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Sepsis by using Cecal Ligation and Single Puncture Causes Alveolar Space Enlargement in LPA_2 Knockout Mice

Yutong Zhao[1]*, Panfeng Fu[2], Jing Zhao[1], Rachel K. Mialki[1], Jianxin Wei[1], Longshuang Huang[2], Jerold Chun[3], Adriana S. Leme[1], Steven D. Shapiro[1] and Viswanathan Natarajan[2,4]

[1]Department of Medicine, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA
[2]Department of Pharmacology, University of Illinois at Chicago, Chicago, IL, USA
[3]Department of Molecular Biology, The Scripps Research Institutes, San Diego, CA, USA
[4]Department of Medicine, University of Illinois at Chicago, Chicago, IL, USA

Abstract

Lysophosphatidic acid (LPA) plays a dual-function in lung inflammatory diseases. LPA receptors contribute to the pathogenesis of asthma, acute lung injury, and fibrosis. Here, we investigate the role of LPA receptor type 2 (LPA_2) in sepsis-induced lung inflammation and injury. Sepsis was induced using cecal ligation and single puncture (CLP) with 27 gauge needle. Plasma interleukin-6 (IL-6) and KC levels were elevated in septic wild type and LPA_2-/- mice, while septic LPA_2-/- mice reduces plasma KC, not IL-6 levels, compared to septic wild type mice. Bronchoalveolar lavage (BAL) KC levels increased in septic wild type and LPA_2-/- mice, while the sepsis had no effect on BAL IL-6 levels, protein leak, and inflammatory cell infiltration in the lungs in wild type and LPA_2-/- mice. Hematoxylin and eosin (H&E) staining revealed that septic LPA_2-/- mice aggravated alveolar space enlargement. Western blotting analysis of lung tissues demonstrate that the level of cortactin, an F-actin binding protein, was decreased in septic LPA_2-/- mice, when compared to wild type mice. The level of immunoglobulin G (IgG) in BAL fluids significantly increased in septic LPA_2-/- mice, when compared to septic wild type mice and sham mice. Furthermore, we found that sham and septic LPA_2-/- mice increased surfactant proteins B, C, and D (SP-B, SP-C, and SP-D) expression in lungs, while SP-A levels in lungs was decreased in sham and septic LPA_2-/- mice. These results suggest LPA_2 may regulate cortactin and surfactant protein expression in the lung. LPA_2 and its downstream signaling may play a protective role against sepsis induced emphysema like disease.

Keywords: LPA receptor; Sepsis; Alveolar space enlargement; Surfactant protein; Cortactin

Abbreviations: LPA: Lysophosphatidic Acid; CLP: Cecal Ligation and Puncture; IL-6: Interleukin-6; BAL: Bronchoalveolar Lavage; H & E: Hematoxylin and Eosin; SP-A-D: Surfactant Protein-A-D; ARDS: Acute Respiratory Distress Syndrome; ELISA: Enzyme-Linked Immunosorbent Assay; IgG: Immunoglobin G

Introduction

Lysophospholipids have been known as vital components in the organization of membrane structure; however the increasing evidences suggest that lysophospholipids also induce various cellular responses through ligation to their receptors on cell surface. Among the lysophospholipids, lysophosphatidic acid (LPA), a simple biophospholipid, has been detected in various biological fluids, such as plasma [1] and bronchoalveolar larvage (BAL) fluids [2-4]. LPA induces both pro and anti-inflammatory responses in inflammatory lung diseases. Evidence of the pro-inflammatory effect of LPA has been observed by it increasing interleukin-8 (IL-8) production and secretion in lung epithelial cells [5-7]. Intratracheal administration of LPA for 6 h induces neutrophil infiltration into the alveolar spaces, while at 24 h, the effect of LPA on neutrophil infiltration returns to the basal level [5]. Recent studies have shown that intratracheal administration of LPA at 24 h or intravenous injection of LPA attenuates endotoxin-induced lung inflammation, suggesting that exogenous LPA exhibits an anti-inflammatory property [8]. Furthermore, LPA increases IL-13 decoy receptor (IL-13Rα) [9] and IL-33 decoy receptor (sST2) [10] release in human bronchial epithelial cells. In addition to the modulation of inflammatory responses, LPA plays a protective role against lung injury by enhancing lung epithelial barrier integrity and remodeling [8,11].

