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RvD1 ameliorates LPS-induced acute lung injury via the suppression of neutrophil infiltration by reducing CXCL2 expression and release from resident alveolar macrophages☆

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ABSTRACT

Acute lung injury (ALI) and/or acute respiratory distress syndrome (ARDS) are life-threatening critical syndromes characterized by the infiltration of a large number of inflammatory cells that lead to an excessive inflammatory response. Resolvin D1 (RvD1), an endogenous lipid mediator, is believed to have anti-inflammatory and proresolving effects. In the present study, we examined the impact of RvD1 on the pulmonary inflammatory response, neutrophil influx, and lung damage in a murine model of lipopolysaccharide (LPS)-induced ALI. Treatment with RvD1 protected mice against LPS-induced ALI, and compared to untreated mice, RvD1-treated mice exhibited significantly ameliorated lung pathological changes, decreased tumor necrosis factor-α (TNF-α) concentrations and attenuated neutrophil infiltration. In addition, treatment with RvD1 attenuated LPS-induced neutrophil infiltration via the downregulation of CXCL2 expression on resident alveolar macrophages. Finally, BOC-2, which inhibits the RvD1 receptor lipoxin A4 receptor/formyl peptide receptor 2 (ALX/FPR2), reversed the protective effects of RvD1. These data demonstrate that RvD1 ameliorates LPS-induced ALI via the suppression of neutrophil infiltration by an ALX/FPR2-dependent reduction in CXCL2 expression on resident alveolar macrophages.

1. Introduction

Acute lung injury (ALI) and/or acute respiratory distress syndrome (ARDS) are characterized by an uncontrolled alveolar inflammatory response, the hallmark of which is the excessive infiltration of inflammatory cells, especially neutrophils [1,2]. A previous study showed that macrophages are necessary for the resolution of lung inflammation [3]. Two populations of alveolar macrophages coexist in the inflamed lung: resident alveolar macrophages populate the lung during embryogenesis and self-renew throughout the whole life cycle [4,5], and recruited macrophages that originate postnatally from circulating monocytes [6]. Previous study showed that resident alveolar macrophages were governed by increased tricarboxylic acid cycle and amino acid metabolism [6]. Another study showed that resident alveolar macrophages could suppress allergic lung inflammation in murine models of asthma [7]. According to results from various experimental models [8–11], resident alveolar macrophages depletion showed a protective effect against ALI/ARDS at an early stage, while it exacerbated lung injury at a later stage. Conversely, recruited macrophages could produce inflammatory cytokines in association with increased glycolytic and arginine metabolism [6].

CXCL2, also known as macrophage inflammatory protein 2-α, is a small secreted cytokine that belongs to the CXC chemokine family [12,13]. The principal role of CXCL2 involves the chemotaxis of neutrophils into inflamed tissues, where they bind to the chemokine receptor CXCR2. CXCL2 is mainly expressed on macrophages, endothelial cells, and glial cells [14,15].

Resolvin D1 (7S,8R,17S-trihydroxy-4Z,8E,10Z,12E,14E,19Z-

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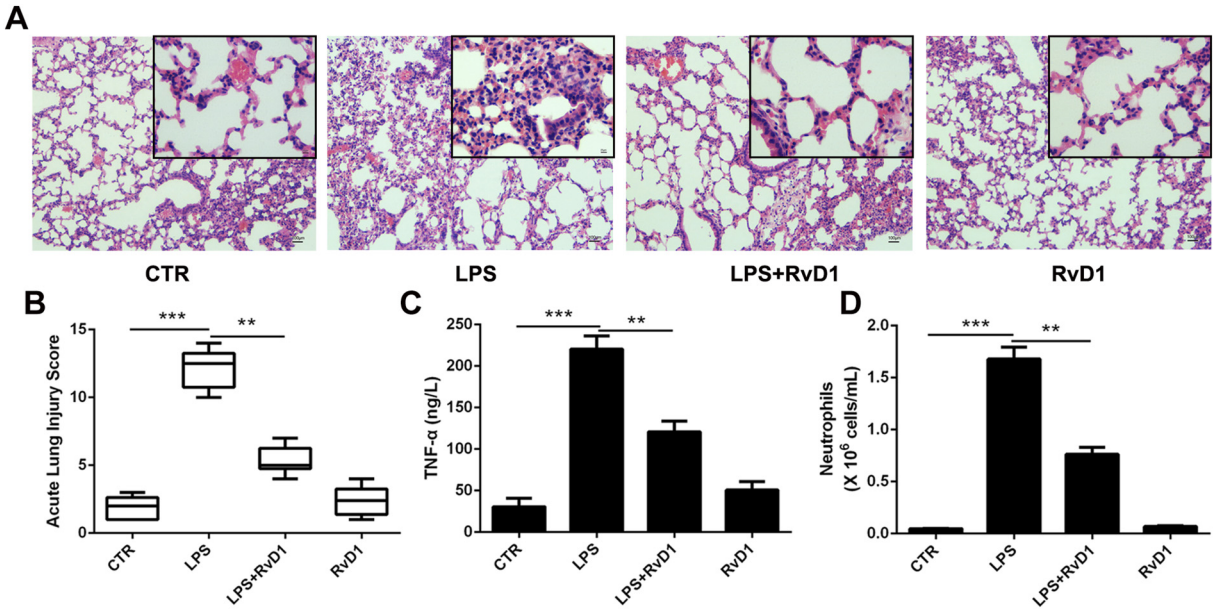

