a similar mechanism. In order to test this hypothesis, we initially studied the effect of LPS on EC Akt1 activity. Intriguingly, LPS decreased Akt activating phosphorylation at both serine-473 and threonine-308 residues at 12 hrs after treatment, which were eventually restored by 24 hrs. On

further analysis of the effect of LPS on EC junction protein expression and EC barrier resistance, we observed that LPS disrupts EC barrier through decreased expression of TJ protein claudin5 and ZO1, two molecules that have been previously shown to regulate paracellular EC barrier permeability [12, 34, 35]. Adding to these Akt1 regulated changes in the EC barrier, another significant finding from our current study is the novel role of Akt1-FoxO1/3a pathway in the regulation of stromelysin1 expression by the endothelial cells in response to LPS treatment.

Changes in the expression and activity of stromelysin1 also may contribute to the cleavage of TJ proteins such as claudin5 and ZO1 in addition to their direct transcriptional repression through FoxO1/3a, subsequently contributing to the lung vascular injury and edema. Literature reporting the involvement of various MMPs, with comparatively very little evidence for stromelysin1, in ALI/ARDS pathology has been available since the early 1990s [36, 37]. Until today, none of these MMPs have been directly implied to have any therapeutic or diagnostic value for ARDS therapy. Although previous studies have used *Stromelysin1*$^{-/-}$ mice to study its role in ALI and other tissue injuries [16, 17, 38], there were intrinsic limitations related to the systemic gene ablation and unknown effects of overcompensating mechanisms. As a prime example, a study showed that *Stromelysin1*$^{-/-}$ mice was beneficial in ameliorating lung injury only in female mice, but not in males [38]. In our study, which is the first of its kind in ARDS, we used stromelysin1 specific inhibitor to surpass such intrinsic compromises in gene knockout models and have performed a highly selective and sensitive method to determine stromelysin1 enzymatic activity in ECs, lung tissues and body fluids. In ischemic stroke, MMP-mediated degradation of claudin5 is responsible for disruption of blood brain barrier [39]. Our *in vitro* results indicated enhanced stromelysin1 expression along with reduced claudin5 expression in HMECs and HPAECs injured with LPS, both of which were rescued upon genetic or pharacological inhibition of FoxO1/3a, indicating these are all different pieces of the same

puzzle. Thus, the enhanced stromelysin1 expression and its proteolytic activity in ECs with LPS treatment might be a contributing factor to the reduced claudin5 expression in the ECs. This was further confirmed *in vivo*, where LPS injured mouse lungs had decreased claudin5 expression, which was restored with treatment by either FoxO1/3a or stromelysin1 inhibitor. Stromelysin1 has been reported to play a role in neutrophil migration from pulmonary circulation into the alveolus [4]. Hence, we considered neutrophil activity (MPO assay) in mice lungs injured with LPS and treated with DMSO or stromelysin1 inhibitor. We observed a significant reduction in neutrophil activity with stromelysin1 inhibition as well as FoxO1/3a inhibition. Overall, the extent of lung damage, lung edema, and inflammatory cell infiltration was enhanced in *EC-Akt1*$^{-/-}$ lungs compared to *WT* lung in response to LPS, both were rescued upon either stromelysin1 inhibition or FoxO1/3a inhibition. The data from the more severely injured *EC-Akt1*$^{-/-}$ mice further confirms what we observed in WT mice, thus revealing immense clinical and translational impact of our findings.

The present diagnostic criteria provided by Berlin definition of ARDS lacks a sensitive biomarker that can be used to diagnose ARDS [40]. Confirmation of the role of stromelysin1 in the pathogenesis of ALI/ARDS must also be demonstrated by its increased activity and not just presence. From our analysis, we demonstrate the potential utility of determining stromelysin1 activity in biological samples, including biological fluids as a diagnostic and prognostic marker (since stromelysin1 activity decreased with the improvement of lung injury with treatments in in vivo mouse model) in ALI/ARDS and to test the efficacy of an investigational therapy via a non-invasive approach. Thus, in addition to our demonstration of the clinical utility of stromelysin1 as a target for ARDS treatment, we also show for the first time that stromelysin1 activity in the blood samples of ARDS patients as compared to healthy controls can be utilized as a screening method. We observed a ~5-fold increase in stromelysin1 activity in ARDS patients and this was not altered by smoking in healthy subjects. Moreover, stromelysin1 activity was significantly

reduced in both the treatment groups involving either FoxO1/3a or stromelysin1 inhibitor in our *in vivo* mouse experiments. This indicates the potential utility of determining stromelysin1 activity in biological samples, including biological fluids as a prognostic marker in ALI/ARDS to test the efficacy of an investigational therapy *via* a non-invasive approach.

## 5. CONCLUSIONS

In conclusion, our study reports several novel findings pertinent to the etiology and molecular characterization of ALI/ARDS disease progression (Fig.8E). To the best of our knowledge, we report for the first time that LPS-induced EC-barrier disruption *in vitro* involves suppression of Akt1, in turn, activating the FoxO1/3a-mediated transcriptional repression of TJ proteins claudin5 and ZO1, and transcriptional activation of stromelysin1 that aggravates EC-injury *in vitro* and pulmonary vascular injury *in vivo,* both of which are blunted by either FoxO1/3a or stromelysin1 inhibition. Our study has identified FoxO1/3a and stromelysin1 as novel therapeutic targets for ARDS. We showed that LPS induced lung edema is further worsened in *EC-Akt1*$^{-/-}$ mice demonstrating the novel role of ECs as a primary source of stromelysin1 that promotes the exudative phase of ALI/ARDS. Moreover, we found that LPS-induced, FoxO-mediated increase in stromelysin1 expression can be enzymatically measured from the lung tissues and the biological fluids such as BALF and blood, suggesting its potential use in the early diagnosis and disease prognosis of ARDS. Importantly, we also provide the first report that plasma/serum samples from the ARDS patients exhibit nearly 5-fold increase in stromelysin1 activity compared to healthy subjects. However, stromelysin1 activity was not altered by smoking in non-ARDS controls ruling out smoking as a confounding factor for ARDS diagnosis. Altogether, our study generates reasonable optimism on developing FoxO and stromelysin1 inhibitors for ARDS therapy and utilizing stromelysin1 activity assay in biological fluids such as BALF and plasma/serum as a simple, non-invasive approach to early diagnosis and prognosis of ALI/ARDS.

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# CHAPTER 3

## AKT INHIBITION PROMOTES RESOLUTION OF LPS INDUCED LUNG INJURY THROUGH $CD4^+/FOXP3^+/CD103^+/CTLA4^+$ ACTIVATED (EFFECTOR) TREGS

### 1. INTRODUCTION

The initial response to injury caused by trauma, pneumonia, sepsis, etc. in lungs is characterized by innate immune cell-mediated damage of the alveolar endothelial and epithelial barrier resulting in the exudation of protein-rich edema fluid into the alveoli. This is a characteristic of the exudative phase of Acute Respiratory Distress Syndrome (ARDS) [1]. The fate of an injured lung can either be the resolution of lung injury or fibroproliferation leading to irreversible damage to lungs. Controlling excessive inflammation can enhance the resolution of lung injury as opposed to fibroproliferation. Hence, the adaptive immune response could play a major role in the resolution of lung injury. Regulatory T cells are an anti-inflammatory subset of T-cells that have been extensively studied to enhance the resolution of lung injury.

The existence of Tregs that actively suppress the function of conventional T cells is a key factor by which immune system maintains immunologic self-tolerance and protect the host against the exacerbated response to foreign antigens. Tregs are a suppressive subset of T cells that mediate peripheral tolerance by antagonizing immune responses. The quantity of Tregs is a crucial determinant of the regulatory burden on the immune system as these cells have the capacity to prevent potentially damaging autoimmune and protective immune responses. The transcription factor FOXP3 directs the suppressive function of Tregs. The major site of Treg induction is the thymus, where FOXP3 expression is initiated through a combination of antigen recognition and microenvironmental influences. In addition, FoxP3 expressing Tregs are induced

peripherally in various tissues, including the colon, placenta, etc., where these peripheral Tregs are required to preserve immune homeostasis [2]. The relative proportion of thymic Tregs is estimated to be around 70-90% based on the markers Helios (or IKZF2) and neuropilin 1 (NRP1) which are enriched within thymus Tregs [3, 4].

Based on function, Tregs can also be divided into central Tregs that are similar to naïve conventional $CD4^+$ T cells, effector Tregs, which have enhanced suppressive function and polarized tissue resident Tregs which are present in most non-lymphoid organs. The central Tregs are $CD62L^{hi}$ $CCR7^+$ (CC-chemokine receptor 7-positive) $CD45RA^{hi}$ $CD25^{low}$ Tregs, the effector Tregs are defined as $CD62^{low}CCR7^{low}CD44^{hi}KLRG1^+$ (Killer cell lectin-like receptor subfamily G member 1- positive) $CD103^+$ or $CD45RA^{low}CTLA4^+CD25^{hi}$ Tregs [2] (Table 1&2).

**Table 4.1** Selected markers for resting, effector and memory Tregs [2, 5].

| **Resting Tregs** | **Activated effector Tregs** | **Memory Tregs** |
|---|---|---|
| $CD25^{hi}$ | CD25 expression variable | $CD25^{hi}$ |
| $CD44^{hi}$ | $CD44^{hi}$ | $CD44^{hi}$ |
| $CD45RA^{hi}$ | $CD45RA^{low}$ | $CD45RA^{low}$ |
| $CD45RO^{low}$ | $CD45RO^{hi}$ | $CD45RO^{hi}$ |
| $CD127^{low}$ | $CD127^{low}$ | $CD127^{hi}$ |
| $\underline{CTLA4^{low}}$ | $\underline{CTLA4^{hi}}$ | $CTLA4^{hi}$ |
| $\underline{CD103^{low}}$ | $\underline{CD103^{hi}}$ | $CD27^{hi}$ |
| $ICOS^{low}$ | $ICOS^{hi}$ | $ICOS^{hi}$ |
| $FoxP3^+$ | $FoxP3^+$ | $FoxP3^+$ |

**Table 4.2** Selected markers associated with activated Tregs at the peripheral sites [2].

| ***Peripheral sites*** | **Skin & lung inflammation** | **Gut homeostasis** | **Germinal centers** | **Adipose metabolism** |
|---|---|---|---|---|
| *Transcription factors* | FOXP3<br>T-bet | FOXP3<br>STAT3 | FOXP3<br>BCL-6 | FOXP3<br>PPARγ |
| *Chemokine receptors* | CXCR3<br>CCR4 | CCR6 | CXCR5 | Unknown |
| *Homeostatic mediators* | CD40L<br>IFNγ<br>IL-27<br>IL-7 | SCFA<br>IL-10<br>IL-6<br>IL-1 | unknown | Lipids<br>LCFA |

Based on their origin, CD4+ Tregs are divided into 'natural' Tregs (nTregs and also known as 'thymus' Tregs or tTregs) and induced Tregs (iTregs). While the nTregs (or tTregs) develop in the thymus during positive and negative selection, iTregs develop in the periphery from conventional CD4+ T cells following antigenic stimulation under a variety of conditions [5]. Both nTregs and iTregs are crucial for maintaining peripheral tolerance by suppressing potential autoimmune responses and also controlling responses to infection.

Similar to all T cells, nTregs arise from progenitor cells in the bone marrow and undergo their lineage commitment and maturation in the thymus. Around ~5-10% of peripheral CD4+ T cells are nTregs. While there are no specific cell surface markers to distinguish nTregs, there are however few surface proteins that are preferentially expressed on nTregs. These include higher expression of PD-1 (programmed cell death-1, pdcd1), neuropilin 1 (Nrp1), Helios (Ikzf2), and CD73 compared with iTregs. Both Tregs express the canonical Treg markers like CD25, Foxp3, GITR and CTLA4 [3, 6].

While the thymus-derived nTregs play a critical role in immune homeostasis, it is now well accepted that regulatory T cells can be induced from conventional, naïve CD4+ T cells in the periphery both *in vivo* and *in vitro*. These iTregs (induced Tregs) play important role in pathological conditions like cancers and inflammation [5]. Studies have described two main subsets of iTregs that are generated in the periphery based on the cytokines that cause their induction: type 1 regulatory T cells (Tr1) are induced by IL-10 and T helper 3 (Th3) are those that are induced by TGFβ [7, 8]. It has also been demonstrated that IL-4 and IL-13 can induce the development of Foxp3+ Tregs from Foxp3- naïve T cells independent of TGFβ and IL-10. Both IL-4 and IL-13 signal through the IL-4Rα chain indicating the importance of this receptor in the generation of Tregs in the periphery [9]. It is also interesting to note that the transcription factor Foxp3 is expressed by Th3 cells following induction but not in Tr1 cells. Finally, generation of nTregs also require co-stimulation through CD28, iTregs can develop in the periphery even without co-stimulation [10].

The various potential suppression mechanisms used by Tregs can be grouped into four basic modes of action:

1. Suppression by inhibitory cytokines (IL-10 & TGFβ)
2. Suppression by cytolysis
3. Suppression by metabolic disruption and
4. Suppression by modulation of dendritic cell (DC) maturation and function [11].

*Suppression by inhibitory cytokines*: The suppressive cytokines TGFβ and IL-10 have been implicated in the effector suppressive function of Tregs. The key role of TGFβ in lymphocyte homeostasis has been demonstrated by TGFβ KO mice that develop multi-organ immune pathology [12]. Tregs could also induce TGFβ production from other cells like antigen presenting cells (APCs) [13]. IL-10 production by Tregs has been shown to be essential for the prevention of colitis in mouse models of IBD [14, 15].