The biological effects of LPA are through LPA receptors on the cell surface. So far, seven LPA receptors have been cloned [12]. The role of LPA receptors in lung inflammatory diseases have been investigated using LPA receptor deficient mice. LPA receptor 1 (LPA_1) mice show a reduction of lung inflammation in murine models of pulmonary fibrosis and acute lung injury [3]. LPA_1 heterozygous knockout mice reduce goblet cell hyperplasia and mucus generation in a murine model of asthma [4]. Down-regulation of LPA_2 reduces pathogen induced eosinophil infiltration into airway lumen [4], suggesting that endogenous LPA and its receptors may exhibit pro-inflammatory properties.

Sepsis is a life-threatening systemic disease caused by bacterial infection. Here, we investigate the effect of down-regulating LPA_2 in sepsis-induced lung inflammation. This study is the first report to demonstrate that LPA_2 deficient mice show alveolar space enlargement with a reduction of cortactin, an increase in the BAL IgG level, and changes of surfactant proteins in the lungs of a murine model of cecal ligation and puncture (CLP)-induced sepsis. These findings may provide a new therapeutic target against septic lung injury.

Materials and Methods

LPA_2-/- mice – LPA_2-/- mice were generated as previously described

***Corresponding author:** Yutong Zhao, Department of Medicine, University of Pittsburgh, 3459 Fifth avenue, MUH NW628, Pittsburgh, PA, 15213, USA, Tel: 412 648 9488; E-mail: zhaoy3@upmc.edu

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[13]. Mice were bred and housed in a specific pathogen-free barrier facility maintained by the University of Illinois at Chicago Animal Resources Center. The studies reported here conform to the principles outlined by the Animal Welfare Act and the National Institutes of Health guidelines for the care and use of animals in biomedical research. Extract-N-Amp Tissue PCR kit (Sigma Aldrich, S. Louis) was utilized for isolating genomic DNA from mouse tail and amplifying DNA fragments. The primers for LPA_2 knockout mice were described in previous studies [13].

Sepsis model by CLP - CLP was used to induce sepsis. Briefly, a 3-cm midline laparotomy was made first through the skin and then the cecum with the adjoining intestine was exteriorized and ligated at 0.5 cm from its end with a 3.0 silk. Then the ligated cecum was punctured with a 27-gauge needle, allowing entrapped fecal material to leak into the normally sterile peritoneal cavity. The cecum was then repositioned in the peritoneal cavity and the abdomen was closed. Sham-operated animals received laparotomy only. After 24 h, plasma, BAL fluids, and lung tissues were collected. After incubation with red cell lysis buffer, cell numbers in BAL fluids were counted by TC10™. Automated Cell Counter (Bio-Rad, Hercules, CA) and cell differentiation was performed by cytospin with HEMA3 staining kit (Fisher Scientific, Kalama, MI). H&E staining of lung tissues was performed by histology co-facility in University of Pittsburgh.

Cytokine measurement - BAL fluids and plasma were centrifuged at 500 g for 10 min to remove cell debris. IL-6 and KC levels were measured with ELISA kits for mouse IL-6 or KC according to the manufacture's instruction (Invitrogen).

Alveolar space measurement

Chord length, which measures the average distance between alveolar walls, is proportional to the amount of emphysema, as prevously described [14,15]. Three randomly selected ×10 fields per slide (3 slides /group) were photographed and the images analyzed using Scion Image software (Scion Corporation, Frederick, MD).

RNA isolation and Real-time RT-PCR - Total RNA was isolated from lung tissues using TRIzol® reagent (Life Technology, Rockville, MD) according to the manufacturer's instructions. RNA was quantified spectrophotometrically and 1 µg of RNA was reversed transcripted using cDNA synthesis kit (Bio-Rad) and Real-time PCR and quantitative PCR were performed to assess expression of the LPA_2 using primers designed based on mouse mRNA sequences. Amplicon expression in each sample was normalized to its 18S RNA content. The relative abundance of target mRNA in each sample was calculated as 2 raised to the negative of its threshold cycle value times 106 after being normalized to the abundance of its corresponding 18S [e.g., $2^{-(\text{Target Gene Threshold Cycle})} / 2^{-(\text{18S Threshold Cycle})} \times 10^6$]. Primers for PCR: $mLPA_2$: Forward: 5'-ATATTCCTGCCGAGATGCTG-3', Reverse: 5'-AAGCTGAGTAACGGGCAGAC-3'; 18S: Forward: 5' -GTAACCCGTTGAACCCCATT-3', Reverse: 5'-CCATCCAATCGGTAGTAGCG-3'.