Fig. 1. RvD1 attenuated LPS-induced lung tissue damage and neutrophil infiltration. Mice received 1 mg/kg LPS by intratracheal atomization and then RvD1 (0.1 μg/mouse) by intraperitoneal injection. Lung histological changes were assessed 24 h later. Representative images of lung tissue sections stained for CTR, LPS, LPS + RvD1 and RvD1 with hematoxylin and eosin are shown and indicate that treatment with RvD1 strongly ameliorates LPS-induced lung edema, hemorrhage and alveolar collapse (A), and acute lung injury score (B). The aerosol inhalation of LPS significantly increased the TNF-α concentration in lung tissue homogenates and BAL fluid neutrophil counts; these effects were markedly attenuated by RvD1 treatment. Data are presented as the mean ± SEM. n = 6–8. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Docosaheaenoic acid) (RvD1) is a lipid mediator derived from both eicosapentaenoic acid and docosahexaenoic acid that decreases excessive polymorphonuclear neutrophil (PMN) infiltration and transmigration [16,17]. A recent study showed that RvD1 improves the survival rate and attenuates lung inflammation [18]. Furthermore, RvD1 was recently shown to inhibit cytokine release at sites of inflammation. RvD1 directly binds to lipoxin A4 receptor/formyl peptide receptor 2 (ALX/FPR2) with high affinity and activates it [19].

The present study tested the hypothesis that RvD1 administration protects against liposaccharide (LPS)-induced lung injury and inhibits neutrophil infiltration. Our secondary hypothesis was that neutrophil infiltration associated with resident alveolar macrophage CXCL2 expression. Finally, we determined the effect of the RvD1 receptor on CXCL2 expression in resident alveolar macrophages to gain a better understanding of the mechanisms of RvD1.

2. Materials and methods

2.1. Materials

RvD1 was obtained from Cayman Chemical Company (Ann Arbor, MI). BOC-2 (ALX inhibitor) was obtained from Biomol-Enzo Life Sciences (Farmingdale, NY). Tumor necrosis factor-α (TNF-α), GolgiPlug and a CXCL2 ELISA kit were purchased from R&D Systems (Minneapolis, MN). LPS (*Escherichia coli* serotype 055:B5) was purchased from Sigma (St. Louis, MO). Mouse BD Fc Block (anti-mouse CD16/CD32) was purchased from BD Pharmingen(San Diego, CA). A CXCL2 inhibitor, CXCR2i was purchased from MedChem Express (Monmouth Junction, NJ). Antibodies against CXCL2 (clone 40605 and polyclonal goat anti-mouse antibody, R&D Systems), Ly6C-FITC, F4/80-PE-Cyanine7, CD11c-PerCP-Cyanine5.5, CD11b-APC, and Siglec F-PE were purchased from Invitrogen (Carlsbad, CA). CXCR2-PE was purchased from BioLegend (San Diego, CA). An RT-PCR kit was obtained from Thermo Scientific (Rockford, IL). SYBR Green Real-time PCR Master Mix was obtained from Toyobo (Osaka, Japan).

2.2. Animal preparation

Specific pathogen-free adult C57BL/6 mice weighing 20–25 g were obtained from Slac Laboratory Animal (Shanghai, China). The mice were housed under controlled temperature and humidity with a standard day-night cycle and had free access to food and water. All procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals. This study was approved by the Animal Studies Ethics Committee of Wenzhou Medical University.