*Suppression by cytolysis*: Cell surface molecules implicated in suppression include cytolytic molecules (Fas and Granzyme B), LAG3 and cytotoxic T-lymphocyte antigen 4 (CTLA-4) [16, 17]. This involves Treg – cell-mediated target-cell killing mediated by granzyme A and perforin, through the adhesion of CD18; and Treg - cell-induced apoptosis of effector T cells mediated by granzyme B which is perforin independent. More recently, it has been shown that Tregs also kill B cells, NK (natural killer) cells and CTLs (cytotoxic T lymphocytes) in the tumor microenvironment in a granzyme B dependent and perforin-dependent manner [11].

*Suppression by metabolic disruption*: Few intriguing Treg suppressive mechanisms have been described recently and these together can be referred to as mechanisms that mediate effector T-cell metabolic disruption. These include IL-2 deprivation-mediated apoptosis of effector T cells. Since Tregs have enhanced CD25 (IL2Rα) expression, they consume local IL-2 and starve actively divided effector T cells. Moreover, Tregs enhance the production of adenosine through the concordant expression of ectoenzymes CD39 and CD73 which suppress effector T-cell function through activation of adenosine receptor 2A ($A_{2A}R$) [18, 19].

*Suppression by DC maturation and function*: Tregs interact with DCs through CTLA4 and suppressor DC-mediated activation of effector T-cells [20]. It was also shown that Tregs could condition DCs to express indoleamine 2,3-dioxygenase (IDO), a potent regulatory molecule which induces the production of pro-apoptotic metabolite from the catabolism of tryptophan, resulting in the suppression of effector T cells through a mechanism dependent on interactions between CTLA4 and CD80 and/or CD86 [21, 22].

Tregs play a prominent role in the resolution of lung injury. Depletion of Tregs in WT mice delayed recovery from lung injury and lymphocyte-deficient recombinase activating gene-1-null ($Rag1^{-/-}$) mice exhibited a profound impairment in the resolution of lung injury that was reversed by administration of isolated Tregs. Even though the resolution of lung injury was impaired in $Rag1^{-/-}$ mice, the pathogenesis of injury was similar in WT and $Rag1^{-/-}$ mice indicating that

lymphocytes play a role only during resolution of lung injury [23]. Several mechanisms by which Tregs mediate a resolution of lung injury have been studied. These include, Treg mediated resolution of fibroproliferation as seen in ALI is attributed to the reduction of fibrocyte recruitment by epithelial cells along the CXCL12-CXCR4 axis (in epithelial cells) [24]. Foxp3+ Tregs enhanced repair of lung epithelium after ALI through directly inducing alveolar type 2 (AT2) epithelial cell proliferation in a CD103-dependent manner [25]. Ultrafine particles in the airway that aggravate proinflammatory immune response mechanistically were responsible for this due to suppression of Treg function in LPS induced lung injury [26]. Strategies to enhance the resolution of lung injury also include phosphodiesterase antagonist pentoxifylline (PTX) induced increase in cAMP that may have partly restored the Treg/Th17 balance by modulating the transcription of Foxp3 and RORγt through the STAT3 pathway, thus enhancing Treg mediated resolution of lung injury [27]. More recently, Tregs role in protecting from transfusion-related acute lung injury (TRALI) was shown to occur via IL-10 [28].

It is important to note that most of the studies adopted the transfer of Tregs to enhance the resolution of lung injury. However, adoptive transfer of foreign cells can result in complications such as Graft vs Host Disease (GVHD) and immune intolerance. Hence, molecular mechanisms to enhance the quantity of self Tregs after lung injury in order to enhance the resolution of lung injury is required from a therapeutic point of view.

Results from two simultaneously conducted studies, recently published, have demonstrated that PTEN-mediated control of PI3K-Akt activity is critical for the function and stability of mouse Foxp3+ Treg cells. Interestingly, silencing PTEN in Tregs resulted in reduced CD25 expression leading to accumulation of Foxp3+CD25- cells, and ultimately, the loss of Foxp3 expression [29, 30]. Loss of PTEN in Tregs exacerbated $T_{FH}$ (T- follicular helper) and GC (germinal center) B cell response resulting in disrupted immune tolerance and homeostasis. PTEN controls the transcriptional program and metabolic balance in Tregs mainly via inhibition of

mTORC2 mediated Akt activity. Mechanistically, PTEN signaling pathway is likely to orchestrate both metabolic and transcriptional programs like those regulated by Foxo1 and Blimp1 and thus regulate Treg stability as well as Treg mediated suppression of $T_H1$ and $T_{FH}$ cells [30]. Another study at the same time identified PTEN signaling in Tregs as an important driver of the immunosuppressive milieu in tumors. Ablation of PTEN in Tregs resulted in the loss of their suppressive phenotype and conversion into proinflammatory helper cells (ex-Tregs) [31].

## 2. RATIONALE & HYPOTHESIS

Regulatory T cell-mediated resolution of ALI has been shown in previous studies. However, this resolution occurs only at later stages (after day 4 from the onset of ALI) where extensive damage has already occurred [23]. $T_{regs}$ have been shown to have enhanced expression of PTEN, a negative regulator of Akt. Moreover, $T_{regs}$ also decrease fibrocyte recruitment and thus inhibit fibro-proliferation in ALI [31]. Based on literature review and the work published from our lab, we hypothesize that Akt inhibition (from day 2 after the onset of ALI) could promote $T_{reg}$ induction, inhibit myofibroblast differentiation and thus enhance the resolution of Lung Injury.

## 3. METHODS AND MATERIALS

### 3.1 Experimental protocol for LPS induced acute lung injury model

Both male and female mice of equal quantity were randomly divided into 2 groups and each group contained 10 mice (equal number of males and females). The groups were as follows: 1. Control group (LPS injury with i.p. DMSO), 2. Treatment group (LPS injury with i.p. TCBN). LPS (5 mg/kg) was administered intra-tracheally (i.t.). All the mice in each group were euthanized and sacrificed after 7 days of LPS injury. Mice weights were monitored daily over the course of injury. The BAL, lung tissue specimens were collected at the time of sacrifice. Lower lobes of lungs were snap frozen for further molecular analysis and the left lung was used for sectioning and staining, while

the upper lobes of right lung was used for lung W/D ratio. Separate batch of animals were used for flowcytometry analysis of mice lung.

### 3.2 Lung wet/dry weight ratio

The wet/dry (W/D) ratio was assayed in the upper lobe of right lung to assess lung edema. The upper lobes of the right lung were excised, and the wet weights were determined. Then, the lung lobes were placed on sterile nonenzymic paper. After incubation at 80°C for 96 hours to remove all moisture, the dry weights were measured, and the W/D ratios were calculated.

### 3.3 Lung injury scoring

Lung injury was scored based on predefined criteria [23] by 3 blinded reviewers. Average of the three reviewers score was considered for analysis. Briefly, all lung fields at ×20 magnification were examined for each sample. Assessment of histological lung injury was performed by grading as follows [scoring from 1 to 5, with 1 being the best (normal lung) and 5 being the worst (severe most lung injury)]: 1, normal; 2, focal (<50% lung section) interstitial congestion and inflammatory cell infiltration; 3, diffuse (>50% lung section) interstitial congestion and inflammatory cell infiltration; 4, focal (<50% lung section) consolidation (Combining into a solid mass without the alveoli structure) and inflammatory cell infiltration and 5, diffuse (>50% lung section) consolidation and inflammatory cell infiltration. Therefore, score from 1 to 5; with 1 being the best (normal lung) and 5 being the worst (most injured lung).

### 3.4 BAL analysis

At the time of sacrifice, animals were anesthetized using i.p. ketamine/xylazine (100 mg/kg & 10 mg/kg respectively). When deep anesthesia was attained, the abdominal content and thorax were exposed through an abdominal midline incision, and exsanguination was performed through excision of the inferior vena cava. A tracheostomy tube was subsequently inserted and secured in the trachea, the diaphragm cut, and the chest wall opened via a midline incision to expose the

lungs. Lungs were then lavaged with 3 × 1 mL aliquots of saline, and each aliquot was instilled and withdrawn three times. The total volume of lavage fluid collected from each mouse was recorded. Lung lavage fluid was immediately centrifuged at 380 g for 10 minutes at 4°C to isolate the cell pellet; the supernatant was collected, and aliquots were frozen at −80°C for subsequent analyses.

### 3.5 Mice weight data

Daily weights of mice were measured to assess the recovery from lung injury. The weights of the mice were normalized to day 0 weights before comparison.

### 3.6 MPO activity assay

MPO activity assay was performed using Mouse MPO ELISA kit from Hycult biotech (Cat # HK210-02) according to manufacturer's protocol.

### 3.7 Western blot analysis

Western blot analysis was performed as described previously [32, 33]. Antibodies used include, P-Akt (s473) (Cat # 9271L), P-FoxO1/3a (Cat # 9464S) and FoxO3a (Cat # 2497S) from Cell Signaling, anti-b-actin from Sigma, St. Louis, MO, USA and mice Foxp3 from Biolegend (Cat # 126402).

### 3.8 Flow Cytometry

Mice lung lobes were digested with collagenase/Dispase (Sigma-Aldrich) 1mg/ml concentration and single cells were obtained by filtering through sterile 40uM gauge. Ack lysis buffer was used to disrupt RBC followed by fixation and permeabilization with Fix/perm (eBioscience Cat # 00-5223-56 & 43). The following Abs were used for surface staining: AlexFluor 700 conjugated anti-CD45 (Biolegend Cat # 103128), FITC conjugated anti-CD4 (eBioscience Cat # 11-0043-85), APC conjugated anti-Foxp3 (for internal staining, eBioscience Cat # 17-5773-82), PE-conjugated anti-CD103 ( & PE-conjugated anti-CTLA4 (eBioscience Cat # 61-1522-80). Lymphocytes were

gated with characteristic low forward scatter/side scatter, using BDFortessa and BDLSR II Flow cytometers. FlowJo was used for analysis of the data.

## 4. RESULTS

### 4.1 Time course of LPS-induced ALI and tissue pathology:

LPS induced lung injury is observed as early as 48hrs after i.t. LPS administration and peaks at day 4 after a single dose of i.t. LPS instillation. Resolution of lung injury starts after day 7 and majority of the mice completely recover by day 10 (Figure 1).

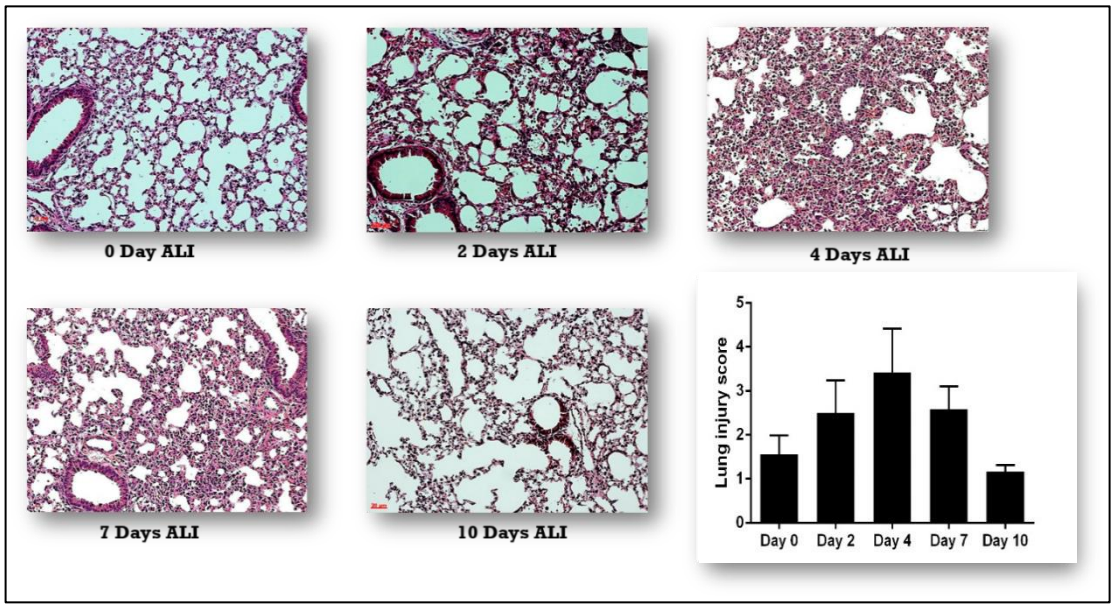
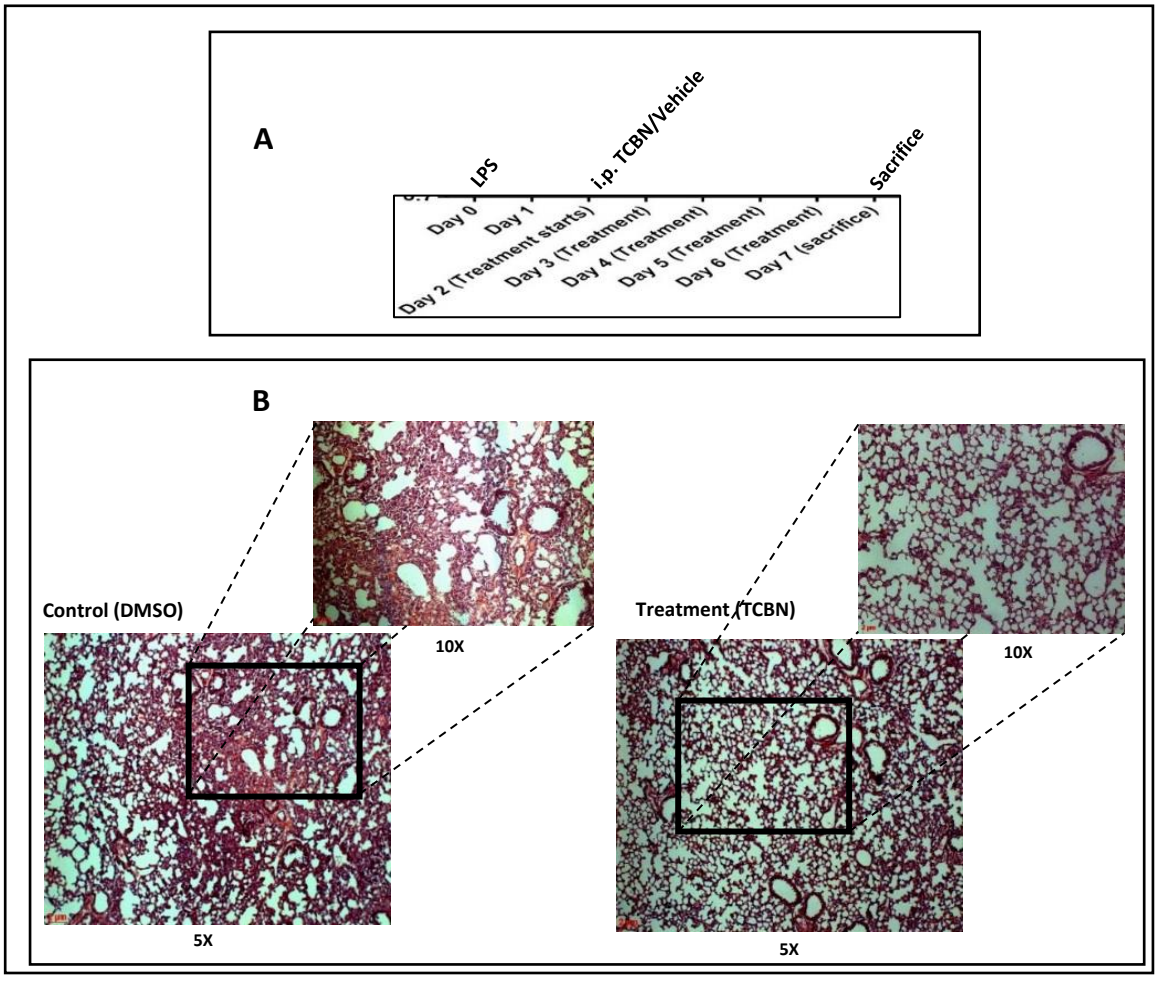



