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Inhibition of spleen tyrosine kinase signaling protects against acute lung injury through blockade of NADPH oxidase and IL-17A in neutrophils and γδ T cells respectively in mice

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ABSTRACT

Acute lung injury (ALI) is one of the most serious complications in critically ill patients which often leads to morbidity and mortality. ALI characterized by severe inflammation of lungs occurs due to uncontrolled inflammatory immune response. However, the immunological mechanism(s) are far from being understood. The spleen tyrosine kinase (SYK), a key component of immune receptor signaling, plays a critical role in the modulation of inflammatory signaling in different immune cells. However, its role in ALI remains to be explored. Therefore, in this study, we investigated the effect of R406, a SYK inhibitor in lipopolysaccharide (LPS)-induced ALI mouse model. LPS led to increased SYK expression in neutrophils and gamma delta (γδ) T cells. This was associated with increased neutrophilic airway inflammation, vascular permeability, myeloperoxidase activity in the lung with upregulated expression of NADPH oxidase (NOX2)/MCP-1/TNF-α in neutrophils and IL-17A in γδ T cells/lung. Pulmonary inflammation was associated with higher mortality in mice with ALI. Inhibition of SYK signaling using R406 in the lung led to blockade of neutrophilic airway inflammation, vascular permeability, pro-inflammatory cytokine release and oxidative stress in innate immune cells, *i.e.* γδ T cells and neutrophils and the lung. R406 administered LPS group had better survival rate than LPS group. This suggests that SYK upregulation in γδ T cells and neutrophils plays an important role in inflammatory process during ALI. In conclusion, R406 exhibited a great potential to block the LPS-induced airway inflammation and mortality which could be developed as a potential future therapy in ALI.

1. Introduction

Acute lung injury (ALI) is an inflammatory lung disease characterized by increased vascular permeability, diffuse pulmonary interstitial edema, hypoxemia which may lead to respiratory failure in severe cases [1,2]. ALI is one of the most serious complications in critically ill patients which often leads to morbidity and mortality. However, the immunological mechanism(s) are far from being understood and the therapies developed as of date have not been able to make any significant difference in mortality rate [3–5]. Consequently, it becomes vital to develop better understanding of the mechanism(s) involved in ALI.

ALI is caused when the innate immunity, triggered in response to lung infections, becomes uncontrolled and results in the disruption of the lung alveolar-capillary membrane barrier, an increased expression of proinflammatory cytokines, and the infiltration of neutrophils and other immune cells ([4,6,7]). Lipopolysaccharide (LPS), an endotoxin, is a known agent for induction of ALI in mice. Inhalation of LPS has been shown to engender release of pro-inflammatory cytokines and chemotactic factors by stimulating innate immune cells eventually causing airway inflammation and injury to the lung tissue [8,9]. Therefore, controlling the innate immunity might be a promising approach against ALI.

Innate immunity is the first line of defense at the time of infection and thus plays a central role in regulation of inflammatory responses during different infectious stimuli [10,11]. Neutrophils and gamma

Abbreviations: ALI, Acute lung injury; BAL, Bronchoalveolar lavage; NOX2, NADPH oxidase; i.n., Intranasal; ROS, Reactive oxygen species; SYK, Spleen tyrosine kinase

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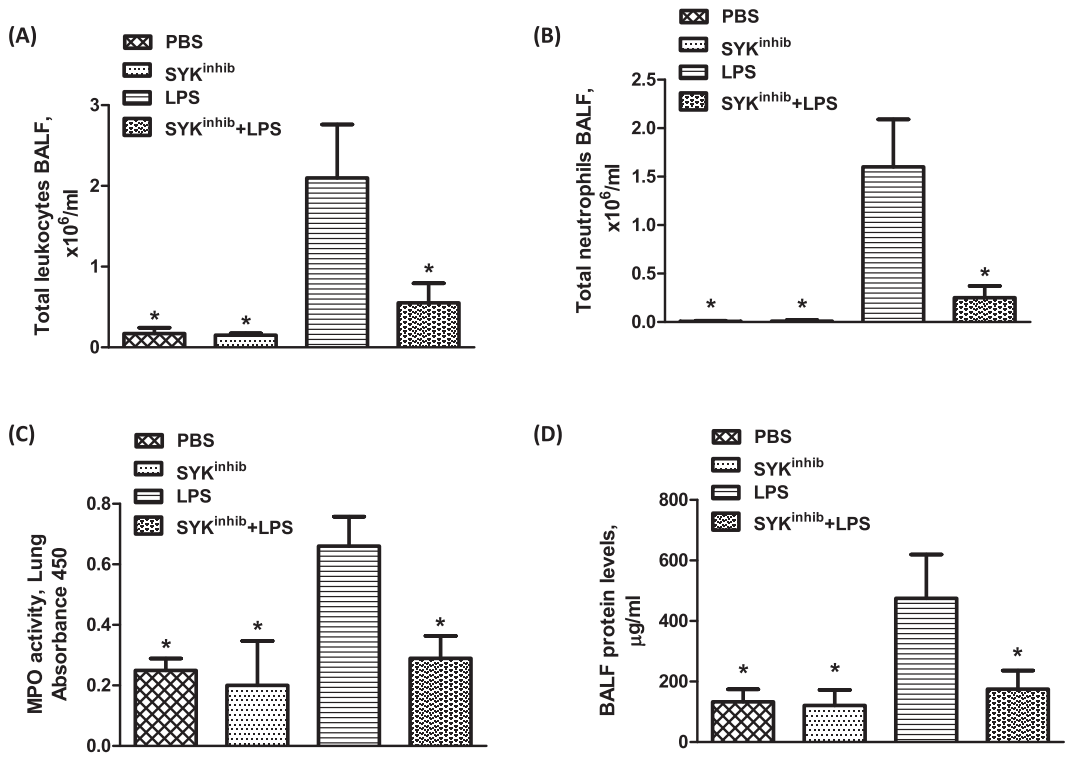


Fig. 1. Effect of SYK inhibitor, R406 on LPS-induced airway inflammation in mice. A) Total leukocyte count in BAL, B) Total neutrophil count in BAL, C) Lung MPO activity, D) Protein concentration in BAL fluid, E) Histopathological examination through H&E staining of the lung sections (×100), and F) survival rate in different groups at the end of five-day period represented as Kaplan-Meier curves. Values are expressed as mean ± SD, n = 8–10/group. *P < 0.05, *vs.* LPS.