Mice were randomized into eight groups (n = 6–8): the control (CTR) group, LPS group, RvD1 group, LPS + RvD1 group, LPS + RvD1 + CXCR2 inhibitor (CXCR2i) group, LPS + CXCR2i group, LPS + RvD1 + BOC-2 group, and LPS + BOC-2 group. An LPS-induced lung injury model was created by atomization inhalation of 1 mg/kg LPS. Mice in the RvD1 group and the CTR group received RvD1 (0.1 μg/mouse) or an equivalent volume of saline via intraperitoneal injection. Mice in the LPS + RvD1 group received RvD1 via intraperitoneal injection 10 min after LPS administration. Mice in the LPS + RvD1 + CXCR2i and LPS + CXCR2i groups received CXCR2 inhibitor (2 mg/kg) in the presence or absence of RvD1 via intraperitoneal injection 10 min after LPS administration. Mice in the LPS + RvD1 + BOC-2 and LPS + BOC-2 groups received BOC-2 (50 μg/kg) via intraperitoneal injection 2 h before LPS administration in the presence or absence of RvD1. Twenty-four hours later, the mice were anesthetized, and bronchoalveolar lavage (BAL) fluid and lung tissues were collected.

2.3. Histology

The lung lobes were harvested, fixed in 4% paraformaldehyde for 24 h, embedded in paraffin and stained with hematoxylin and eosin (H &E) for light microscopy. A semiquantitative scoring system that took into account alveolar congestion, alveolar hemorrhage, neutrophil infiltration or aggregation in the airspace or vessel wall, alveolar wall/hyaline membrane thickness and inflammatory cell infiltration was adopted to evaluate lung injury. The pathological scoring system used