**Figure 3.1** *Single dose i.t. LPS 5mg/kg instillation on day 0 results in lung injury & edema from day 2 and peaks at day 4, lasting up to day 7. Injury resolution is enhanced after day 7 and mice recover from injury by day 10.*

### 4.2 Inhibition of Akt activity 48hrs after lung injury enhances injury resolution by day 7 post LPS instillation

Our preliminary studies investigating the alveolar inflammatory cell infiltration and histological evidence of lung injury, showed significant lung injury until day 7 after i.t. LPS instillation in C57BL6 mice. Resolution of lung injury was observed after Day 7. The lung injury was completely

resolved in mice by day 10 after a single i.t. LPS instillation at day 0 [5]. To test whether Akt inhibition enhances the resolution of lung injury through regulatory T-cells at day 7 after injury, C57BL6 lungs were administered with LPS and treated with Triciribine or vehicle (DMSO) 48 hrs after injury until sacrifice on day 7 (Figure 2A). Assessment of lung injury on day 7 including lung histology (Figure 2B-C), mice weights (Figure 2D), lung edema (Figure 2E), BAL protein (Figure 2F) and Lung MPO activity (Figure 2G) was performed. Significant improvement in lung injury scores was seen with the treatment accompanied by an improvement in body weights as well as reduced lung edema with treatment. BAL cell counts were increased with Akt inhibition indicating the enhanced resolution of lung injury with Akt inhibition in C57BL6 mice.



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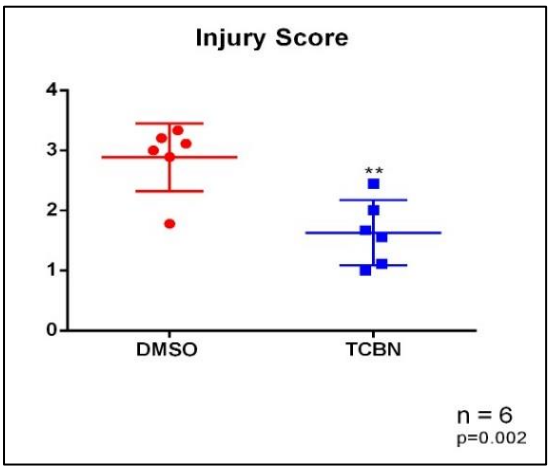
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3
2
1
0
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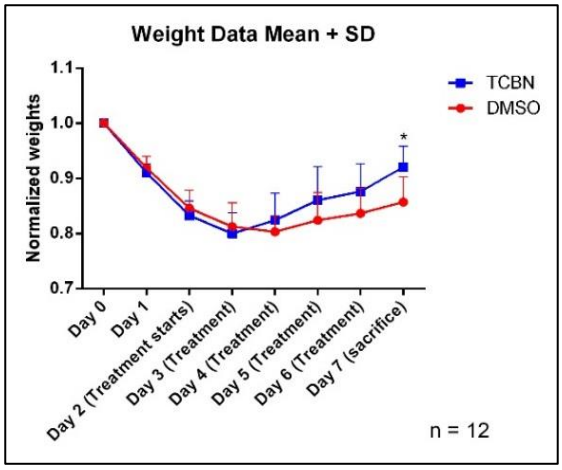
DMSO
TCBN
n = 6
p=0.002

D


Weight Data Mean + SD
Normalized weights
1.1
1.0
0.9
0.8
0.7
TCBN
DMSO
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Day 0
Day 1
Day 2 (Treatment starts)
Day 3 (Treatment)
Day 4 (Treatment)
Day 5 (Treatment)
Day 6 (Treatment)
Day 7 (sacrifice)
n = 12

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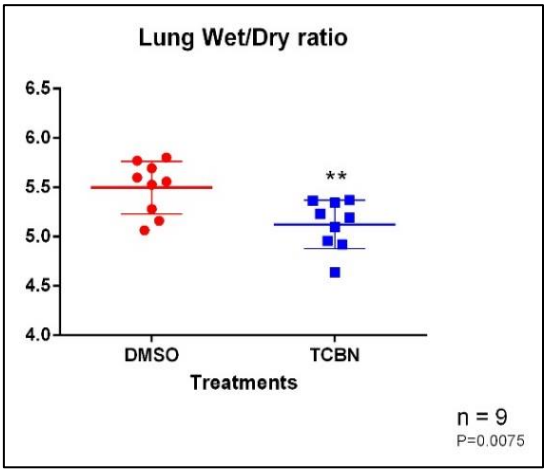
Lung Wet/Dry ratio
6.5
6.0
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4.5
4.0
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DMSO
TCBN
Treatments
n = 9
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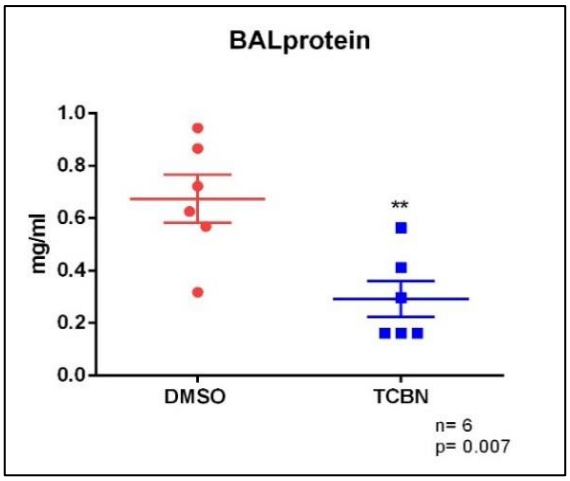
F


BALprotein
mg/ml
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DMSO
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n= 6
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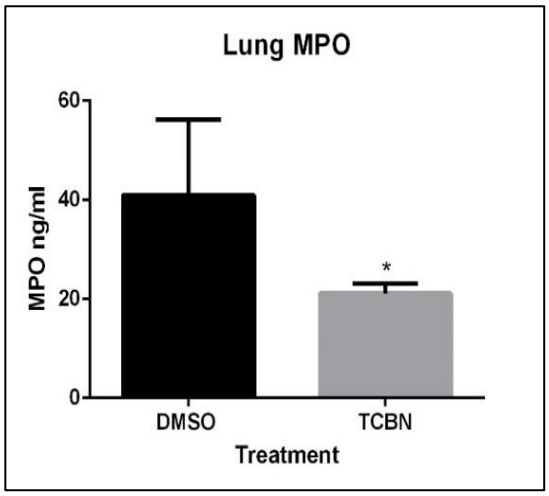
Lung MPO
MPO ng/ml
60
40
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DMSO
TCBN
Treatment

***Figure 3.2*** *Akt inhibition enhances the resolution of lung injury. A. The depiction of the experimental design. B. Representative H&E staining images of mice lung sections from control and treatment mice showing enhanced resolution of lung injury at day 7 with the TCBN treatment (n=6). C. Lung injury score (blindly scored by 3 reviewers) depict a significant reduction in lung injury at day 7 with TCBN treatment (n=6). D. Mice body weight measured over the period of 7 days after LPS injury shows a significant improvement (increase) in weight with the treatment (n=12). E. Lung wet/dry ratio as a measure of lung edema showed a significant reduction with TCBN treatment. F. Bronchoalveolar Lavage (BAL) protein was significantly decreased with treatment (n=6). G. Lung MPO levels were lower in the treatment group compared to control (n=6).*

### 4.3 Resolution of lung injury with Akt inhibition was associated with a significant increase in Lung $CD4^+/FoxP3^+/CD103^-/CTLA4^-$ resting as well as $CD4^+/FoxP3^+/CD103^+/CTLA4^+$ activated effector Tregs

Since Akt inhibition was associated with enhanced resolution of lung injury, we next sort to determine whether regulatory T-cells were involved in mediating the resolution. A single cell suspension of mice lungs and spleen cells were stained and subjected to flow cytometric analysis for effector regulatory T-cell markers. Akt inhibition enhanced $CD4^+/FoxP3^+/CD103^-/CTLA4^-$ resting Tregs in mice lungs (Figure 3A & 3C) as well as effector Tregs that were both $CD4^+/FoxP3^+/CD103^+$ (Figure 3A&3D) and $CD4^+/FoxP3^+/CTLA4^+$ Tregs (Figure 3B&3D). On the other hand, analysis for Tregs in lymphoid organ spleen with Akt inhibition after lung injury exhibited enhanced $CD4^+/FoxP3^+/CD103^-/CTLA4^-$ resting Tregs (Figure 4C) but not enhanced effector Tregs that were positive for either CD103 (Figure 4A&4D) or CTLA4 (Figure 4B&4D) surface markers. This indicates that Akt inhibition enhances the resolution of lung injury by specifically and consistently enhancing effector Tregs that were $CD4^+/FoxP3^+/CTLA4^+$ only at the site of injury (Figure 5A&B). Western blot analysis of mice lung lysates showed significant

decrease in p-FoxO1/3a (Figure 6A, C&D) and increase in Foxp3 expression (Figure 6A-B) with Akt inhibitor treatment.