Western blotting

Equal amounts of protein (20 µg) or equal volumes of BAL fluids were subjected to 10% SDS/PAGE gels, transferred to polyvinylidene difluoride membranes, blocked with 5% (w/v) BSA in TBST (25 mM Tris-HCl, pH 7.4, 137 mM NaCl and 0.1% Tween-20) for 1 h and incubated with antibodies (dilute 1:1000) in 5% (w/v) BSA in TBST for overnight at 4°C. The membranes were washed at least three times with TBST at 15 min intervals and then incubated with a rabbit or mouse horseradish peroxidase-conjugated secondary antibody (1: 3,000) for 1 h at room temperature. The membrane was developed with an enhanced chemiluminescence detection system according to Manufacturer's instructions.

Statistical analyses

All results were subjected to statistical analysis using one-way ANOVA and, where appropriate, analyzed by Student–Newman-Keuls test. Data are expressed as means ± S.D. of samples (n = 3-7) and level of significance was taken as $P < 0.05$.

Results

Septic wild type and LPA_2-/- mice increase plasma IL-6 levels -

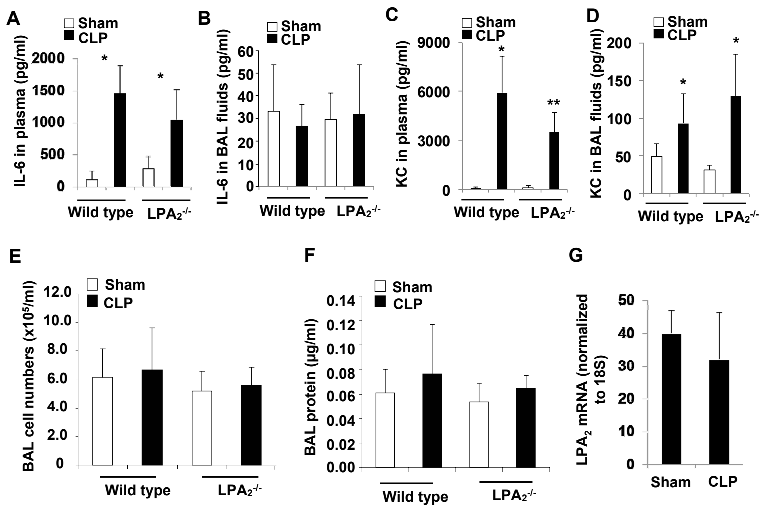

Figure 1: Septic LPA_2-/- mice showed similar manner as wild type in plasma IL-6 level, BAL IL-6 level, cell numbers, and protein levels in lungs, except reduction of plasma KC levels – A, C. Wild type and LPA_2-/- mice were challenged with CLP for 24 h. Plasma IL-6 and KC levels were measured by a ELISA kits. Data represent mean ± SD and n = 4-7. **p* < 0.01 vs sham mice, ***p* < 0.05 *vs* sham wild type. B, D. Wild type and LPA_2-/- mice were challenged with CLP for 24 h and BAL fluids were collected. BAL IL-6 and KC levels were measured by mouse cytokine ELISA kits. Data represent mean ± SD and n = 4-7. **p* < 0.01 *vs* sham mice. E. BAL cell number was accounted. Data represent mean ± SD and n = 4-7. F. BAL protein concentration was measured with BSA as series standard. Data represent mean ± SD and n = 4-7. G. mouse LPA_2 mRNA expression was examined by Real-time PCR. Data represent mean ± SD and n = 3-4.