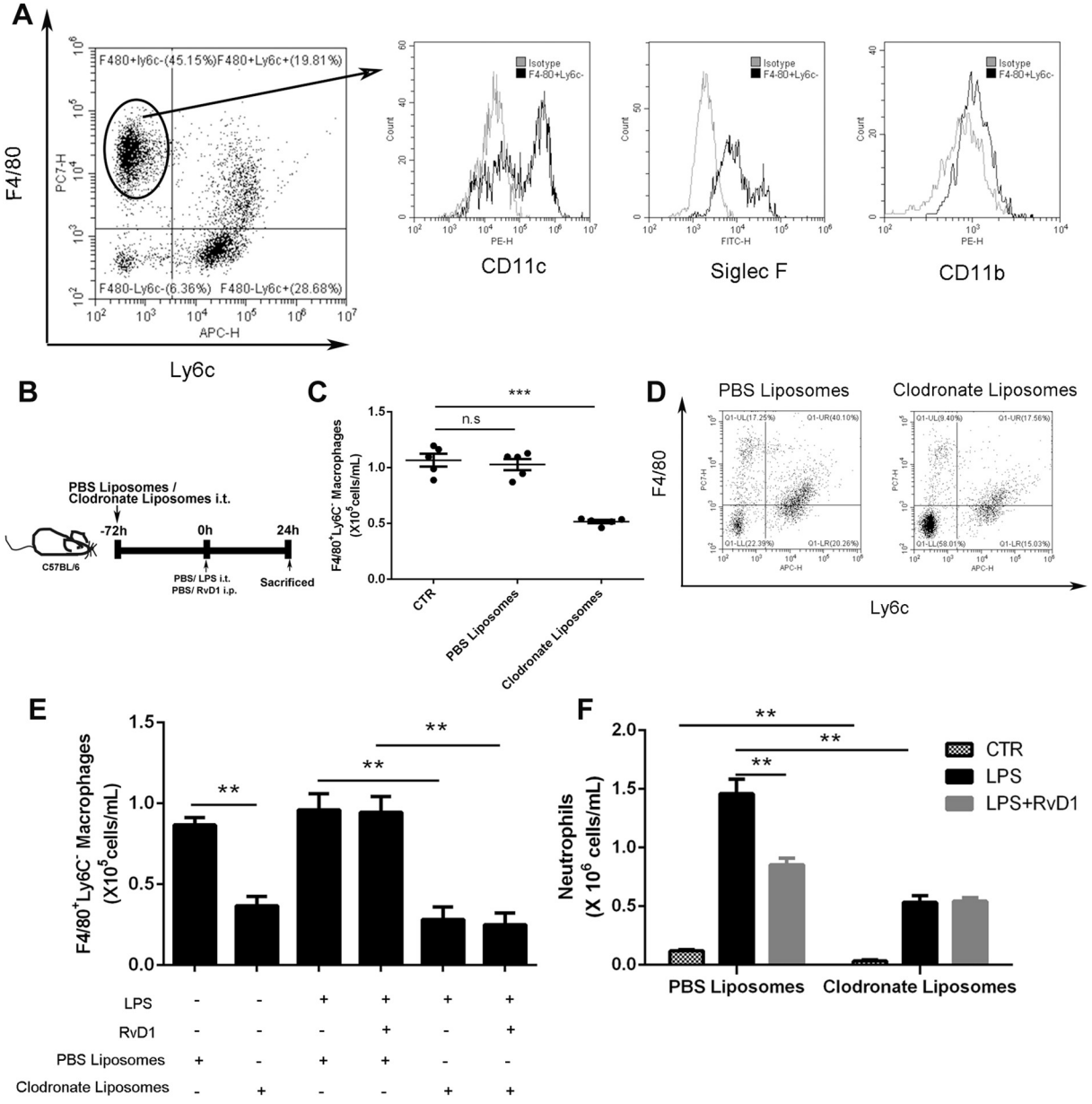

Fig. 2. Inhibition of LPS-induced neutrophil infiltration by RvD1 was dependent on resident alveolar macrophages. Resident alveolar macrophages in the BAL fluid were determined by flow cytometry 24 h after LPS (1 mg/kg) inhalation. F4/80$^+$Ly6C$^-$CD11c$^+$Siglec F$^+$CD11b$^-$ cells were defined as resident alveolar macrophages (A). Resident alveolar macrophages were depleted in lung 24 h after the intratracheal administration of 50 μL clodronate liposome for 72 h in the normal control mice (B, C) and in the LPS-stimulated lung (D). Then, the RvD1 (0.1 μg/mouse) was administration 10 min after LPS (1 mg/kg) stimulation, the numbers of resident alveolar macrophages (E) and neutrophils (F) in the BAL fluid were measured by flow cytometry. The data are presented as the mean ± SEM. n = 6–8. **p < 0.01.

for light microscopy was as follows: 0 = no injury; 1 = slight injury (25%); 2 = moderate injury (50%); 3 = severe injury (75%); and 4 = very severe injury (almost 100%). Each item was scored from 0 to 4, as described previously. The four scores were summed to determine the lung injury score (total score: 0–16).

2.4. Flow cytometry

Single-cell suspensions were generated by digestion with 1 mg/mL collagenase IV (Sigma) and 100 mg/mL DNaseI (Sigma) in FCS-containing RPMI for 30 min at 37 °C under mild stirring. Freshly collected and isolated cells were incubated with antibodies against F4/80-PE-Cyanine7, Ly6C-FITC, CD11b-APC, CD11c-PerCP-Cyanine5.5 as appropriate for 30 min. Afterwards, fluorescence-activated cell sorting (FACS) lysis solution (BD Biosciences, Heidelberg, Germany) was added, and the cells were incubated for another 10 min. Cells were centrifuged for 5 min (400 ×*g*) and washed with phosphate-buffered saline (PBS). Finally, the cells were resuspended in PBS and analyzed on a FACS Calibur using CytExpert 2.0 (Beckman Coulter).

For intracellular staining, cells were cultured for 4 h with 1 μL/mL Golgi-Plug, treated with Fc Block for 20 min, fixed for 10 min with 4% paraformaldehyde and then permeabilized with BD Perm/wash. Cells were then incubated with titrated antibodies. A FITC-labeled secondary goat anti-mouse antibody was used to detect CXCL2.