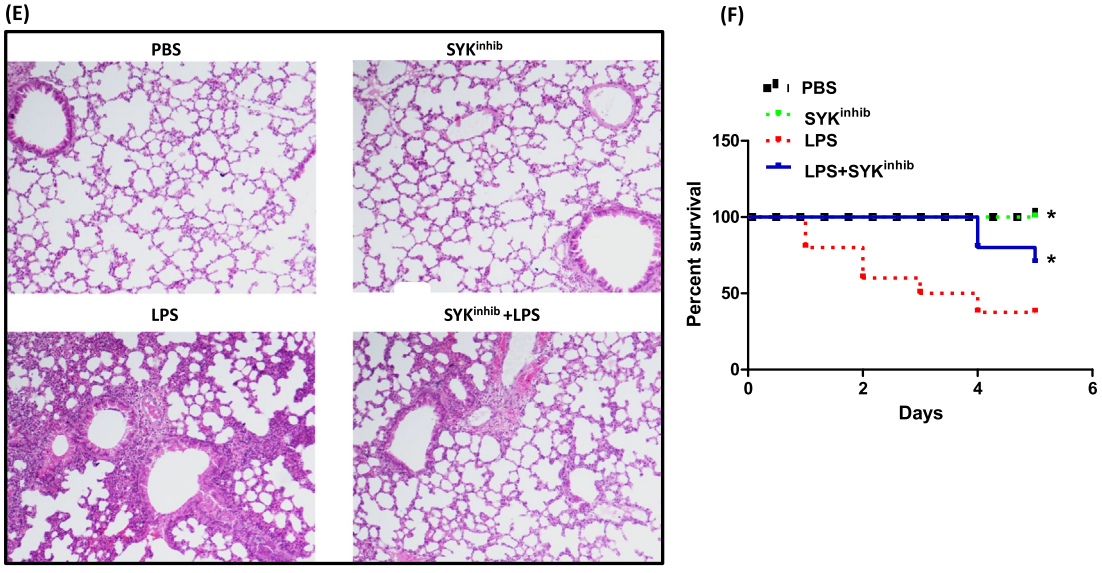


Fig. 1. (*continued*)

delta (γδ) T cells are two important types of cells through which innate immunity functions. Phagocytic cells such as neutrophils are an important component of the inflammatory response against different infections but are also found to be crucial contributors to pulmonary inflammation during ALI [12,13]. The inflammation mediated by neutrophils is caused primarily by two mechanisms – inflammatory cytokines and reactive oxygen species (ROS) generation. Release of inflammatory cytokines such as TNF-α, IL-17A, MCP-1 leads to amplification of lung injury through binding to their respective receptors [6,14–16]. The other mechanism by which neutrophils induce inflammation is generation of ROS through activation of NADPH oxidase 2 (NOX2) ([16]; [27]).

γδ T cells, a distinct subset of T cells, produces several inflammatory cytokines including IL-17A, whose increased expression is known to be a critical event in ALI [17]. IL-17A is also one of the key cytokines involved in neutrophil inflammation [18]. Several studies have evinced that IL-17A operates by inducing the airway epithelial cells to secrete chemokines (IL-6, CXCL1, CXCL5, CXCL8, GCSF, and GM-CSF) which subsequently recruit neutrophils to the airways leading to inflammation [19,20]. Recent research shows that elevated level of IL-17A in circulation as well as alveolar neutrophils leads to elevated alveolar permeability and increased lung injury/inflammation [17,21].