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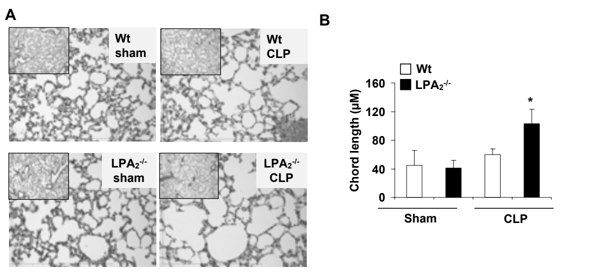

Figure 2: Septic LPA2-/- mice showed enlargement of alveolar spaces – A. Four group mice were sacrificed at 24 h and lung tissues were fixed and stained with H&E. B. Chord length of alveolar spaces were measured and quantified by Scion Image software. Data represent mean ± SD. **p* < 0.01 *vs* sham wild type, sham, and septic LPA_2-/- mice.

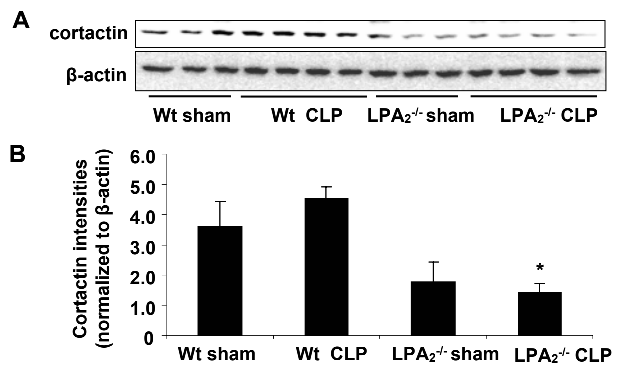

Figure 3: BAL IgG level increased in septic LPA_2-/- mice - Wild type and LPA_2-/- mice were challenged with CLP for 24 h and BAL fluids were collected. A. BAL fluids from sham wild type and LPA_2-/- mice were subjected to 10 % SDS/PAGE gel and mouse IgG levels were detected by an anti-mouse IgG antibody. B. IgG bands were quantified with ImageJ software. C. BAL fluids from all the four groups were subjected to 10 % SDS/PAGE gel and mouse IgG levels were detected by an anti-mouse IgG antibody. D. IgG bands were quantified with ImageJ software. Data represent mean ± SD and n = 5-6. **p* < 0.01 *vs* sham wild type, sham LPA_2-/-, and septic wild type mice.

We have shown that LPA_2 heterozygous (LPA_2+/-) mice reduce mucus generation in a mouse model of Th2-dominant inflammatory diseases [4]. Sepsis is a clinical syndrome that complicates severe infection. Treating sepsis during its mild stage is critical because the likelihood of multisystem organ dysfunction increases as it progresses. To investigate the role of LPA_2 in the pathogenesis of sepsis-induced lung injury, we selected a CLP-induced sepsis mouse model. Sepsis was induced by CLP with a 27 gauge needle. Septic wild type and LPA_2-/- mice survived after a 24 h period (data not shown). Blood samples were collected and plasma IL-6 and KC levels were determined by ELISA kits. Plasma IL-6 and KC levels significantly increased in both septic wild type and LPA_2-/- mice when compared to sham mice (Figure 1A-1C), suggesting that CLP induced a systemic inflammatory response.

Plasma KC levels were significantly reduced in septic LPA_2-/- mice (Figure 1C) when compared to septic wild type mice, however, plasma IL-6 in septic LPA_2-/- mice had no statistical difference when compared to septic wild type mice (Figure 1A).

Sepsis increases KC levels, not lung leak and neutrophil influx in wild type and LPA_2-/- mice

To investigate the lung inflammatory responses under sepsis, we measured the BAL IL-6 and KC levels from septic wild type and LPA_2-/- mice. BAL KC levels, but not the BAL IL-6 levels, were increased in both in septic wild type and LPA_2-/- mice, while there was no statistical difference between in the two groups (Figure 1D). These results suggest that LPA / LPA_2 axis contributes to KC production in plasma. We further determined whether sepsis induces inflammatory cell infiltration to the lung alveolar spaces. The BAL cell numbers were accounted. As shown in Figure 1E, there was no significant difference in BAL cell numbers within these four groups. Cytospin showed that macrophage is the dominant cell type in BAL fluids in the all four groups (data not shown). In addition, there was no significant change in BAL protein concentration within all the four groups (Figure 1F). To investigate whether CLP affects lung LPA_2 expression, LPA_2 mRNA expression in lung tissues were examined by Real-time PCR. As shown in Figure 1G, LPA_2 mRNA was not changed after CLP. These results suggest that sepsis by CLP with a 27-gauge needle increased KC levels in BAL fluids, but the procedure has limited effects on inflammatory cells influx into lung and lung leak in wild type and LPA_2-/- mice.