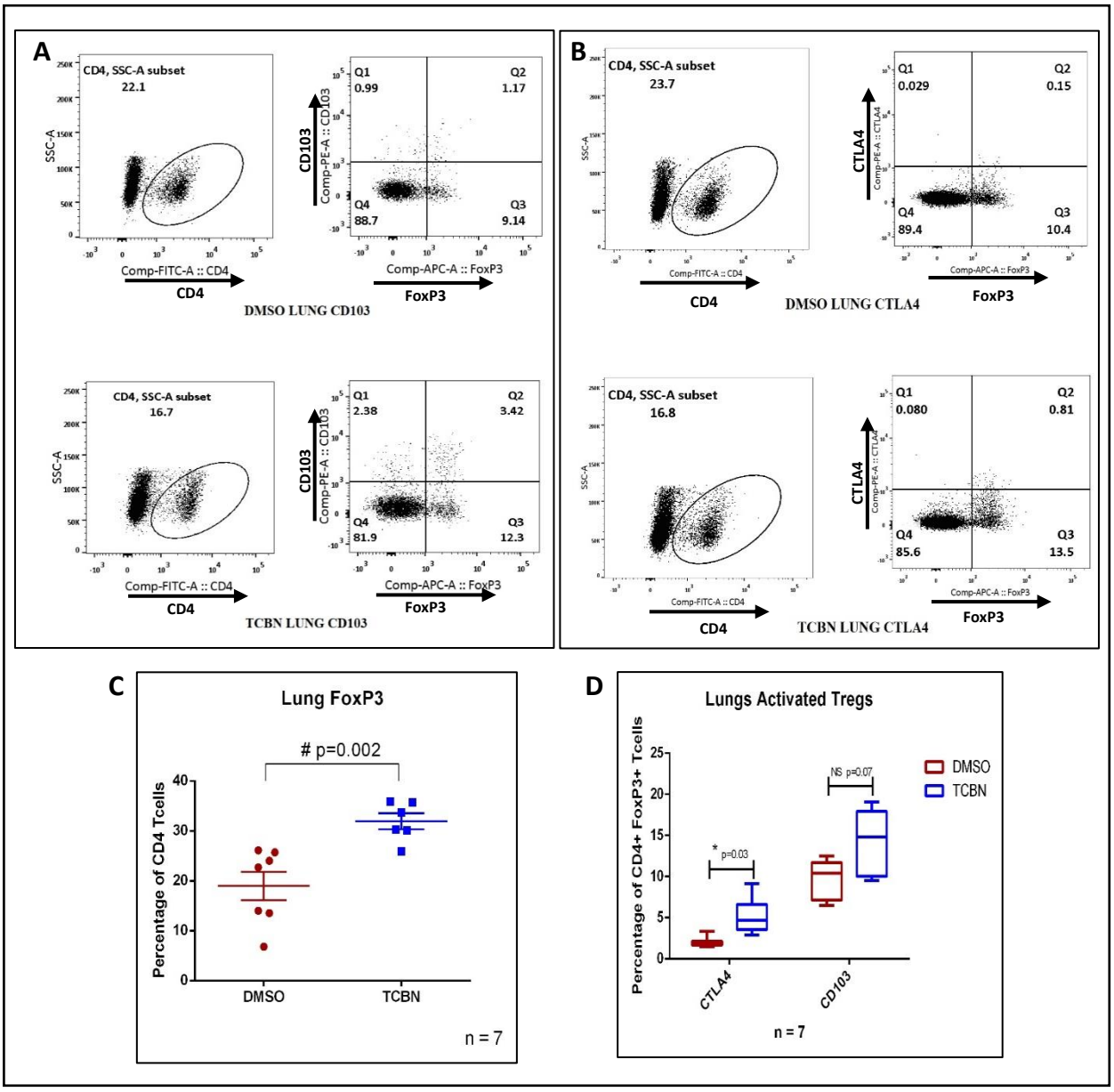


***Figure 3.3*** *A. Representative images of single cell suspension and staining of mice lungs for $CD4^+/FoxP3^+/CD103^+$ effector Tregs showed a significant increase in $CD4^+/FoxP3^+$ T-cells as well as $CD4^+/FoxP3^+/CD103^+$ Tregs with treatment (lower graph) as compared to control (upper graph). B. Single cell suspension and staining of mice lungs for $CD4^+/FoxP3^+/CTLA4^+$ Tregs showed a significant increase in $CD4^+/FoxP3^+$ T-cells as well as $CD4^+/FoxP3^+/CTLA4^+$ Tregs with*

*treatment (lower graph) as compared to control (upper graph). C. Analysis of lung Tregs showed a significant increase in the percentage of FoxP3$^+$ Tregs with the treatment compared to control. D. Analysis of activated Tregs in lungs showed a significant increase in CD4$^+$/FoxP3$^+$/CTLA4$^+$ effector Tregs and a strong trend for an increase in CD4$^+$/FoxP3$^+$/CD103$^+$ effector Tregs with the treatment compared to control.*

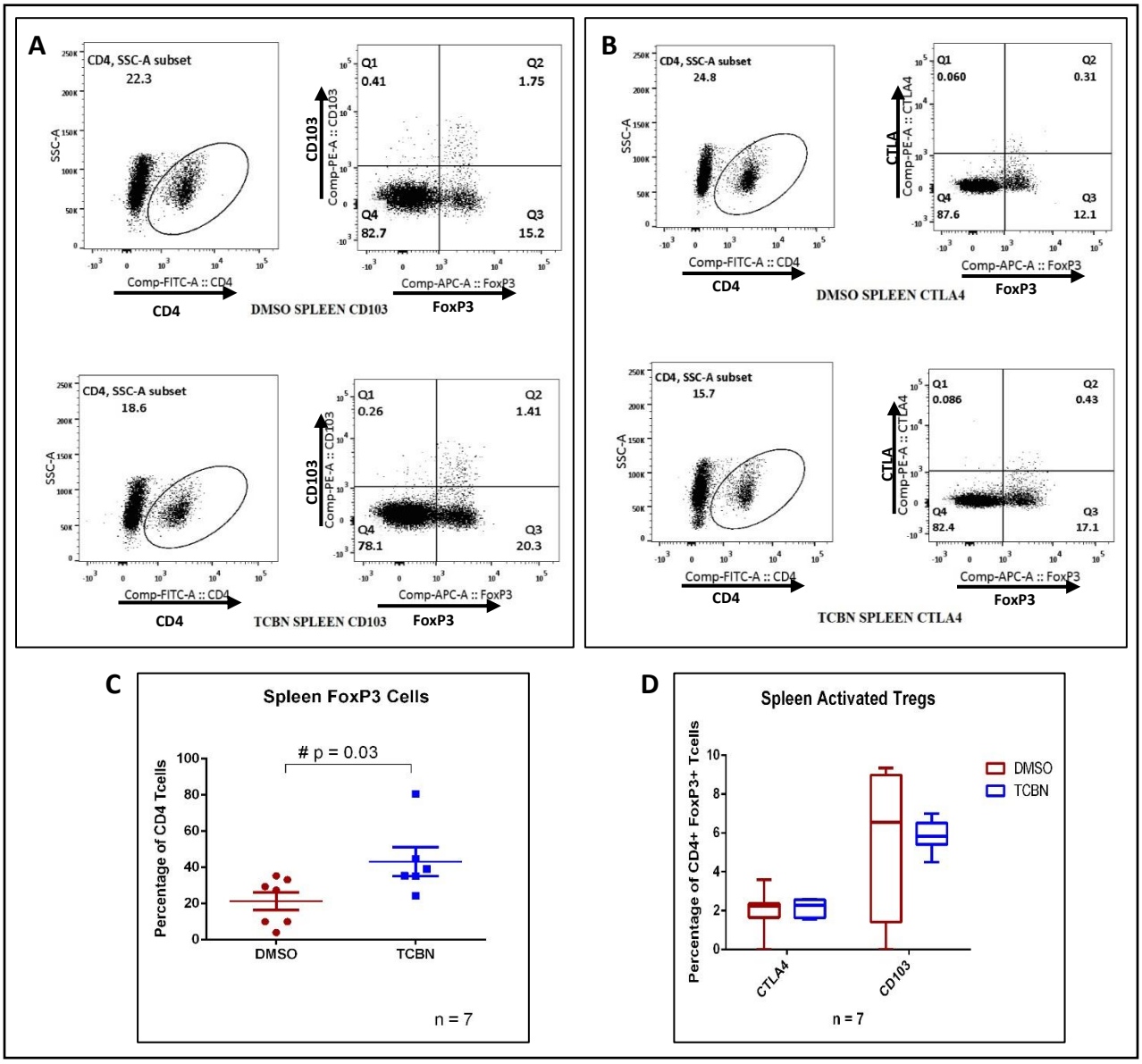


***Figure 3.4*** *A. Representative images of single cell suspension and staining of mice spleen for CD4$^+$/FoxP3$^+$/CD103$^+$ Tregs showed a significant increase in CD4$^+$/FoxP3$^+$ T-cells but not CD4$^+$/FoxP3$^+$/CD103$^+$ Tregs with treatment (lower graph) as compared to control (upper graph).*

*B. Single cell suspension and staining of mice spleen for $CD4^+/FoxP3^+/CTLA4^+$ Tregs showed a significant increase in $CD4^+/FoxP3^+$ T-cells but not $CD4^+/FoxP3^+/CTLA4^+$ Tregs with treatment (lower graph) as compared to control (upper graph). C. Analysis of spleen Tregs showed a significant increase in the percentage of $CD4^+/FoxP3^+$ Tregs with treatment compared to control, but not in activated/effector Tregs (Fig 4D) expressing activation markers $CTLA4^+$ or $CD103^+$.*

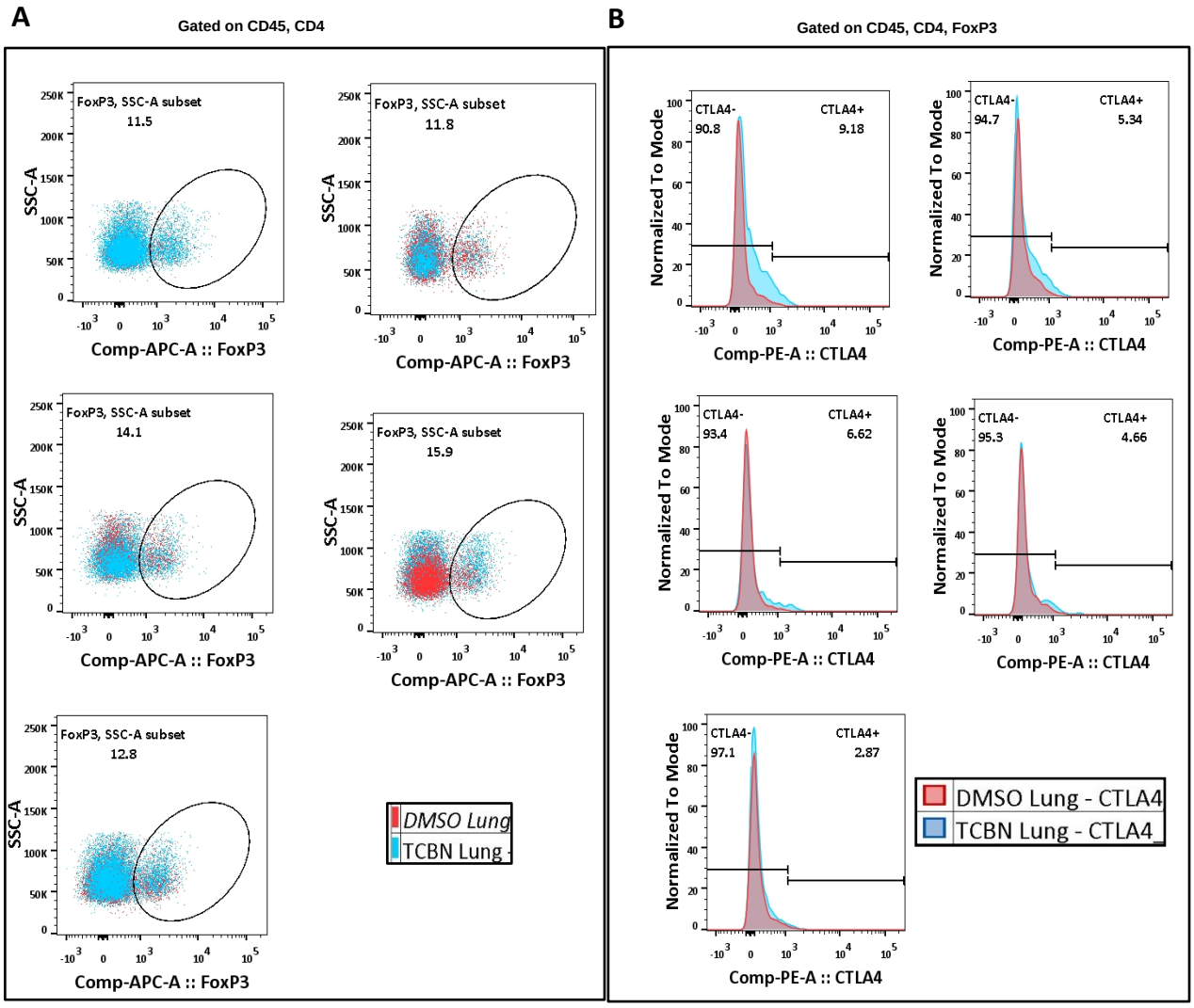


***Figure 3.5*** *A. A direct comparison (Control vs Treatment) of mice lung $CD4^+$ T-cells shows greater $CD4^+/FoxP3^+$ Tregs with TCBN treatment (blue) as compared to control (red). B. Direct*

*comparison of mice lung cells shows greater CD4$^+$/FoxP3$^+$/CTLA4$^+$ activated/effector Tregs with TCBN treatment (blue) as compared to control (red). (n=5)*