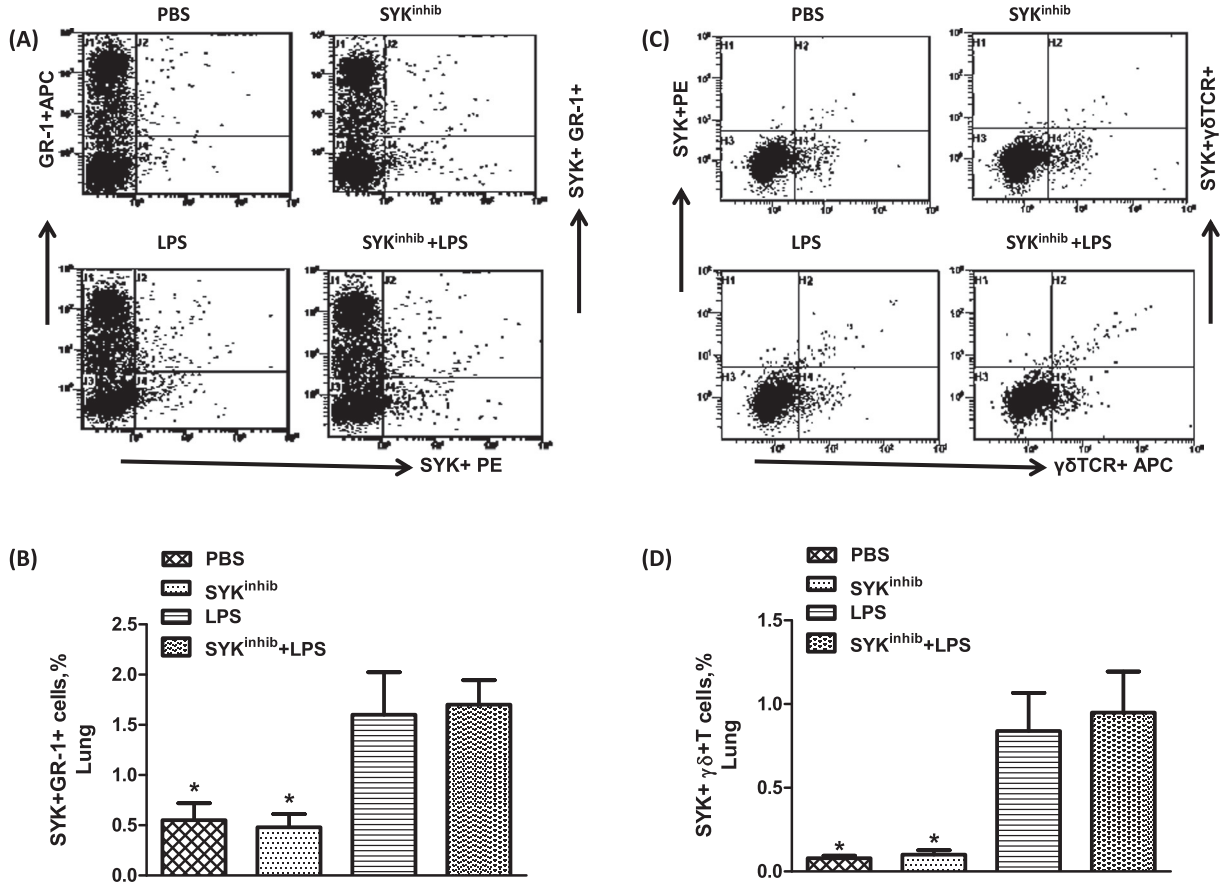


Fig. 2. Expression of SYK in pulmonary neutrophils and γδ T cells in SYK inhibitor and LPS treated mice. A) Representative flow plot showing immunostaining for SYK+ and GR-1+ cells, B) % SYK + GR-1+ neutrophils, C) Representative flow plot showing immunostaining for SYK+ and γδTCR+ T cells, and D) % SYK+ γδTCR+ T cells. Values are expressed as mean ± SD, $n = 8$/group. *$P < 0.05$, *vs.* LPS.

Currently, the information on drug targets and their clinical potential in the treatment of ALI is limited. Targeting the overactivity of the innate immunity inflammatory response has received much attention lately as a potential therapeutic strategy against ALI ([3,22]). In this regard, spleen tyrosine kinase (SYK) has been shown to be involved in the regulation of pro-inflammatory signaling pathways associated with alcoholic liver disease, infection, and rheumatoid arthritis [23–25]. SYK also plays a critical role in the regulation of innate immune cells such as neutrophils and γδ T cells [22,26]. Therefore, it was hypothesized that SYK may have a role in regulating ALI through modulation of immune responses in neutrophils and γδ T cells. Further, SYK inhibition might provide a therapeutic strategy to block LPS-induced ALI through downregulation of inflammatory mediators in these innate immune cells. Our study shows that LPS-induced ALI is associated with increased SYK expression and neutrophilic airway inflammation. R406, a SYK inhibitor demonstrates its role against airway inflammation through downregulation of over-responsive neutrophils and γδ T cells during lung injury.

2. Materials and methods

2.1. Animals

Male BALB/c mice, weighing 25–30 g and 10–12 weeks of age, were used in all the experiments. Mice were maintained in specific pathogen-free conditions at 25 °C under a 12-h light-dark cycle and received food and water *ad libitum*. Animal protocols were approved by the Experimental Animal Care Center, College of Pharmacy, King Saud University. All the experiments were performed in accordance with the relevant guidelines and regulations.

2.2. ALI model

The method for establishing the LPS-induced ALI model was performed as previously described [27]. Lightly anaesthetized mice were instilled a single dose of LPS (50 μg/25 μl/mouse) intranasally (i.n.) to induce acute lung injury. Control mice were administered phosphate buffer saline (PBS) intranasally.

The effect of SYK inhibitor on LPS-induced acute lung injury in mice was investigated by administration of 100 μg, R406 (Cayman Chemical, USA), i.n. 1 h before and 12 h after LPS instillation in each mouse. To examine the effect of SYK inhibitor against ALI, mice models were divided into four groups. 1) Control group (PBS): mice were administered PBS (i.n) and drug vehicle (i.n.); 2) Control group administered R406 (SYK^{inhib}): mice were administered both PBS (i.n.) + R406 (i.n.); 3) LPS administered group (LPS): mice received LPS (i.n.) and drug vehicle (i.n) as described above; and 4) LPS group administered R406 (R406): mice were administered both LPS (i.n.) + R406 (i.n.) as described above. The drug vehicle was also administered i.n. in the same manner as described above before and after LPS/PBS. Mice were sacrificed 24 h after LPS administration for various analyses.

A pilot study was also done to assess the effect of R406 on mortality in ALI model. In this study, mice were followed for 5 days after different treatments as stated above and the survival of mice in each treatment group ($n = 10$/group) was recorded every day for 5 days.