Septic LPA_2-/- mice show alveolar space enlargement

Further, we examined the lung histology by H&E staining. All the four groups did not show significant inflammatory cell influx into the lung alveolar spaces. These data are consistent with the results from cell number accounts (Figure 1E). To estimate the morphological change in response to CLP, we measured the chord length, which measures the average distance between alveolar walls and is proportional to the amount of emphysema. Chord length was not significantly changed in septic wild type mice when compared to sham wild type (60.2 ± 7.6 to 44.6 ± 21.3 μm, $p > 0.05$). Chord length of sham LPA_2-/- mice was similar to sham wild type mice (44.6 ± 21.3 to 40.9 ± 11.0 μm, $p > 0.05$), however, chord length was significantly increased in septic LPA_2-/- mice when compared to septic wild type mice (102.9 ± 20.0 to 60.2 ± 7.6 μm, 41.5%, $p < 0.01$) (Figure 2A and 2B). These results suggest that LPA_2 and its downstream signaling protect against alveolar space enlargement.

LPA_2-/- mice reduced cortactin expression in lung

To investigate the mechanisms by which septic LPA_2-/- mice show emphysema phenotype, we determined the lung expression levels of an F-actin binding protein, cortactin, since it plays a critical role in maintaining both lung epithelial [11] and endothelial barrier function [16,17]. As shown in Figure 3, cortactin levels in lung tissues slightly decreased in sham LPA_2-/- mice, when compared to sham wild type mice, but this was not statistically significant. Cortactin levels in the lungs from septic LPA_2-/- mice were significantly reduced, when compared to sham and septic wild type mice (Figure 3). The entirety of the data indicates level of cortactin is less in LPA_2-/- mice, compared to wild type mice. The reduction of cortactin levels in the lungs from septic LPA_2-/- mice may contribute to pathogenesis of emphysema.

Septic LPA_2-/- mice increased BAL IgG levels

IgG levels in BAL fluids are usually very low, whereas BAL IgG levels increase in lung inflammatory diseases. The local BAL IgG production is an index of an increase in invading bacteria or pathogen into the lungs. We measured the BAL IgG levels from sham and septic wild type and LPA_2-/- mice by Western blotting. BAL IgG levels were similar between sham wild type and LPA_2-/- mice (Figure 4A and 4B), while BAL IgG levels increased in septic LPA_2-/- mice, but not in septic wild type mice (Figure 4C and 4D). These results suggest that down-regulation of LPA_2 or its mediated signaling increases IgG levels in BAL fluids in CLP-induced sepsis.

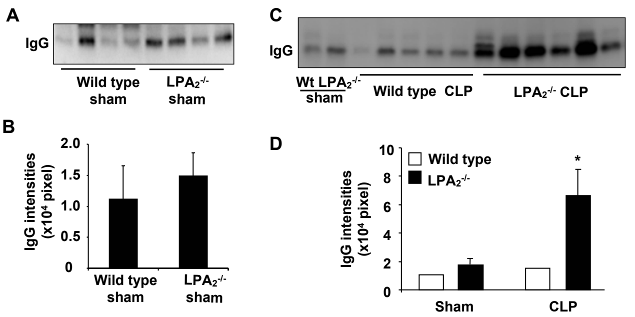

Figure 4: Septic LPA_2-/- mice reduced cortactin expression in the lungs – A. Four group mice were sacrificed at 24 h and lung tissues were lysed. Equal amount of lung tissue proteins (20 μg) were subjected to 10 % SDS/PAGE gel and cortactin levels were determined by Western blotting with antibodies to cortactin and β-actin. B. Intensities of cortactin and β-actin bands were analyzed by ImageJ software. Data represent mean ± SD and n = 3-4. *p < 0.01 *vs* sham and septic wild type mice.