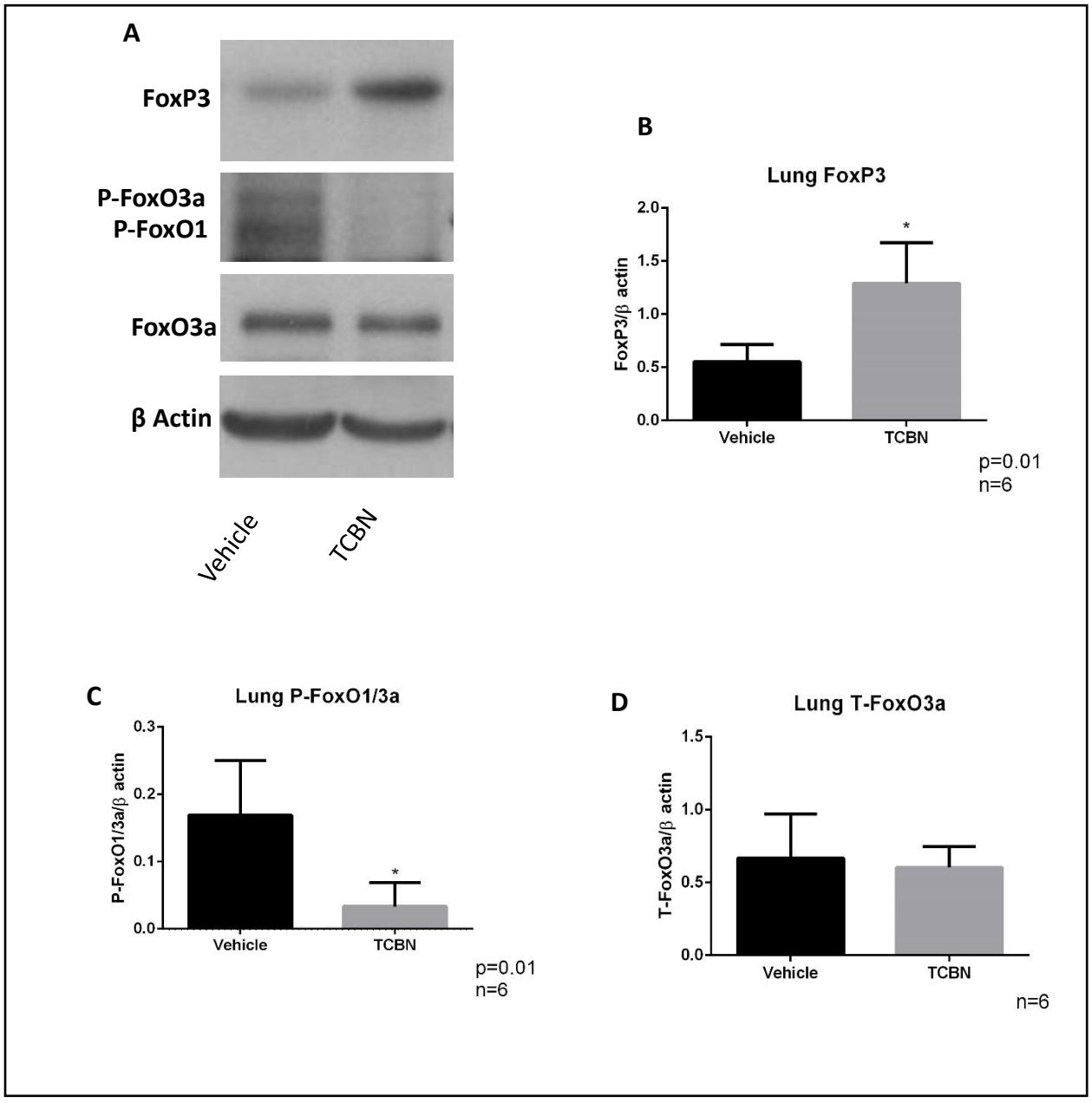


***Figure 3.6*** *Akt inhibitor activated FoxO1/3a and enhanced lung FoxP3 expression. A. Representative western blot images showing lung FoxP3, P-FoxO1/3a & FoxO3a expression. B-D. Quantitative analysis showing a significant increase in lung FoxP3 expression associated with decreased P-FoxO1/3a expression while total FoxO3a was comparable in both groups.*

### 4.4 *In vitro*, Akt inhibition in mouse T-lymphocytes enhanced FoxP3 expression

To confirm *in vivo* results, mice T-lymphocyte cell line (EL-4) were treated with two Akt inhibitors (TCBN & MK-2206) or vehicle for 72 hrs. These cells were then subjected to western blot analysis for protein expression. Both the Akt inhibitors reduced Akt phosphorylation and the more prominent Akt inhibitor MK-2206 enhanced FoxP3 expression after 72 hrs. of treatment (Figure 7). Note that 24 hrs. of treatment were not sufficient to produce this effect (data not shown) indicating that this is a long-term process. Flowcytometry analysis of these cells showed a significant increase in FoxP3 expressing Tregs with TCBN treatment and MK (2,5&10uM) treatment (Figure 8 A-E). The activated CD4+/FoxP3+/CTLA4+ Tregs (Fig.8F) and CD4+/FoxP3+/CD103+ Tregs (Fig. 8G) were significantly enhanced with a higher dose of MK compound.

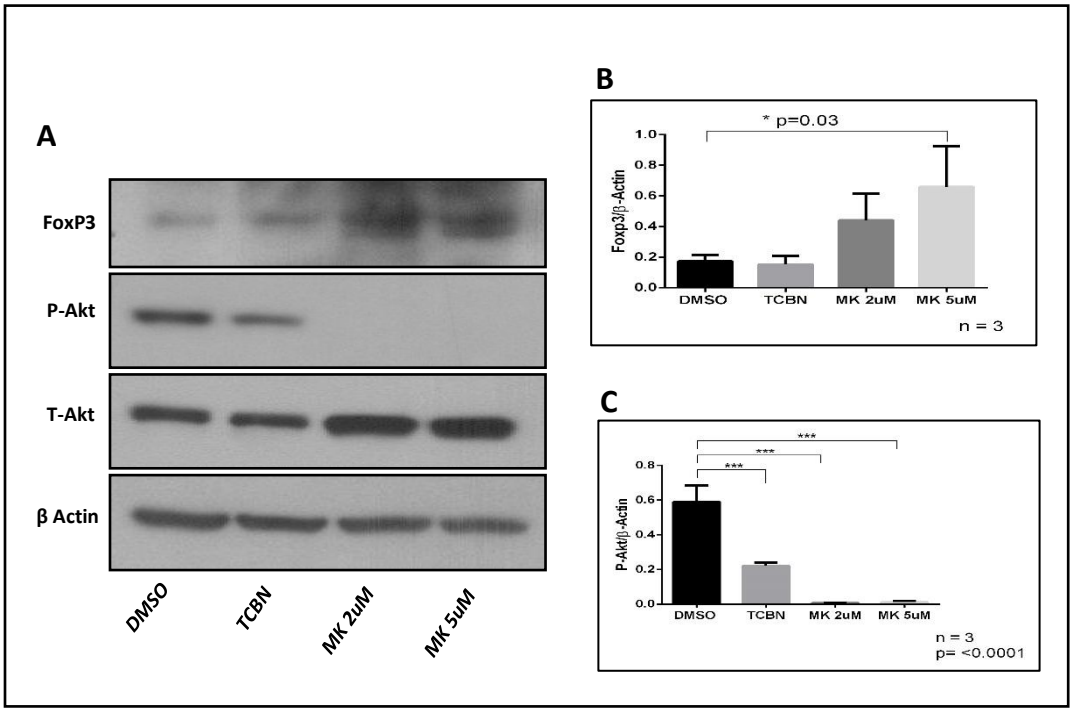


***Figure 3.7*** *In vitro Akt inhibition induced iTregs from mouse T-Lymphocytes. A. EL-4 cells were treated with 2 Akt inhibitors (TCBN & MK-2206) for 72 hrs. B-C. Both treatments suppressed Akt phosphorylation at S473 (Fig 7c) but MK 5uM also enhances FoxP3 expression significantly (Fig*

*7b) in these cells compared to vehicle treatment. MK had a greater effect on inhibiting Akt phosphorylation and enhancing FoxP3 expression compared to* TCBN.

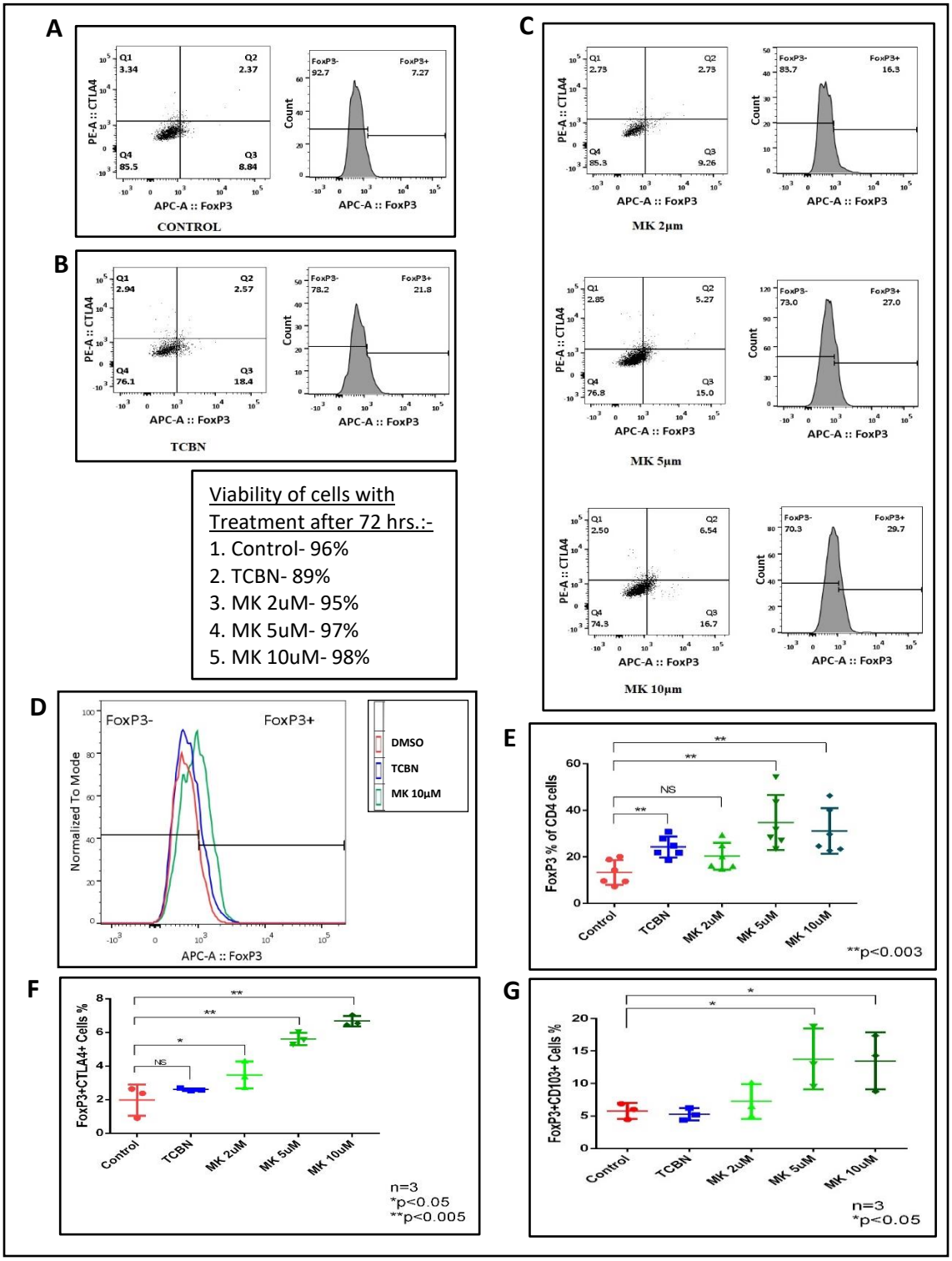

***Figure 3.8*** *A-C. Representative Flow cytometry graphs showing enhanced FoxP3 expression in EL4 T-lymphocytes after 72 hours of treatment with 2 Akt inhibitors – TCBN 10uM & MK-2206 (2uM, 5uM & 10uM). D-E. Akt inhibition significantly enhanced the percentage of FoxP3 cells in EL-4 lymphocytes with the effect being greater with a higher dose of MK. F. Akt inhibition with MK treatment increased $CD4^+$/$FoxP3^+$/$CTLA4^+$ effector Tregs. G. Akt inhibition with MK (only 5 & 10 uM) treatment increased $CD4^+$/$FoxP3^+$/$CD103^+$ effector Tregs.*

### 4.5 FoxP3 specific PTEN KO mice had greater lung edema, neutrophil activity, and lesser FoxP3+ Tregs at the site of injury as well as fewer activated CTLA4 expressing Tregs

To confirm the hypothesis that Akt signaling in Tregs is responsible for Treg activation, expression of activated markers like CTLA4 and resolution of lung injury; Foxp3 specific PTEN KO mice (Foxp3-PTENcre) that possess enhanced Akt activity in Tregs were obtained from collaborator and homozygous KO was generated. Foxp3PTENCre mice were then subjected to i.t. LPS injury and compared with respective WT littermates. Injury resolution at day 7 was impaired in these mice. Pulmonary edema as measured by Lung wet/dry ratio was enhanced in these mice (Figure 9A) while the CD4+/FoxP3+/CTLA4- resting Tregs were significantly decreased (Figure 9B). Neutrophil activity was enhanced in Foxp3PTENCre mice further indicating impaired injury resolution at day 7 in these mice (Figure 9C). Foxp3 expression in lung lysates was significantly reduced in Foxp3PTENCre mice indicating that enhanced Akt activity in these Tregs suppresses Foxp3 expression (Figure 9D&E). Activated Tregs that were $CD4^+$/$FoxP3^+$/$CTLA4^+$ cells were absent (Figure 9F&G). Therefore, impairment of lung injury resolution in Foxp3PTENCre (KO mice) was associated with decreased effector Tregs and since the only difference between WT and Foxp3PTENcre is the enhanced Akt activity in Tregs, this indicates the importance of inhibiting Akt activity in Tregs to enhance the resolution of lung injury.