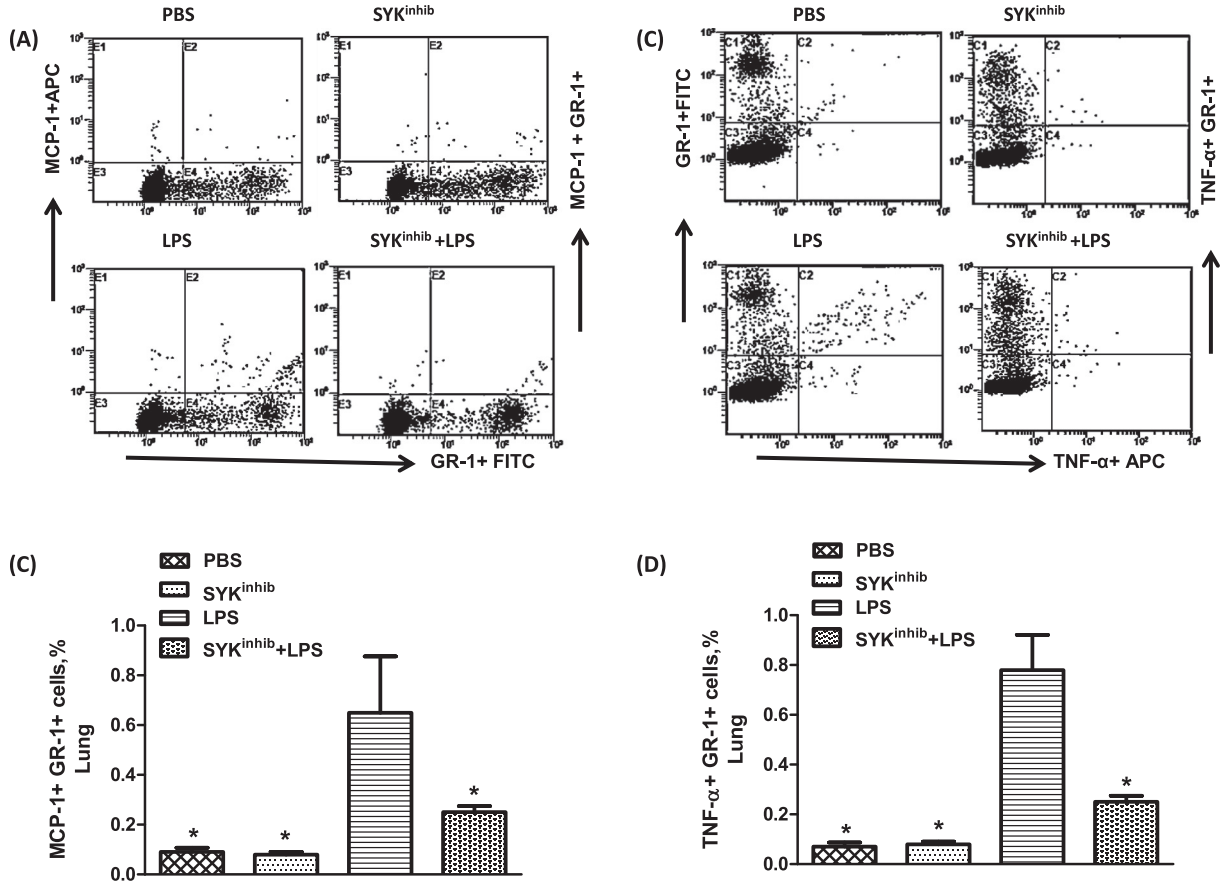


Fig. 3. Effect of SYK inhibitor, R406 on inflammatory cytokines in neutrophils of LPS-treated mice. A) Representative flow plot showing immunostaining for TNF-α + and GR-1 + cells, B) % TNF-α + GR-1 + neutrophils, C) Representative flow plot showing immunostaining for MCP-1 + and GR-1 + neutrophils, and D) % MCP-1 + GR-1 + neutrophils. Values are expressed as mean ± SD, $n = 8$/group. *$P < 0.05$, *vs.* LPS.

2.3. Assessment of inflammation and histological analysis

Bronchoalveolar lavage (BAL) was performed 1 day after LPS challenge by lavaging the lungs for BAL fluid (BALF) collection. Differential count for neutrophils, macrophages, lymphocytes was done after cytocentrifugation of recovered cells on the slide. Cell number was expressed as mean ± SEM per ml for each group. Cell-free BALF protein levels were also quantified. To evaluate the histological alterations, lung tissues were fixed in 10% formalin, and sectioned at 5 μm thickness. The sections were the stained with haematoxylin and eosin (H&E) using standard histological techniques for examination under light microscopy.

2.4. Measurement of myeloperoxidase (MPO) activity

Lung MPO activity was determined as a measure of neutrophil inflammation according to our previous studies (Nadeem et al., 2017c).

2.5. Measurement of inflammatory cytokines by ELISA

Protein levels of inflammatory cytokines, IL-17A/MCP-1/TNF-α in the lung were measured using ELISA kits from Biolegend, USA as per manufacturer's protocol.

2.6. Real-time PCR

Total RNA was extracted from the lung as described previously [27,28]. mRNA levels were measured by real-time PCR analysis on ABI PRISM 7500 sequence detection system (Applied Biosystems) as described earlier [27,28]. Using cDNA and carboxyfluorescein (FAM)-labelled primers, expressions for IL-17A, NOX2, MCP-1, TNFα the lung were quantified using Taqman Universal Mastermix (Applied Biosystems, USA). Real-time PCR was carried out on a 7500 real-time PCR system (Applied Biosystems). mRNA levels was expression as fold change using the relative gene expression method [29].

2.7. Flow cytometry

For flow cytometry, whole lungs were utilized to create single cell suspensions in RPMI-1640 as described previously [28]. Single-cell suspensions were labelled with APC/FITC-conjugated anti-GR-1/γδTCR antibody (BioLegend, USA) for surface labeling followed by FITC/APC/PE-conjugated anti-nitrotyrosine/IL-17A/NOX2/MCP-1/TNF-α/SYK monoclonal antibodies (Santa Cruz Biotech, USA and BioLegend, USA). The stained cells were acquired on a flow cytometer (Beckman Coulter, Brea, CA, USA) and analyzed for the expression of protein of interest using Cytomics FC 500, as described earlier ([27], and Nadeem et al., 2017c).

2.8. Chemicals

Highest-grade chemicals/reagents were purchased from Sigma Chemicals (St Louis, MO, USA) unless stated otherwise.

2.9. Statistical analysis

The data were expressed as mean ± SD. Comparisons among different groups were analyzed by ANOVA (analysis of variance) followed