2.5. Depletion of resident alveolar macrophages

Alveolar macrophages were depleted by the intratracheal

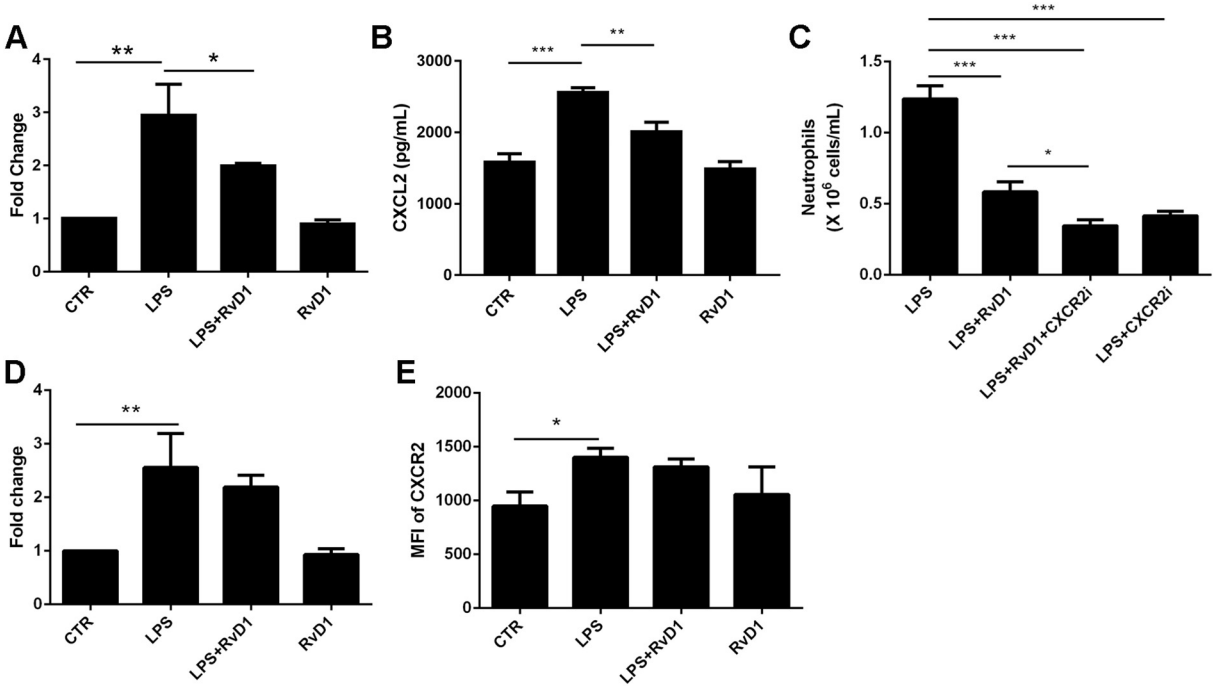

Fig. 3. RvD1 downregulated LPS-stimulated CXCL2 expression and release to inhibit neutrophil infiltration. Mice received 1 mg/kg LPS by intratracheal atomization and then RvD1 (0.1 μg/mouse) by intraperitoneal injection. CXCL2 mRNA expression in lung tissue homogenates (A) and CXCL2 levels in BAL fluid (B) were measured 24 h later. Remarkably, treatment with RvD1 downregulated LPS-stimulated expression of the chemokine CXCL2 in lung tissue homogenates and attenuated its release into the BAL. A CXCR2 inhibitor was administered, and the number neutrophils in the BAL fluid was measured by flow cytometry. The CXCR2 expression on lung tissue homogenates was measured by RT-PCR(D), and the CXCR2 level on BAL fluid neutrophils was measured by flow cytometry (E). CXCR2i = CXCR2 inhibitor. Data are presented as the mean ± SEM. n = 6–8. *p < 0.05, **p < 0.01, ***p < 0.001.