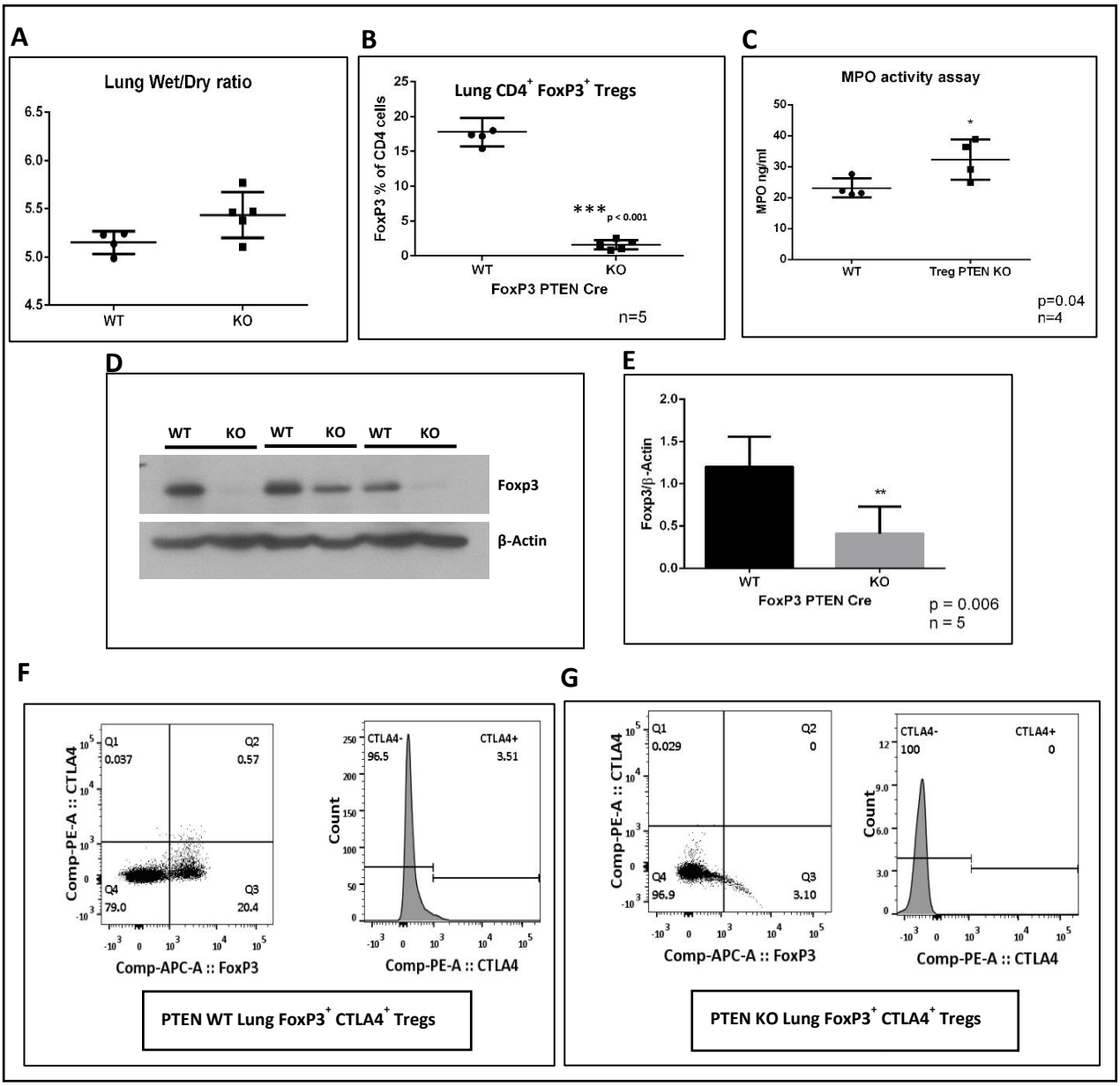


**Figure 3.9** *A. Lung wet/dry ratio measured after 7 days of lung injury showed greater edema in KO mice compared to littermate WT. B. Lung FoxP3+ Tregs percentage was greater in WT littermates compared to KO among the Foxp3-PTENCre mice. C. MPO levels in mice lungs were enhanced in KO mice. D&E. Western blot of mice lung lysates showed decreased Foxp3 expression in KO mice compared to WT. F&G. Analysis for activated Tregs showed null $CD4^+/FoxP3^+/CTLA4^+$ Tregs in KO mice while the WT littermates expressed $CD4^+/FoxP3^+/CTLA4^+$ Tregs at day 7 after lung injury.*

## 5. DISCUSSION

Extensive progress has been made in understanding the pathophysiology of ARDS, but we still lack a therapy to completely prevent mortality in ARDS patients. Supportive care including ventilation & prone positioning is the current management for ARDS as specific pharmacological therapies have not been successful. ARDS has a mortality of 32-45%. Since 2010, the overall mortality rates in-hospital is 45%, ICU 38%, 28/30-day mortality of 30% and 60-day mortality was found to be 32% [34]. The cause of death in patients with ARDS is not lung pathology per se but mostly concurrent extra-pulmonary organ dysfunction. However, early mortality is mostly associated with lung injury alone and the late mortality is associated with complications such as multiple organ failure [34, 35]. Irreversible damage to the lung associated with multiple organ failure is due to uncontrolled injury, inflammation, and fibro-proliferation in ARDS. It is therefore important to prevent persistent inflammation and enhance the resolution of lung injury in order to reduce mortality.

$CD4^+/CD25^+/Foxp3^+$ Tregs mediate the resolution of lung injury by modulating innate immune responses. The role of Tregs in the resolution of lung injury has been shown recently. Intriguingly, while Tregs do not play a role in the pathogenesis of lung injury, they play a prominent role in the resolution of injury. However, LPS induced lung injury and inflammation results in a decrease in the percentage of alveolar $Foxp3^+$ Treg [23]. Molecular mechanisms that enhance $Foxp3^+$ Tregs at the site of injury (iTregs) are not clear. In the current study, we show the importance of inhibiting Akt activity and activating downstream FoxO1/3a in enhancing the number of iTregs at the site of injury. Akt inhibitor TCBN enhanced the resolution of lung injury 7 days after i.t. LPS instillation. Akt inhibitor reduced lung injury score, decreased lung wet/dry ratio (pulmonary edema), improved mice body weights, and decreased neutrophil activity in mouse lungs on day 7 as compared to vehicle treatment. Further, to investigate the beneficial effects of Akt inhibitor given 48 hrs after injury, we studied the total number of $CD45^+/CD4^+/Foxp3^+/CD103^+$

effector Tregs & $CD45^+/CD4^+/Foxp3^+/CTLA4^+$ effector Tregs in mice lungs and spleen. Interestingly, while Akt inhibitor enhanced $CD4^+/FoxP3^+/CD103^-/CTLA4^-$ resting Tregs in both lungs and spleen of mice, the activated or effector Tregs that were $CD4^+/Foxp3^+/CTLA4^+/CD103^+$ Tregs were enhanced only in mice lungs (site of injury) as opposed to the spleen. Western blot analysis of mice lung treated with Akt inhibitor showed a simultaneous decrease in p-FoxO1/3a along with increased Foxp3 expression. This indicates the role of activated FoxOs downstream of inhibited Akt in the expression of Foxp3.

Previous studies have indicated that co-stimulation through CD28, although required for generation of nTregs, was not necessary for the development of iTregs [5, 10, 36]. Moreover, a recently published study used EL4 T lymphocytes to induce Tregs in absence of co-stimulation [37]. In our study, we used two Akt inhibitors TCBN 10uM and MK-2206 (2uM,5uM & 10uM) on EL4 cells to test our hypothesis that Akt inhibition and downstream FoxO activation *in vitro* alone is sufficient to induce Foxp3 expression. After 72 hrs. of Akt inhibition (while 24 hrs. did not have any effect) in mouse EL-4 T-lymphocytes, enhanced Foxp3 expression was observed. The more potent Akt inhibitor MK-2206 compound treatment also enhanced $CD4^+Foxp3^+CTLA4^+CD103^+$ effector Tregs at higher doses as measured by flow cytometry. While partial inhibition of Akt phosphorylation was not sufficient to enhance effector Treg markers, prominent inhibition of Akt phosphorylation was required to enhance effector Treg markers expression. These results confirm the role of Akt inhibition in enhancing the number of effector iTregs.

Loss of PTEN, a negative regulator of Akt, in Tregs results in uncontrolled Akt activity leading to loss of Foxp3 expression and disrupted immune tolerance [29-31]. Since injury resolution is dependent on Treg activity, we sort to determine the effect of lung injury in Foxp3PTENCre mice that lack PTEN expression in Foxp3 expressing Tregs. Disruption of PTEN in Tregs resulted in reduced CD25 expression, eventual loss of Foxp3 expression and lymphoproliferative disease in mice at age of 15 weeks or later [31]. Here, 8-12 weeks old mice

were used for LPS induced lung injury. Lung injury resolution was impaired in Foxp3PTENCre mice in comparison to WT mice, as evidenced by increased pulmonary edema, enhanced lung MPO activity and enhanced lung injury. Lack of activated/effector $CD4^+/Foxp3^+/CTLA4^+$ Tregs was associated with impaired injury resolution in these mice. Since the only difference in these two groups was enhanced Akt activity in Tregs, these results indicate the importance of inhibiting Akt activity to prevent excessive inflammation and enhance injury resolution in LPS induced lung injury in mice. Moreover, inhibition of Akt activity not only enhanced Treg quantity but is also required for Treg activity and its effect on the resolution of lung injury.

In conclusion, our study provides a potential therapeutic target to enhance Tregs mediated resolution of lung injury and prevent irreversible damage to the lung occurring during the fibroproliferative phase of ARDS. Akt inhibitor could be used after the exudative stage of ARDS to enhance the resolution of lung injury and restore lung function.

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# CHAPTER 4

# INTEGRATED DISCUSSION AND TRANSLATIONAL IMPACT

## Discussion

Acute Respiratory Distress Syndrome is an acute inflammatory lung injury, characterized by enhanced pulmonary vascular permeability, edema, increased lung weight and loss of aerated lung tissue [1]. Among ICUs in 50 countries, the period prevalence of ARDS was 10.4% of ICU admissions [2]. Unfortunately, because no validated diagnostic biomarkers are yet available, ARDS diagnosis and definition heavily relies on clinical features and chest imaging as surrogates [3]. On the other hand, the priority in the treatment of ARDS is identification and treatment of underlying cause of lung injury [4]. Other than treating the cause for damage, there is no specific pharmacological treatment for preventing further lung injury or enhancing resolution of injury. Supportive care remains the mainstay for limiting further lung damage and improving lung function. Supportive care includes lung-protective ventilation to prevent ventilator-associated lung injury and conservative fluid therapy to prevent lung edema formation and promote lung edema resorption. However, the optimal approach to lung protective ventilation is unknown.

Recent studies indicate that ARDS continues to have a high mortality despite the advances in supportive care. There was a significant increase in mortality with increase in the ARDS severity category with an overall average of 40% mortality in ARDS patients admitted to hospital [2, 5]. Another reason for high mortality could be the heterogeneity in the physiology and response to therapy where the universal application of certain treatment strategies may not be expected or advisable. For instance, low tidal volume ventilation might result in hypercapnia that may further exacerbate intracranial hypertension in brain-injured patients and patients with unstable spines or major fractures cannot safely be ventilated in the prone position [5]. Hence the two major ARDS supportive care (low tidal volume ventilation & prone positioning) might be ineffective or unreasonable in several patients. This high mortality rate supports the need for new and effective therapies that can reduce ARDS mortality.

Overall, this project aims to provide stage specific therapeutic targets for ARDS. No single animal model reproduces all the complex characteristics of ARDS in humans. We used LPS-

induced lung injury for several reasons. This model is highly reproducible, extensively characterized with distinct severities of lung injury and injury resolution observed over a period of time. Moreover, this model has been widely employed by several studies that scrutinized pathophysiology of the disease. Finally, LPS is a potent activator of innate immune pathways and can produce diffuse lung injury without prohibitive mortality thus facilitating a focused evaluation on resolution of acute lung injury. Here, using LPS induced lung injury as a model for ARDS, we show a reciprocal regulation of Akt-FoxO signaling in the initial vs later phases of ARDS (Figure 1).

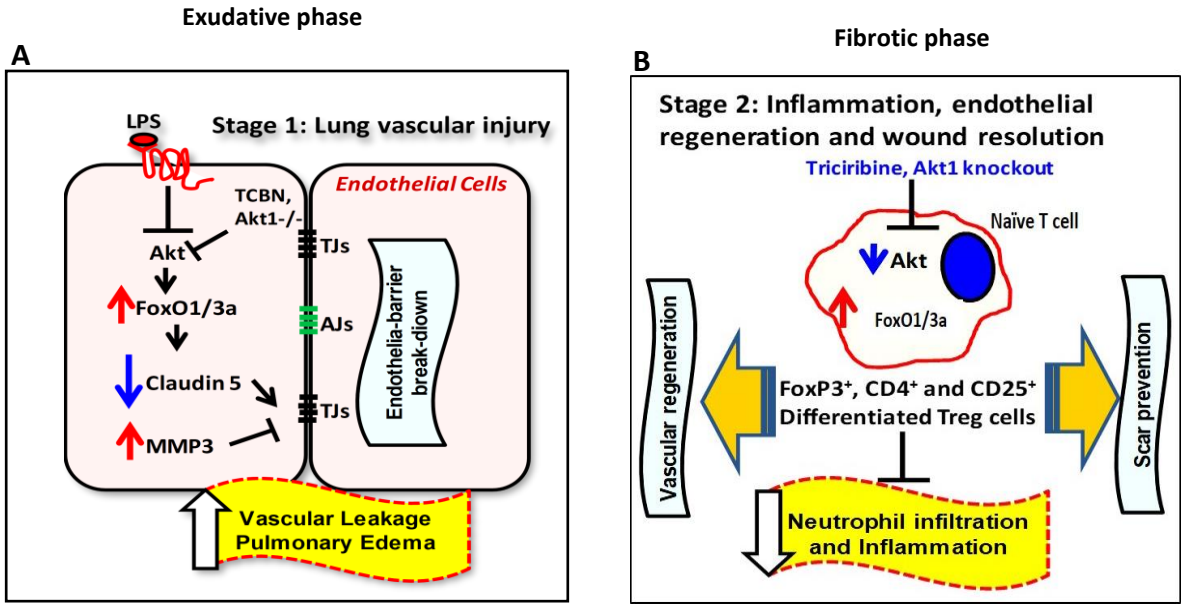


***Figure 4.1*** *A. During the initial exudative stage of LPS induced lung injury, Inhibition of Akt and downstream activation of FoxOs in EC is deleterious as it enhances pulmonary edema. B. During the later fibrotic stage of lung injury, Inhibition of Akt and downstream activation of FoxOs in T-lymphocytes is beneficial as it enhances $CD4^+Foxp3^+CTLA4^+$ effector Treg mediated resolution of lung injury.*

In the initial exudative stage, LPS induced lung injury is associated with reduced Akt1 phosphorylation in lung endothelial cells which results in FoxO1/3a mediated suppression of claudin-5 expression, enhanced MMP3 expression and activity along with barrier disruption ensuing pulmonary edema. Intriguingly, inhibition of Akt and activation of downstream FoxO1/3a results in enhanced expression of $CD4^+$/$FoxP3^+$/$CD103^+$/$CTLA4^+$ effector Tregs at the site of

injury, thus suppressing excessive inflammation and enhancing resolution of lung injury in the later stage. While the exudative phase could be potentially prevented by targeting FoxOs locally in the lungs for high risk patients in ICU, the more severe fibrotic phase could benefit from Akt inhibition leading to increased iTregs mediated resolution of lung injury. Thus, our study provides stage specific therapeutic targets for ARDS.