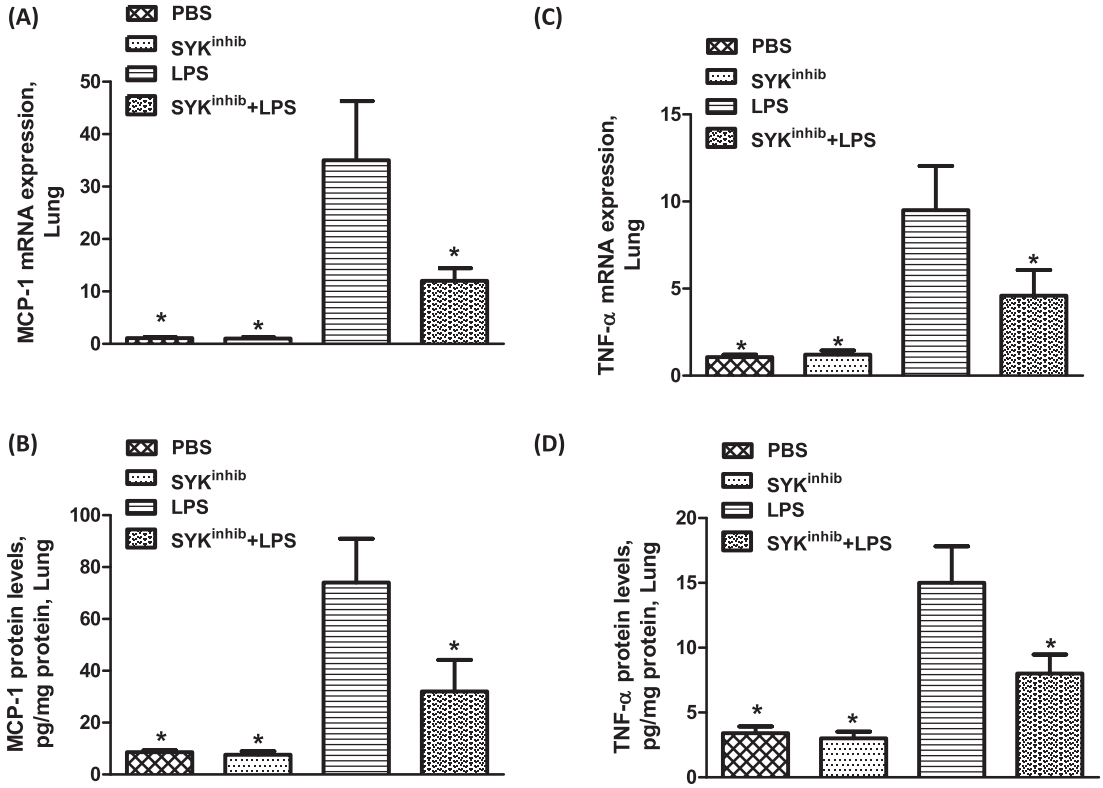


Fig. 4. Effect of SYK inhibitor, R406 on inflammatory cytokines in lungs of LPS-treated mice. A) mRNA expression of TNF-α, B) mRNA expression of MCP-1, C) Protein levels of TNF-α, and D) Protein levels of MCP-1 levels. Values are expressed as mean ± SD, $n = 8$/group. *$P < 0.05$, *vs.* LPS.

by Tukey's multiple comparison tests. Survival of the mice was presented in the form of Kaplan-Meier curves and the log-rank test was employed to test differences in the study groups. The significance was set at $P < 0.05$ for all the statistical analyses. All the statistical analyses were performed using Graph Pad Prism statistical package.

3. Results

3.1. SYK inhibition limited lung inflammation in ALI mouse model

SYK inhibitors have been utilized previously in several autoimmune/inflammatory diseases, therefore we tested whether SYK inhibition would result in prevention of airway inflammation during ALI in this study. Our data show that administration of LPS causes ALI in mice as depicted by increased airway inflammation (total leucocytes/neutrophils count, and histopathology), vascular permeability (protein concentration, an indicator of alveolar-capillary leak) and MPO activity (Fig. 1A–E). LPS-induced increase in all of these inflammatory parameters was blocked by SYK inhibitor, R406 (Fig. 1A–E). Mice which underwent LPS-induced ALI had higher mortality rate at the end of 5-day period as compared to other groups, *i.e.* PBS group, SYK^{inhib} group, and LPS + SYK^{inhib} group (Fig. 1F). These findings show that SYK inhibition results in amelioration of LPS-induced ALI which is associated with reduced mortality.

3.2. Effect of SYK inhibitor on inflammatory cytokines in neutrophils, γδ T cells and lung

Innate immune cells, neutrophils and γδ T cells are found to be involved in the pathogenesis of ALI. In this study, we attempted to explore the pathway through which SYK mediates inflammation in innate immune cells during ALI. Our data show that both neutrophils and γδ T cells show increased expression of SYK after LPS administration which is not affected by SYK inhibitor, R406 (Fig. 2A–D). Upregulated SYK expression was concomitant with increased expression of inflammatory cytokines, MCP-1/TNF-α in neutrophils (Fig. 3A–D). Further, MCP-1/TNF-α protein expression in LPS-treated mice was downregulated by treatment with R406 (Fig. 3A–D). Furthermore, mRNA/protein levels of these cytokines were also elevated in lung by LPS which were attenuated by treatment with R406 (Fig. 4A–D).

Next we determined if γδ T cells had increased IL-17A expression as it is known for neutrophilic inflammation. Our data show increased RORC and IL-17A expression in γδ T cells of LPS treated mice (Fig. 5A–D). Further, LPS-induced increase in RORC and IL-17A expression was attenuated by R406 treatment in γδ T cells (Fig. 5A–D). Furthermore, IL-17A mRNA/protein levels also in LPS-treated mice which were downregulated by treatment of R406 (Fig. 5E–F). These data suggest that pro-inflammatory cytokines in innate immune cells such as neutrophils and γδ T cells could be due to activation of SYK. Further, R406-mediated anti-inflammatory response might be partly attributed to inhibition of these inflammatory cytokines in neutrophils and γδ T cells.

3.3. Effect of SYK inhibitor on NOX2 expression/oxidative stress in neutrophils/lung

Next it was determined if R406 administration is associated with modulation of oxidative stress in neutrophils as they are crucial contributors in ALI. For this purpose, we assessed effect of SYK inhibition on NOX2/nitrotyrosine expression in neutrophils. Our data show that neutrophils and lung have increased expression of NOX2 after LPS administration (Fig. 6A–B and G). Upregulated NOX2 expression was concomitant with increased oxidative stress as depicted by increased nitrotyrosine expression in neutrophils (Fig. 6C–D), and pulmonary lipid peroxides (Fig. 6H) in LPS group. Dual protein expression of NOX2 and nitrotyrosine in neutrophils also increased in LPS treated group (Fig. 6E–F). NOX2, nitrotyrosine and their dual expression in neutrophils and pulmonary lipid peroxides were downregulated by