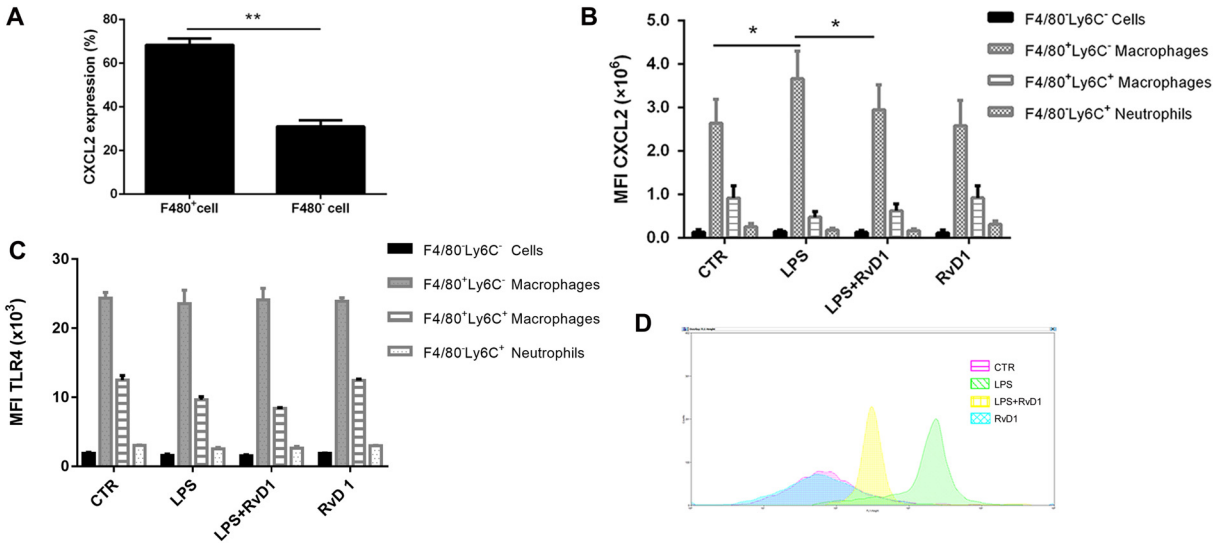

Fig. 4. RvD1 inhibited LPS-stimulated CXCL2 expression on resident alveolar macrophages without altering TLR4 expression. The percentage of CXCL2 expression on the F4/80$^+$ cells and F4/80$^-$ cells was measured by flow cytometry (A). Then, mice received 1 mg/kg LPS by intratracheal atomization and then RvD1 (0.1 μg/mouse) by intraperitoneal injection. Single-cell suspensions from lung homogenates was collected 24 h later. CXCL2 expression was assessed by flow cytometry and expressed as mean fluorescence intensity (MFI) (A). CXCL2 was mainly expressed on resident alveolar macrophages, which was strongly downregulated by RvD1 (A, C). TLR4 surface expression was determined by flow cytometry and expressed as mean fluorescence intensity (MFI) (B). Data are presented as the mean ± SEM. n = 6–8. *p < 0.05, **p < 0.01, ***p < 0.001.

instillation of 50 μL liposome clodronate (5 mg/mL). Control mice received empty (saline-containing) liposomes. Then, 3 days later, the mice were administered LPS (1 mg/kg) via aerosol inhalation and RvD1 (0.1 μg/mouse) via intraperitoneal administration. BAL fluid was collected 24 h later.

2.6. Cell sorting by flow cytometry

Cells were sorted by MoFlo XDP (Beckman Coulter, Miami, FL)with a 100 μm nozzle directly into 5 mL tubes containing 3 mL RPMI. Cells (100,000–300,000) of each population of interest were sorted at a speed of 10,000 cells/s. The sorted resident macrophages (1×10^6) were cultured into four groups (n = 6–8): LPS group, LPS + RvD1

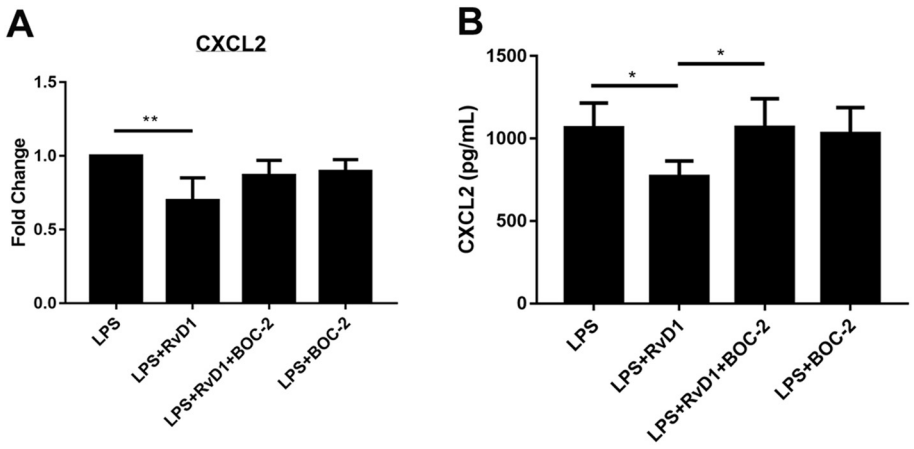

Fig. 5. RvD1 attenuated LPS-stimulated CXCL2 expression on resident macrophages by BOC-2. Sorted resident alveolar macrophages were incubated with LPS (1 μg/mL), LPS + RvD1 (100 nmol/mL), LPS + RvD1 + BOC-2 (10 μM), and LPS + BOC-2. CXCL2 mRNA (A) and protein levels of CXCL2 (B) were assessed by PCR and ELISA, respectively. RvD1 strongly downregulated LPS-stimulated CXCL2 expression on resident alveolar macrophages at the mRNA and protein levels in the supernate; this effect was abrogated by BOC-2, which inhibits the RvD1 receptor ALX/FPR2. The data are presented as the mean ± SEM. n = 6–8. *p < 0.05, **p < 0.01.