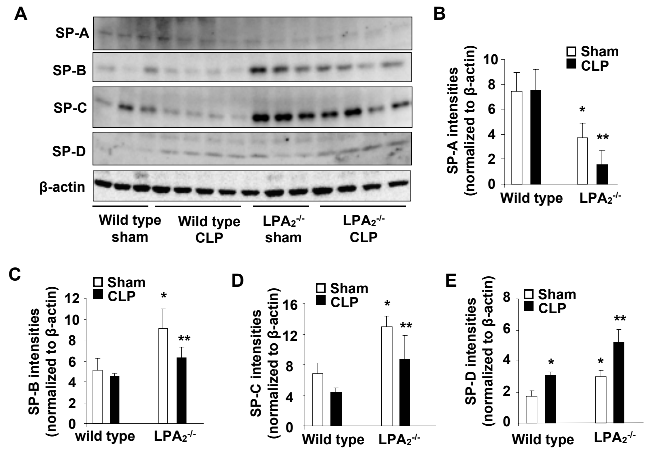

Figure 5: Septic LPA_2-/- mice altered surfactant protein levels in the lung – A. Four group mice were sacrificed at 24 h and lung tissues were lysed. Equal amount of lung tissue proteins (20 μg) were subjected to 12 % SDS/PAGE gel and surfactant protein levels were determined by Western blotting with antibodies to surfactant protein A-D and β-actin. B-E. Intensities of surfactant protein and β-actin bands were analyzed by ImageJ software. Data represent mean ± SD and n = 3-4. *p < 0.05 *vs* sham wild type mice. *p < 0.05 *vs* septic wild type mice.

Septic LPA_2-/- mice changed surfactant proteins levels in the lungs

In addition to cytoskeleton associated proteins, surfactant proteins play a critical role in maintaining alveolar structure by reducing surface tension and preventing collapse of the lung [18]. The changes of surfactant protein (SP-A, SP-B, SP-C, and SP-D) level in the lungs from the septic wild type and LPA_2-/- mice were examined by Western blotting (Figure 5). SP-A levels in the lungs were reduced in sham and septic LPA_2-/- mice, when compared to sham and septic wild type mice. SP-B levels in lungs were unaltered between sham and septic wild type mice, while SP-B levels in lungs increased in sham and septic LPA_2-/- mice, when compared to sham and septic wild type mice. SP-B levels in lungs from septic LPA_2-/- mice were lower than that from sham LPA_2-/- mice; however it was not statistically significant. SP-C levels in lungs increased in sham and septic LPA_2-/- mice, when compared to sham and septic wild type mice. SP-D levels in lungs increased in septic wild type mice and sham LPA_2-/- mice, when compared to sham wild type mice. Furthermore, when compared to septic wild type mice, SP-D levels further increased in septic LPA_2-/- mice. Overall, changes of surfactant protein are more significant between LPA_2-/- and wild type mice, as compared to sham vs. sepsis.

Discussion

Sepsis is characterized as an inflammatory infection that if not treated promptly can prove to be fatal. Active intracellular signaling and cellular responses are associated with sepsis. The current study focuses on determining the role of a bioactive lysophospholipid receptor, LPA_2 in CLP-induced septic lung injury. We found that septic LPA_2-/- mice show a significant reduction in plasma KC levels, an increase in BAL IgG level, enlargement of alveolar spaces, reduction of cortactin, and changes of surfactant proteins expression in the lungs. Septic LPA_2-/- mice exhibit same manner as septic wild type mice regarding the plasma and BAL IL-6 levels, inflammatory cell infiltration, and protein leak in BAL fluids. These results suggest that LPA_2 may protect against emphysema by maintaining alveolar structure.