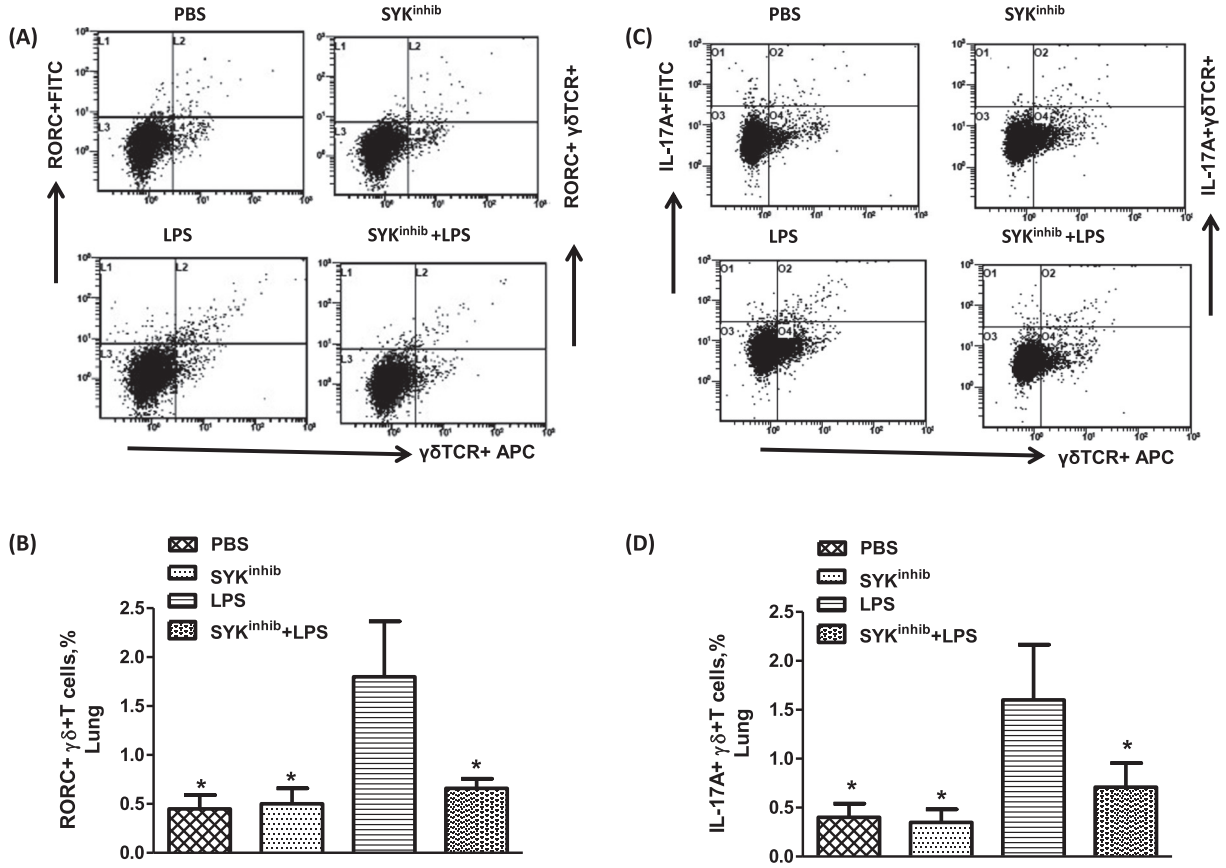
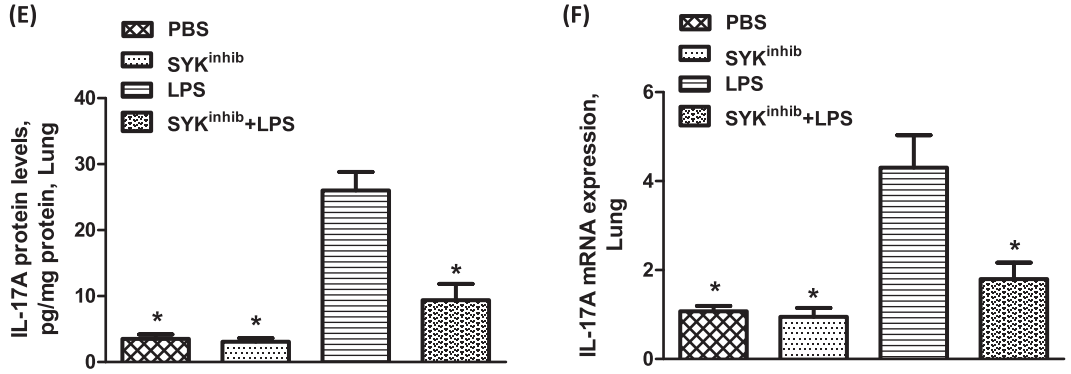


Fig. 5. Effect of SYK inhibitor, R406 on IL-17A in γδ T cells, and lung of LPS-treated mice. A) Representative flow plot showing immunostaining for RORC+ and IL-17A+ γδTCR+ T cells, B) % RORC+ γδTCR+ T cells, C) Representative flow plot showing immunostaining for IL-17A+ and IL-17A+ γδTCR+ T cells, D) % IL-17A+ γδTCR+ T cells, E) IL-17A mRNA expression, and F) IL-17A protein levels in the lung. Values are expressed as mean ± SD, $n = 8$/group. *$P < 0.05$, *vs*. LPS.



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treatment of R406 (Fig. 6A–H). These data suggest that R406-induced decrease in oxidative stress in neutrophils/lung also contributes to the anti-inflammatory effect in ALI. If summarized, these results show that SYK inhibition causes potent anti-inflammatory effects during LPS-induced ALI by downregulation of inflammatory cytokines and oxidative stress in pulmonary neutrophils/γδ T cells.

4. Discussion

SYK is an immunologically important kinase but its role during ALI and the mechanism(s) through which it acts are not well characterized. SYK has been shown to be important regulator of innate immunity which gets activated during ALI [22,30]. SYK inhibition has been shown to be anti-inflammatory by reducing the expression of pro-inflammatory genes in various immune/non-immune cells [23,31,32]. Our results corroborate these earlier findings since blockade of SYK signaling using R406 produced a protective effect as depicted by reduction in the airway inflammation, MPO activity, and inflammatory/oxidative mediators in innate immune cells/lung during ALI. Enhanced survival of R406-treated LPS group could be owing to attenuation of inflammation in neutrophils and γδ T cells.

LPS is a potent activator of TLR4 which is expressed on the host immune cells [33]. In various immune cells such as neutrophils and macrophages, SYK has been shown to play a critical role in the signal transduction of TLR4 which is activated by LPS [34,35]. The activation of TLR4 is mediated by recruiting Toll-interleukin 1 receptor adaptor which can associate with cytosolic domains known as MyD88, TIRAP, TRAM and TRIF to initiate either TIRAP-MyD88 or TRAM-TRIF-