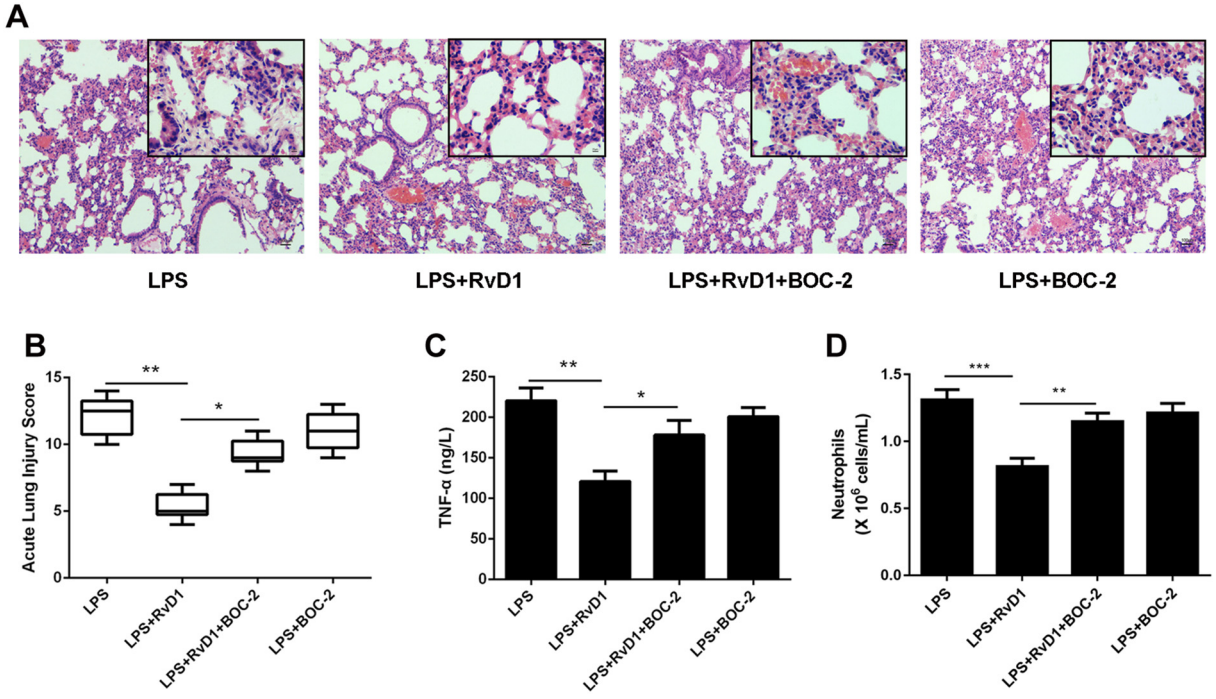

Fig. 6. The beneficial effects of RvD1 on LPS-induced ALI were abrogated by BOC-2.
Mice were intraperitoneally injected with BOC-2 (50 μg/kg) 2 h before LPS (1 mg/kg) administration in the presence or absence of RvD1 (0.1 μg/mouse). Lung tissues and BAL fluid were collected 24 h later respectively. The beneficial effects of RvD1 on LPS-induced ALI were abrogated by BOC-2, as assessed by (A) histological analysis of hematoxylin and eosin stained sections, (B) acute lung injury score, (C) TNF-a protein expression in lung tissue homogenates, and (D) the number of neutrophils in BAL fluid. Data are presented as the mean ± SEM. n = 6–8. *p < 0.05, **p < 0.01, ***p < 0.001.

group, LPS + RvD1 + BOC-2 and LPS + BOC-2 group. Cells in the LPS and LPS + RvD1 groups were incubated with LPS (1 μg/mL) in the presence or absence of RvD1 (100 nmol/mL) for 24 h. Cells in the LPS + RvD1 + BOC-2 and LPS + BOC-2 groups were pretreated with BOC-2 (10 μM) 30 min before incubation with LPS in the presence or absence of RvD1 for 24 h.

2.7. Quantitative real-time RT-PCR

Briefly, total RNA was isolated from lung tissues and resident alveolar macrophages using TRIzol reagent (Invitrogen, Carlsbad, CA) followed by phenol-chloroform extraction and ethanol precipitation (Fisher Scientific, Houston, TX). Purified RNA sample concentrations were determined on a NanoDrop 1000 (Thermo Scientific). RNA samples were reverse-transcribed into cDNA using an RT-PCR kit, according to the manufacturer's instructions. Gene expression was assessed utilizing Taq-Man Gene Expression Assays-On-Demand primer/probe sets and TaqMan Universal Master Mix (Life Technologies) on the Applied Biosystems 7500 real-time PCR system complete with SDS software. Twenty-microliter PCR reactions were performed using 1 μL of cDNA, 3.6 μL of primer/probe set, 10 μL of master mix, and 5.4 μL of DEPC-treated water by initially heating the samples to 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of heating to 95 °C for 15 s and 60 °C for 1 min. Target gene expression levels were normalized to the housekeeping gene GAPDH and the fold change was calculated using the $2^{-\triangle\triangle}$Ct method.