The lack of recognition of ARDS by clinicians may also limit the implementation of effective therapies and enhance mortality. According to recent clinical study, ARDS was underdiagnosed, with 39.8% of all patients with ARDS being clinician-unrecognized [2]. The probability of clinician recognition of ARDS increased with increase in severity of the disease, higher nurse-to-patient ratios, higher physician-to-patient ratios, younger patient age, lower PaO2/FIO2 ratio and the presence pf pneumonia or pancreatitis [2]. This indicates the need for a reliable diagnostic marker that is convenient to measure, can be used by clinicians to recognize ARDS at an early stage and thus can reduce mortality by decreasing the percentage of underdiagnosed patients.

During our study in pursuit of potential therapeutic targets for ARDS, we made an ‘astute observation’ that MMP3 activity correlated with the extent of lung injury in mice. Since, MMP3 activity can be measured in body fluids, we compared MMP3 activity in serum & plasma samples of ARDS patients with that of healthy volunteers. A significant 3-fold increase in MMP3 activity was observed in the ARDS patient samples. In accordance to these results, with reasonable optimism we believe that these results indicate the utility of MMP3 activity as a diagnostic marker for ARDS. This needs further confirmation.

## Translational impact

Our study indicates that MMP3 activity could be used as an early diagnostic marker and prognostic marker for ARDS. While FoxO inhibitor and MMP3 inhibitors would be beneficial in the initial exudative stage of ARDS, Akt inhibitor could be beneficial to enhance resolution of lung injury during the fibrotic stage of ARDS.

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# Appendix

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# TNFα induces inflammatory stress response in microvascular endothelial cells via Akt- and P38 MAP kinase-mediated thrombospondin-1 expression

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**Abstract** Tumor necrosis factor-α (TNFα) and thrombospondin-1 (TSP-1) are well-known mediators of inflammation. However, a causal relationship between TNFα stimuli and TSP-1 expression in endothelial cell stress, and the underlying mechanisms has not yet been investigated. In our study, human microvascular endothelial cells (hMEC) were treated with TNFα and analyzed for endothelial dysfunction, TSP-1 expression, and associated mechanisms. TNFα treatment induced a dose-dependent increase in TSP-1 expression in hMEC associated with increased endothelial permeability, apoptosis, and reduced proliferation. Whereas TNFα activated Akt, ERK, and P38 mitogen-activated protein kinase (P38 MAPK) simultaneously in hMEC, inhibitors of Akt and P38 MAPK, but not ERK blunted TNFα-induced TSP-1 expression. Silencing of NFκB gene had no significant effect on TNFα-induced TSP-1 expression. Our study demonstrates the novel role of TNFα in inducing inflammatory stress response in hMEC through Akt- and P38 MAPK-mediated expression of TSP-1, independent of NFκB signaling.

**Keywords** TNFα · TSP-1 · Inflammation · Endothelium · P38 MAPK · Akt

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**Abbreviations**

| | |
|---|---|
| TNFα | Tumor necrosis factor-α |
| P38 MAPK | P38 mitogen-activated protein kinase |
| TSP-1 | Thrombospondin-1 |
| MAPK | Mitogen-activated protein kinase |
| ERK | Extracellular regulated kinase |
| hMEC | Human microvascular endothelial cells |
| ECIS | Electric cell-substrate impedance sensing |

## Introduction

Tumor necrosis factor alpha (TNFα), a mediator of inflammation, acts on the endothelium, the first line of action during inflammatory stimuli [1]. Activated macrophages, the major source of TNFα, have the ability to lyse many cell types including tumor cells [2]. As a result of the cellular stress inflicted by TNFα, endothelial cells undergo apoptosis [3] leading to barrier breakdown and increased vascular permeability aiding migration of inflammatory cells into the tissue environment [2, 4]. TNFα deregulation has been reported in many human diseases such as Alzheimer's disease [5], cancer [6], depression [7], and inflammatory bowel disease [8]. Although TNFα signaling pathway is considered as a new therapeutic strategy for inflammatory diseases [9], lack of knowledge on the precise molecular and cellular events in TNFα-induced stress response limits its application in anti-inflammation therapy.

Thrombospondin-1 (TSP-1) is a multifunctional 450 kDa glycoprotein, which is secreted by many cell types including endothelial cells, and are also stored in the platelet α-granules [10]. Whereas macrophages, fibroblasts, and endothelial cells are known to lodge cell surface TSP-1

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Cellular and Molecular Life Sciences

ORIGINAL ARTICLE

# Akt1 promotes stimuli-induced endothelial-barrier protection through FoxO-mediated tight-junction protein turnover

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**Abstract** Vascular permeability regulated by the vascular endothelial growth factor (VEGF) through endothelial-barrier junctions is essential for inflammation. Mechanisms regulating vascular permeability remain elusive. Although 'Akt' and 'Src' have been implicated in the endothelial-barrier regulation, it is puzzling how both agents that protect and disrupt the endothelial-barrier activate these kinases to reciprocally regulate vascular permeability. To delineate the role of Akt1 in endothelial-barrier regulation, we created endothelial-specific, tamoxifen-inducible Akt1 knockout mice and stable ShRNA-mediated Akt1 knockdown in human microvascular endothelial cells. Akt1 loss leads to decreased basal and angiopoietin1-induced endothelial-barrier resistance, and enhanced VEGF-induced endothelial-barrier breakdown. Endothelial Akt1 deficiency resulted in enhanced VEGF-induced vascular leakage in mice ears, which was rescued upon re-expression with Adeno-myrAkt1. Furthermore, co-treatment with angiopoietin1 reversed VEGF-induced vascular leakage in an Akt1-dependent manner. Mechanistically, our study revealed that while VEGF-induced short-term vascular permeability is independent of Akt1, its recovery is reliant on Akt1 and FoxO-mediated claudin expression. Pharmacological inhibition of FoxO transcription factors rescued the defective endothelial barrier due to Akt1 deficiency. Here we provide novel insights on the endothelial-barrier protective role of VEGF in the long term and the importance of Akt1-FoxO signaling on tight-junction stabilization and prevention of vascular leakage through claudin expression.

**Keywords** VEGF · Angiopoietin-1 · Akt · VE-cadherin · Claudin · Vascular permeability

**Abbreviations**

| | |
|---|---|
| VEGF | Vascular endothelial growth factor |
| Ang-1 | Angiopoietin-1 |
| VECad-Cre-Ak1 | Vascular endothelial cadherin-cre recombinase-Akt1 knockdown |
| HMEC | Human microvascular endothelial cells |
| FoxO | Forkhead box protein O |
| ECIS | Electric cell-substrate impedance sensing |
| WT | Wild type |
| GFP | Green fluorescent protein |
| eNOS | Endothelial nitric oxide synthase |

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RAPID COMMUNICATION

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# Modulation of long-term endothelial-barrier integrity is conditional to the cross-talk between Akt and Src signaling

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Although numerous studies have implicated Akt and Src kinases in vascular endothelial growth factor (VEGF) and Angiopoietin-1 (Ang-1)-induced endothelial-barrier regulation, a link between these two pathways has never been demonstrated. We determined the long-term effects of Akt inhibition on Src activity and vice versa, and in turn, on the human microvascular endothelial cell (HMEC) barrier integrity at the basal level, and in response to growth factors. Our data showed that Akt1 gene knockdown increases gap formation in HMEC monolayer at the basal level. Pharmacological inhibition of Akt, but not Src resulted in exacerbated VEGF-induced vascular leakage and impaired Ang-1-induced HMEC-barrier protection in vitro at 24 hr. Whereas inhibition of Akt had no effect on VEGF-induced HMEC gap formation in the short term, inhibition of Src blunted this process. In contrast, inhibition of Akt disrupted the VEGF and Ang-1 stabilized barrier integrity in the long-term while inhibition of Src did not. Interestingly, both long-term Akt inhibition and Akt1 gene knockdown in HMECs resulted in increased Tyr416 phosphorylation of Src. Treatment of HMECs with transforming growth factor-β1 (TGFβ1) that inhibited Akt Ser473 phosphorylation in the long-term, activated Src through increased Tyr416 phosphorylation and decreased HMEC-barrier resistance. The effect of TGFβ1 on endothelial-barrier breakdown was blunted in Akt1 deficient HMEC monolayers, where endothelial-barrier resistance was already impaired compared to the control. To our knowledge, this is the first report demonstrating a direct cross-talk between Akt and Src in endothelial-barrier regulation.

**KEYWORDS**
Akt, endothelial-barrier, Src, vascular permeability, VE-cadherin

## 1 | INTRODUCTION

Vascular permeability is a complex yet a highly coordinated process that not only regulates vesicular trafficking but also integrates complex junction rearrangements, and refined cytoskeletal dynamics (Goddard & Iruela-Arispe, 2013). The endothelium plays a key role in regulating vascular integrity. Recent studies suggest that impairment of endothelial function, as observed in the presence of cardiovascular risk factors, is not only a marker but also contributes to the pathogenesis of cardiovascular diseases (Landmesser, Hornig, & Drexler, 2004). Thus, improving endothelial function is an important therapeutic target for reducing vascular diseases (Bonetti, Lerman, & Lerman, 2003; Melo et al., 2004). It is evident that Akt1 is highly involved in the vascular endothelial growth factor (VEGF)-mediated vascular permeability as the phosphorylation of Akt1 increases considerably in VEGF-stimulated endothelial cells (Chen et al., 2005). However, since Akt1 is activated by not only vascular permeability-inducing agents such as VEGF and tumor necrosis factor-α (Fairaq, Goc, Artham, Sabbineni, & Somanath, 2015; Gao et al., 2016) but also by agents that promote barrier integrity such as angiopoietin-1 (Ang-1), roundabout guidance receptor-4, and sphingosine-1-phosphate, the precise role of Akt1 in regulating vascular permeability was not clear until recently (Daly et al., 2004; De Palma, Meacci, Perrotta, Bruni, & Clementi, 2006; Somanath, Kandel, Hay, & Byzova, 2007). Studies from our laboratory have demonstrated that Akt1 is important for vascular maturation and that suppression of Akt1

Fei Gao and Harika Sabbineni contributed equally to this work.

  

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ARTICLE

Translational Therapeutics

# Endothelial Akt1 loss promotes prostate cancer metastasis via β-catenin-regulated tight-junction protein turnover

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**BACKGROUND:** Cancer research, in general, is focused on targeting tumour cells to limit tumour growth. These studies, however, do not account for the specific effects of chemotherapy on tumour endothelium, in turn, affecting metastasis.
**METHODS:** We determined how endothelial deletion of Akt1 promotes prostate cancer cell invasion in vitro and metastasis to the lungs in vivo in endothelial-specific Akt1 knockdown mice.
**RESULTS:** Here we show that metastatic human PC3 and DU145 prostate cancer cells invade through Akt1-deficient human lung endothelial cell (HLEC) monolayer with higher efficiency compared to control HLEC. Although the endothelial Akt1 loss in mice had no significant effect on RM1 tumour xenograft growth in vivo, it promoted metastasis to the lungs compared to the wild-type mice. Mechanistically, Akt1-deficient endothelial cells exhibited increased phosphorylation and nuclear translocation of phosphorylated β-catenin, and reduced expression of tight-junction proteins claudin-5, ZO-1 and ZO-2. Pharmacological inhibition of β-catenin nuclear translocation using compounds ICG001 and IWR-1 restored HLEC tight-junction integrity and inhibited prostate cancer cell transendothelial migration in vitro and lung metastasis in vivo.
**CONCLUSIONS:** Here we show for the first time that endothelial-specific loss of Akt1 promotes cancer metastasis in vivo involving β-catenin pathway.

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## INTRODUCTION

Currently, research in the development of cancer therapy more focused on the pathways promoting tumour cell growth and invasion. Studies that address the specific role of a pathway in stromal cells and how drugs affect stroma when used for cancer therapy are fewer. Among the cells in the tumour microenvironment, tumour endothelium plays a significant role not only in tumour angiogenesis, perfusion and metastasis[1–3] but also as the first line of defense in a patient's fight against cancer cell metastasis to other vital organs. Hence, it is important to determine the specific role of a pathway and the effect of a drug on tumour vasculature alone so as to improve the efficacy and minimise the side effects of cancer chemotherapy.