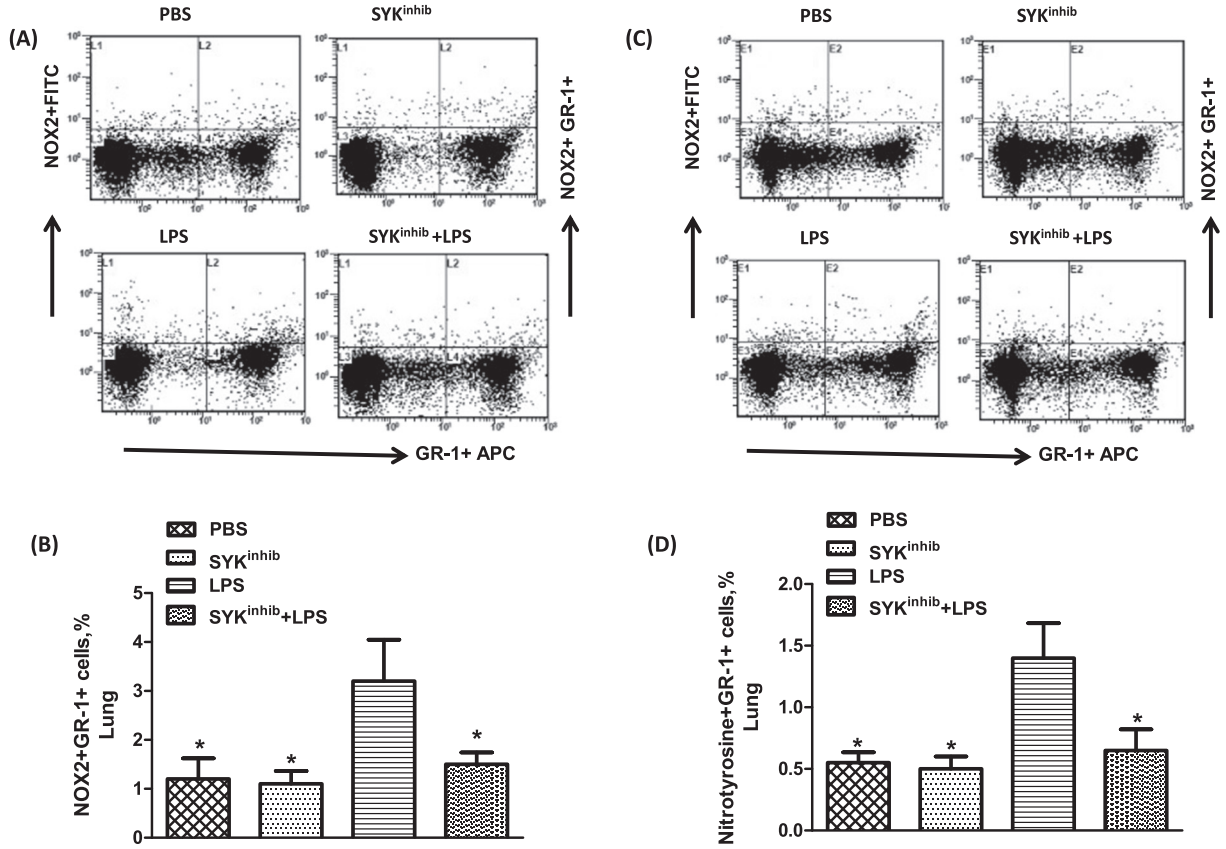

Fig. 6. Effect of SYK inhibitor, R406 on NOX2/oxidative stress in neutrophils/lung of LPS-treated mice. A) Representative flow plot showing immunostaining for NOX2+ and GR-1+ cells, B) % NOX2 + GR-1+ neutrophils, C) Representative flow plot showing immunostaining for nitrotyrosine+ and GR-1+ cells, D) % nitrotyrosine + GR-1+ neutrophils, E) Representative flow plot showing dual immunostaining for nitrotyrosine+ and NOX2+ cells, F) % NOX2+ nitrotyrosine+ neutrophils, G) mRNA expression of NOX2, and E) lipid peroxides levels in the lung. Values are expressed as mean ± SD, $n = 8$/group. *$P < 0.05$, *vs.* LPS.

dependent signaling pathway [36,37]. These pathways trigger the activation of downstream kinases such as mitogen-activated protein kinases and interleukin receptor-associated kinase (IRAK1). IRAK1 induces expression of pro-inflammatory genes by activation of transcription factor, NFκB [31,38,39]. IRAK1 is regulated by SYK and this is how SYK is thought to be an important mediator in the intracellular signal transduction during an inflammatory response [31,35]. Therefore, the approach of targeting SYK downstream of TLR4 signaling could be beneficial to control the resultant inflammatory response during ALI.

SYK signaling is required in different immune cells for various functions. For instance, it is a key molecule in the B-cell receptor signaling which functions as a vital component of adaptive immunity by generating antigen-specific antibodies [40,41]. Similarly, other pivotal components of innate immunity like neutrophils, macrophages, and mast cells are also governed by SYK signaling., SYK regulates integrin signaling cascade resulting in neutrophil migration, sustained leukocyte adhesion, IgE-mediated basophil activation, and cytokine release after mast cells activation [34,42,43]. All of these studies point out to the importance of SYK signaling in different immune cells.

Activation of SYK during an inflammatory response causes activation of downstream signaling and leads to various cellular responses in different immune/non-immune cells that include degranulation, cytokine/chemokines release, and ROS production [43–46]. For example, inflammatory cytokines and ROS are produced after SYK activation in immune cells such as neutrophils/macrophages [15,38,47,48]; in γδ T cells, SYK/PI3K axis drives the proinflammatory program [26]; in human corneal epithelial cells, SYK activation by Aspergillus fumigatus leads to upregulated expression of inflammatory cytokines/chemokines, particularly IL-1β, IL-6, IL-8 and CXCL1 [25]. In agreement with the previous studies, our results also demonstrate that upregulation of SYK in innate immune cells such as neutrophils and γδ T cells plays an important role in ALI. Our study further shows that SYK signaling is important in the regulation of inflammatory/oxidative mediators in these innate immune cells during ALI. This was evident from increased inflammatory cytokines and oxidative stress with concomitant upregulation of SYK in neutrophils and γδ T cells during ALI. Based on these observations, we tested SYK inhibition as an approach to contain overt inflammatory response during ALI.

Neutrophils are a part of innate immunity and play an inflammatory role during lung injury by generation of oxidative stress through activation of NOX2. Several studies have reported that inflammation activates SYK leading to ROS production ([43,45]). Our study also shows an important role of SYK in upregulation of NOX2 in neutrophils which was concomitant with increased oxidative stress in neutrophils/lungs of LPS-treated mice. SYK inhibitor, R406 led to downregulation of NOX2 and associated oxidative stress in the lungs during ALI.