Among the seven LPA receptors, the roles of LPA_1-3 in lung inflammatory diseases have been investigated. LPA receptors contribute to pathogenesis of asthma [2,4], fibrosis [19], and acute lung injury [3]. LPA_1 deficient mice reduce intratracheal LPS [3] and bleomycin [19]-induced acute lung injuries. Mucus generation is attenuated in pathogen-induced LPA_1 and LPA_2 heterozygous knockout mice [4]. To investigate the role of LPA_2 in the pathological changes of the lungs in sepsis, we generated a murine model of sepsis using CLP with 27-gauge needle. The model demonstrates increases in plasma IL-6 and KC levels and BAL KC levels in septic wild type and LPA_2-/- mice. This suggests that the sepsis model used in this study induces a systemic inflammation, such as BAL KC release in the lungs, however, there is no increase in lung protein leak and inflammatory cell influx. LPA plays a pro-inflammatory role by inducing IL-8 [5-7] and PGE2 [20] release in several cell types, including lung epithelial, endothelial [21], and smooth muscle cells [22]. LPA_{1-3} is involved in LPA-induced activation of transcriptional factors. We have shown that LPA1 contributes to LPS-induced signaling via interaction with LPS co-receptor, CD14 [3]. The role of LPA_2 in lung inflammation has been investigated in murine models of asthma [4] and fibrosis (Zhao Y et al. unpublished data). LPA_2 contribute to LPA-induced TGFβ activation [23] and IL-8 production [6]. LPA_2 heterozygous knockout mice reduce mucus generation and eosinophil infiltration into alveolar spaces in the asthma model [4] and lessen inflammation and protein leak in the lung in a bleomycin-induced murine model (Zhao Y et al. unpublished data). The current study suggests that LPA_2 is involved in plasma KC production, but not plasma IL-6 and BAL KC production, in sepsis.

The novel finding in this study is that LPA_2-/- mice exhibit emphysema-like phenotype with reduction of cortactin, increases of BAL IgG, and changes of surfactant protein expression. This is the first report to demonstrate that LPA_2 plays a protective role in maintaining alveolar structure.

LPA exhibits an anti-apoptosis via ligation to LPA_2 [24]. To investigate whether the alveolar space enlargement in LPA_2-/- mice is due to an increase in apoptosis, we examined the apoptotic cells in lung tissues by TUNEL assay and Western blotting with a cleaved caspase 3 antibody. There was no significant increase in apoptosis in septic wild type and LPA_2-/- mice, when compared to sham mice (data not shown). Interestingly, the cortactin expression in the lungs from septic LPA_2-

/- mice was significantly reduced. Cortactin, a F-actin binding protein plays a critical role in maintaining cell structure, cell-cell contact, and migration [25,26]. Cortactin activation is induced by LPA and mediates LPA-induced lung epithelial cell migration [11]. Cortactin deficiency increase vascular permeability [27]. The loss of cortactin might contribute to pathogenesis of alveolar space enlargement in septic LPA_2-/- mice. The current study demonstrates the reduction of cortactin has no association with alveolar permeability in septic LPA_2-/- mice. The disparity may be due to the levels of cortactin in the different systems. The current study demonstrated that CLP-challenged LPA_2-/- mice partially reduce cortactin levels (~52% reduction when compared to wild type). This partial reduction of cortactin may not be sufficient to induce permeability and cell death, while it may contribute to cytoskeleton rearrangement thus causing an increase in alveolar spaces.

Furthermore, we found that LPA_2-/- mice increase the BAL IgG level and change the surfactant protein expression in the lungs. Serum IgG is a biomarker for emphysema [28]. The local BAL IgG production indicates an increase in invading bacteria into the lungs. The increases in BAL IgG in LPA_2-/- mice are not likely from circulation, since there is no endothelial and epithelial barrier disruption in the current model. Intratracheal injection of IgG immune complex induces rat lung injury [29]. These results, at least, in part, suggest that LPA_2-/- mice increase bacterial invasion into the lungs after CLP and that the lack of the LPA/LPA_2 axis might promote bacterial invasionmediated airspace enlargement. Changes of surfactant proteins are associated with respiratory failures [30-32]. For example, serum SP-A and SP-D increase in patients with septic acute respiratory distress syndrome (ARDS) [33] and serum SP-A and SP-B increase in patients with acute respiratory failure [31,32]. A significant decrease in SP-A, SP-B, and SP-C levels in septic adult sheep has been observed [34]. The role of changes of surfactant protein expression in the pathogenesis of alveolar space enlargement in septic LPA_2-/- mice is not clear, whereas LPA2 and its mediated downstream signaling regulates surfactant protein expression in sepsis. Increased SP-D plays a protective role in the development of emphysema, in part by preventing alveolar cell death [31]. Here, the increases in surfactant proteins in sham and CLP-challenged LPA_2-/- mice might be a negative feedback loop in providing host defense for the lungs. The reduction of LPA_2 and cortactin levels in lungs might be developed as biomarkers for the high risk of emphysema. Future studies will focus on the mechanisms by which LPA and LPA_2 regulate cortactin and surfactant protein expression and stability.

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