Preclinical and clinical research evidence has revealed the integral role of phosphatase and tensin homologue (PTEN)-Akt pathway in multiple cancers,[4] including prostate cancer.[5] A number of studies from our laboratory have indicated that pharmacological and genetic inhibition of Akt, particularly Akt1, inhibits prostate and bladder cancer cell function in vitro and tumour xenograft growth in vivo.[6–8] We previously reported that, drugs such as statins and angiotensin receptor blocker candesartan, that have the ability to normalise Akt1 activity in prostate cancer by inhibiting hyperactive Akt1 in prostate cancer cells,[9–11] and activating Akt1 from its basal state in endothelial cells, led to the inhibition of prostate cancer cell transendothelial migration in vitro.[12] We have also reported that Akt1 gene knockout in mice promoted tumour vascular permeability and angiogenesis in a murine B16F10 melanoma model.[13] Most recently, we demonstrated that endothelial-specific knockdown of Akt1 results in increased vascular permeability via FoxO- and β-catenin-mediated suppression of endothelial tight-junction claudin expression, mainly claudin-5.[14] Since many inhibitors of Akt are in different phases of clinical trials for various types of cancers, it is important to understand the effect of Akt1 suppression in endothelial cells of tumour vasculature, and its consequences on tumour growth and metastasis.

In the current study, we investigated the effects of endothelial-specific knockdown of Akt1, a major endothelial isoform of Akt[13] on prostate cancer cell invasion in vitro and metastasis in vivo using murine lung colonisation model of in vivo metastasis. Our analysis revealed that Akt1 deficiency in human lung microvascular endothelial cells (HLECs) enhances the ability of human metastatic PC3 and DU145 prostate cancer cells to migrate across the endothelial monolayer in vitro, and murine RM1 prostate cancer cell metastasis to the lungs in vivo, with no changes in the growth of RM1 tumour xenografts in vivo. The akt1 loss in HLECs resulted in increased translocation of phosphorylated β-catenin from the endothelial-barrier junctions to the cytosol and the nucleus, in turn, suppressing the transcription of endothelial tight-junction proteins such as claudin-5, ZO-1 and ZO-2.

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# Inducible overexpression of endothelial proNGF as a mouse model to study microvascular dysfunction

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ABSTRACT

Impaired maturation of nerve growth factor precursor (proNGF) and its accumulation has been reported in several neurodegenerative diseases, myocardial infarction and diabetes. To elucidate the direct impact of proNGF accumulation identified the need to create a transgenic model that can express fully mutated cleavage-resistant proNGF. Using Cre-Lox technology, we developed an inducible endothelial-specific proNGF transgenic mouse (proNGF$^{Loxp}$) that overexpresses GFP-conjugated cleavage-resistant proNGF123 when crossed with VE-cadherin-CreERT2 (Cre). Expression of proNGF, inflammatory mediators, NGF and VEGF was evaluated by PCR, Western blot and immunohistochemistry. EC-proNGF overexpression was confirmed using colocalization of anti-proNGF within retinal vasculature. EC-proNGF did not cause retinal neurotoxicity or marked glial activation at 4-weeks. Microvascular preparation from Cre-proNGF mice showed significant imbalance of proNGF/NGF ratio, enhanced expression of TNF-α and p75$^{NTR}$, and tendency to impair TrkA phosphorylation compared to controls. EC-proNGF overexpression triggered mRNA expression of p75$^{NTR}$ and inflammatory mediators in both retina and renal cortex compared to controls. EC-proNGF expression induced vascular permeability including breakdown of BRB and albuminuria in the kidney without affecting VEGF level at 4-weeks. Histopathological changes were assessed after 8-weeks and the results showed that EC-proNGF triggered formation of occluded (acellular) capillaries, hall mark of retinal ischemia. EC-proNGF resulted in glomerular enlargement and kidney fibrosis, hall mark of renal dysfunction. We have successfully created an inducible mouse model that can dissect the contribution of autocrine direct action of cleavage-resistant proNGF on systemic microvascular abnormalities in both retina and kidney, major targets for microvascular complication.

## 1. Introduction

ProNGF is the precursor form of the mature nerve growth factor (NGF), which is mainly secreted from glia. ProNGF normally undergoes proteolytic cleavage; intracellularly by plasmin and furin and extracellularly by matrix metalloproteinase to produce the mature form [1,2]. Diabetes-induced alteration in NGF levels has been correlated with various microvascular complications including retinopathy, nephropathy, and neuropathy. Diabetes increased serum and kidney levels of NGF in an experimental model of diabetic nephropathy [3]. A clinical study showed positive correlation of serum NGF levels in patients with diabetic retinopathy with the existence of diabetic nephropathy [4]. We discovered that diabetes causes imbalance of NGF and its precursor proNGF ratio in retina and ocular fluids that were putatively correlated with serum levels in diabetic patients [5,6]. This imbalance was associated with increased expression of p75$^{NTR}$ receptor, retinal inflammation [7], vascular permeability, neurodegeneration [5,8] and formation of occluded capillaries [9,10]. In addition to diabetes, impaired maturation of proNGF and its accumulation has been also reported in neurodegenerative diseases such as Alzheimer [11,12], Pick's disease [13] and diabetic encephalopathy [14].

The main biological function of proNGF is regulating cell death via binding to p75$^{NTR}$ and its co-receptor; sortilin, a member of VPS-10p domain receptor family [2,15–17]. Cell death mechanisms involve direct activation of apoptotic pathways or non-cell autonomous signaling pathways [18]. To further study the role of proNGF away from complex nature of diabetic milieu, we have stably over-expressed a cleavage-resistant isoform of proNGF in rodent retina. Stable overexpression of

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Review Article

# Renin–angiotensin system as a potential therapeutic target in stroke and retinopathy: experimental and clinical evidence

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**Abstract**

As our knowledge expands, it is now clear that the renin–angiotensin (Ang) system (RAS) mediates functions other than regulating blood pressure (BP). The RAS plays a central role in the pathophysiology of different neurovascular unit disorders including stroke and retinopathy. Moreover, the beneficial actions of RAS modulation in brain and retina have been documented in experimental research, but not yet exploited clinically. The RAS is a complex system with distinct yet interconnected components. Understanding the different RAS components and their functions under brain and retinal pathological conditions is crucial to reap their benefits. The aim of the present review is to provide an experimental and clinical update on the role of RAS in the pathophysiology and treatment of stroke and retinopathy. Combining the evidence from both these disorders allows a unique opportunity to move both fields forward.

**Key words:** cognitive impairment, renin–angiotensin system, retinopathy, stroke, traumatic brain injury.

## INTRODUCTION

One of the molecular pathways central to the pathophysiology of different neurovascular diseases is the renin–angiotensin (Ang) system (RAS). There is a strong body of experimental evidence that modulating the RAS is associated with endogenous neurovascular restoration and recovery in disorders of the brain and retina. However, clinical data are still lagging with no RAS modulating drug currently approved for use as a direct treatment for cerebrovascular or retinal disorders so far. The aim of this review is to discuss the different pleiotropic actions of modulating RAS in different neurovascular disorders with a special focus on stroke and retinopathy.

### Brain and retina as integral parts of the central nervous system

Both brain and retina share the neurovascular unit structure, including neurons, vessels and glia. This neurovascular coupling allows brain and retinal neurons to elicit haemodynamic changes to match their metabolic needs [1–4]. Moreover, the brain and inner retina both have a tight blood–tissue barrier that plays a fundamental physiological role in controlling the microenvironment and preserving neuronal function. The blood–retina barrier and blood–brain barrier (BBB) are located in the microvascular endothelium of capillaries and covered by the processes of glia (astrocytes and Müller cells) as well as pericytes [1–4]. Developmentally, the retina is considered as part of the brain that

**Abbreviations:** ACE, angiotensin-converting enzyme; ACEI, angiotensin-converting enzyme inhibitors; AD, Alzheimer's disease; AGE, advanced glycation end products; Ang, angiotensin; ARB, angiotensin type 1 receptor blocker; AT1R/AT2R, angiotensin II type 1/2 receptor; BBB, blood–brain barrier; BDNF, brain derived neurotrophic factor; BP, blood pressure; C21, compound 21; CA, cerebral aneurysms; CBF, cerebral blood flow; CNS, central nervous system; DIRECT, Diabetic REtinopathy Candesartan Trial; DR, diabetic retinopathy; eNOS, endothelial nitric oxide synthase; HA, heat acclimation; i.p., intraperitoneal; ICH, intracerebral haemorrhage; ICV, intracerebroventricular; INTERACT, Intensive blood pressure reduction in acute cerebral haemorrhage trial; INWEST, Intravenous Nimodipine West European Stroke Trial; IRAP, insulin regulated aminopeptidase; KO, knockout; LDH, lactate dehydrogenase; MCA, middle cerebral artery; MCAO, middle cerebral artery occlusion; NEJM, New England Journal of Medicine; OGD, oxygen glucose deprivation; pMCAO, permanent middle cerebral artery occlusion; PPAR-$\gamma$, peroxisome proliferator-activated receptor $\gamma$; PROGRESS, The perindopril protection against recurrent stroke study; RAGE, receptor for AGE; RAS, renin–angiotensin system; ROP, retinopathy of prematurity; ROS, reactive oxygen species; SAH, subarachnoid haemorrhage; SCAST, Scandinavian Candesartan Acute Stroke Trial; SCOPE, Study on COgnition and Prognosis in the Elderly; SHR, spontaneously hypertensive rat; STZ, streptozotocin; TBI, traumatic brain injury; tPA, tissue plasminogen activator; VEGF, vascular endothelial growth factor; WT, wild-type.

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# Vascular protective effects of Angiotensin Receptor Blockers: Beyond Blood pressure

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### Keywords

ARB; candesartan; angiogenesis; vascular protection; ischemic retinopathy; stroke; VEGF

## Introduction

Angiotensin-II (Ang-II) activates two major types of receptors, angiotensin II type one (AT1R) and type two (AT2R) receptors. While AT1R is widely expressed and mediates most inflammatory Ang-II effects, AT2R, is less expressed and has opposite effects, promoting vasodilation and anti-inflammatory effects [1]. Physiologically, AT2R actions are usually masked by the more abundant AT1R. It has been suggested that ARBs can mediate their action through increasing angiotensin II (Ang II) availability to bind to the beneficial angiotensin type 2 receptor (AT2R), thus leading to unopposed AT2R stimulation. AT1R blockers (ARBs) represent a major class of antihypertensive medications. They are considered first line treatment for essential hypertension. Moreover, ARBs are the cornerstone treatment for other cardiovascular diseases especially in patients with diabetic and renal comorbidities. Clinical and experimental evidence have documented the beneficial actions of ARBs beyond the blood pressure lowering effect. Ischemic diseases such as stroke and proliferative retinopathy are characterized by hypoxia-driven release of angiogenic growth factors [2]. However, revascularization of the ischemic areas is inadequate, resulting in impaired neuro-vascular function. ARBs have been shown to exhibit vascular protective and pro- or anti-angiogenic effects depending on the tissue/cell type and disease condition under study [3]. Our group has demonstrated the vascular protective effects of ARBs and candesartan, in particular, in models of ischemic stroke and retinopathy. The positive impact of candesartan was mainly via enhancing the proangiogenic state and stimulation of reparative angiogenesis. This commentary aims to highlight the recently identified pathways engaged as a result of directly blocking the AT1 receptor or indirectly by possible activation of AT2 receptor, in the context of the published literature.

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**Conflicts of Interest**: The authors declare no conflict of interest.

Chapter 12

# Stage-Specific Effects of Hypoxia on Interstitial Lung Disease

Sandeep Artham and Payaningal R. Somanath

Additional information is available at the end of the chapter

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**Abstract**

Interstitial lung disease (ILD) comprises a group of lung diseases principally affecting the pulmonary interstitium, for example, pulmonary fibrosis. Following acute lung injury (ALI), the fate of an injured lung progressing towards either injury resolution or pulmonary fibrosis is dictated by hypoxia at various stages during the disease progression. Hypoxia that is tissue destructive at one stage of lung injury becomes beneficial at a different stage, with each hypoxic stage involving a different scheme of molecular pathways, cellular interplay and tissue remodeling. In this chapter, we provide a detailed account of hypoxia during the different stages of lung injury in ILDs, delineate the cellular and molecular mechanisms mediating tissue remodeling in the hypoxic lungs as well as the basic and clinical findings in this field with an emphasis on future therapeutics to modulate hypoxia to treat ILD.

**Keywords:** acute lung injury, wound resolution, hypoxia, interstitial lung disease, PAH

## 1. Introduction

Interstitial lung disease (ILD) comprises a group of lung diseases principally affecting the pulmonary interstitium, for example, pulmonary fibrosis [1]. An injured lung as a result of infection, inhalation of chemical, and other harmful substances either resolves over time or progresses into irreversible damage and fibrosis. Therefore, lung injury as in acute respiratory distress syndrome (ARDS), due to conditions like hypoxia can progress to interstitial lung damage or fibrosis similar to ILD-associated pulmonary fibrosis. Yet, another important pulmonary pathological condition associated with hypoxia is the pulmonary arterial hypertension (PAH) [2]. The ARDS is a devastating clinical syndrome of acute lung injury (ALI) that affects both medical and surgical patients [3]. The official definition of ARDS was first published in 1994 by