Neutrophils are also reported to synthesize inflammatory cytokines which play a significant role in the augmentation of inflammation under different conditions. SYK signaling is known to control the expression of inflammatory cytokines in immune cells such as neutrophils and macrophages [15,48,49]. Our study shows increased expression of TNF-α/MCP-1 during ALI which was blocked by SYK inhibition. These cytokines have the potential to amplify the inflammation loop by recruiting more neutrophils and other immune cells in the lung during ALI. Therefore, reduction in both oxidative stress and inflammatory cytokines in the neutrophils could partly contribute to the anti-inflammatory effect of R406 during ALI.

Like neutrophils, γδ T cells are also an important part of innate inflammatory response. They have the capacity to produce IL-17A

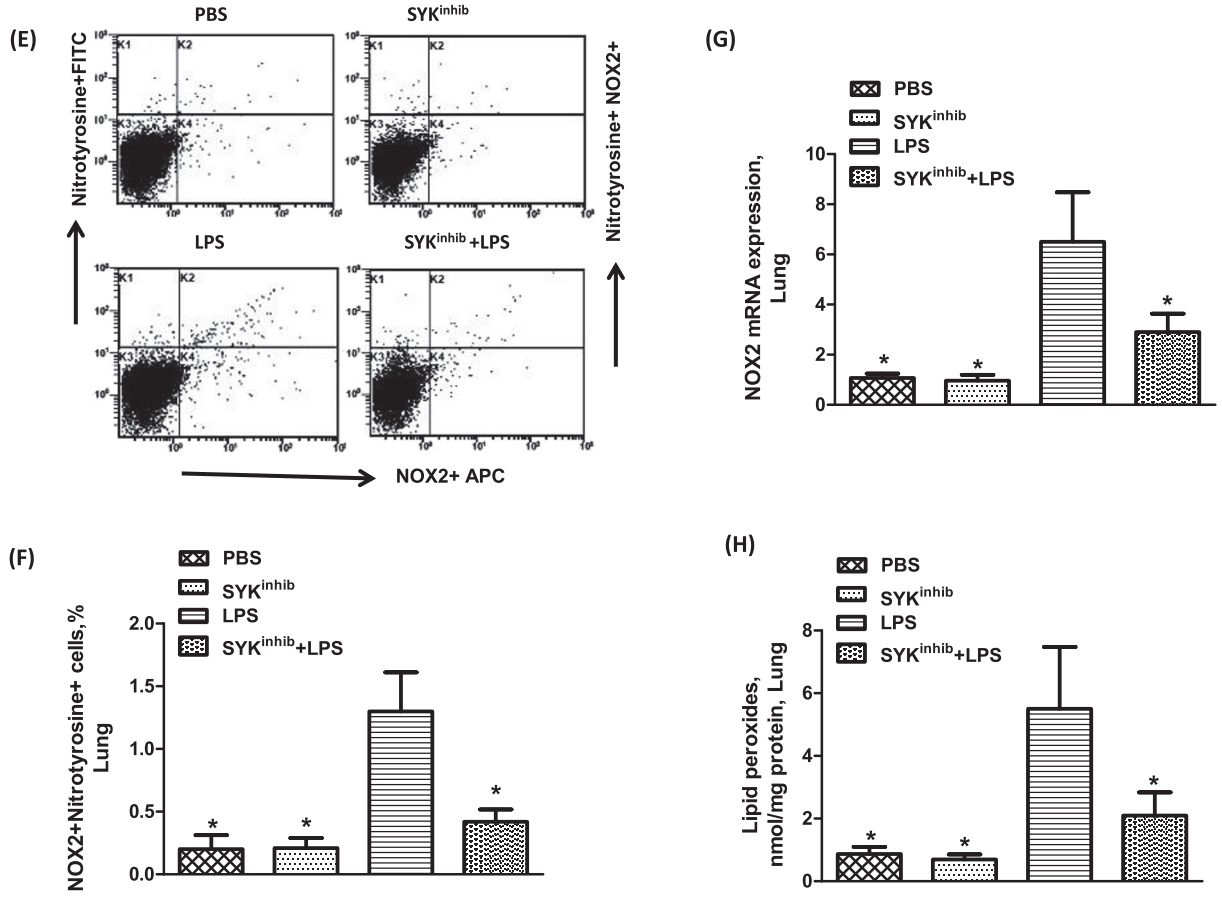

Fig. 6. (*continued*)

which protects the host against fungal/bacterial infections by mounting an inflammatory response under normal conditions [50–52]. However, their overactivation could also pose a threat as increased IL-17A is thought be inflammatory in nature during ALI/airway inflammation ([17,19,53]). It has been shown recently that SYK mediates inflammatory program in γδ T cells through PI3K/Akt signaling pathway that can lead to psoriatic inflammation [26]. SYK inhibition led to blockade of IL-17A expression in γδ T cells/lung in our study. Blockade of IL-17A expression through SYK inhibition could be responsible for attenuation of neutrophilic airway inflammation. It has been shown in the past that IL-17A drives neutrophilic inflammation in the lung [19,21,28,53].

Although LPS-induced model is a frequently used model to mimic ALI/ARDS, it however does not recapitulate all the symptoms and mechanisms observed in human ALI/ARDS as is the case with most disease models in animals which limits their utility in testing potential new therapies. Further, SYK signaling in other cells such as endothelial cells, macrophages and epithelial cells might also contribute to the airway inflammation observed in this study. This might be an interesting future study and is a limitation of the current study.

In line with our study, a number of well-established studies have documented an anti-inflammatory role of SYK inhibition. For example, it has been shown that autoimmune/inflammatory diseases such as rheumatoid arthritis, systemic lupus erythematosus, allergy, and alcoholic liver disease can be ameliorated through SYK inhibition ([23,54]). However, our findings allude to the involvement of SYK in the regulation of innate immune cells such as γδ T cells and neutrophils in the context of ALI for the first time. Further, our study shows that SYK inhibitor, R406 enhances the survival rate of mice with ALI probably by controlling overtly aggressive innate immune cells through down-regulation of inflammatory cytokines and oxidative stress. In this way, SYK inhibition represents an important therapeutic strategy for the treatment of ALI.

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Disclosure

All authors declare no conflicts of interest.

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