The primers used for the detection of the CXCL2 gene were forward 5′-CCACTCACCTGCTGCTACTCATTC-3′ and reverse 5′-CTGCTGCTGGT GATCCTCTTGTAG-3′. The primers used for the detection of the CXCR2 gene were forward 5′-GACAAGCACTTAGACCAGGCCATG-3′ and reverse 5′-AGCTCACTCGATCTGCTGTCTCC-3′.

2.8. ELISA

Protein levels of CXCL2, from the supernatants of the sorted macrophage populations or BAL supernatant, and TNF-α, from lung tissue homogenates, were measured with commercially available ELISA kits (R&D Systems Inc., Minneapolis, MN) according to the manufacturers' instructions.

2.9. Statistical analysis

The data represent the mean ± SEM. All data were analyzed by one-way ANOVA followed by Tukey's post hoc test for multiple comparisons. All tests were two-sided, and significance was determined at the $p < 0.05$ level. Statistical analyses were performed using Prism 6.0 software (GraphPad Software, San Diego, CA).

3. Results

3.1. RvD1 attenuated neutrophil infiltration in the lung and ameliorated lung tissue damage during LPS-induced ALI

First, we evaluated the effect of RvD1 on LPS-induced ALI. The lung tissues from the CTR group exhibited normal pulmonary histology, but those from the LPS group were significantly damaged and exhibited interstitial edema, hemorrhage, thickening of the alveolar wall, and infiltration of inflammatory cells into the interstitium and alveolar spaces. All the morphologic changes observed in the LPS group were less pronounced in the LPS + RvD1 group (Fig. 1A). ALI scores were quantified in parallel with pathophysiological changes (Fig. 1B). The TNF-α concentration in lung tissue homogenate and the number of neutrophils in the BAL were significantly higher in the LPS group than in the CTR group ($p < 0.01$), but substantially lower in the LPS + RvD1 group than in the LPS group ($p < 0.05$) (Fig. 1C, D). However, there was no significant difference in these parameters between the CTR and RvD1 groups ($P > 0.05$).

3.2. RvD1 inhibited neutrophil infiltration in ALI via resident alveolar macrophages

As resident alveolar macrophages play an important role in neutrophil infiltration, we were interested to know whether resident alveolar macrophages contribute to RvD1-mediated ALI resolution. We separated $F4/80^{+}Ly6C^{-}CD11c^{+}Siglec\ F^{+}CD11b^{-}$ resident alveolar macrophages from BAL fluid (Fig. 2A) as previously described [20]. Resident alveolar macrophages were depleted by clodronate liposome administration in the normal control mice, and we found the number of resident alveolar macrophages was decreased in the clodronate liposome group compared with the PBS liposome group ($P < 0.05$) (Fig. 2B, C). In addition, there were fewer resident alveolar macrophage in the LPS + RvD1 + clodronate liposome group than in the LPS + RvD1 + PBS liposome group ($P < 0.05$) (Fig. 2D, E). The number of neutrophils decreased after clodronate liposome administration in the presence or absence of LPS stimulation ($P < 0.05$) (Fig. 2F), but there was no significant difference in the number of neutrophils between the LPS + RvD1 group and LPS group after clodronate liposome administration ($P > 0.05$) (Fig. 2F).

3.3. RvD1 downregulated CXCL2 expression and release during LPS-induced ALI

Chemokine CXCL2 mRNA levels were significantly higher in LPS-treated lung tissue homogenates than in the homogenates of normal, untreated lungs ($P < 0.05$) (Fig. 3A). Moreover, LPS inhalation substantially increased CXCL2 levels in the BAL fluid compared with those of the BAL fluid of the CTR group ($P < 0.05$) (Fig. 3B). Treatment with RvD1 markedly attenuated LPS-stimulated CXCL2 expression in lung tissue homogenates and BAL fluid compared with CXCL2 expression in the LPS group ($P < 0.05$) (Fig. 3A, B).

In addition, there were fewer neutrophils in the BAL fluid of the LPS + RvD1 + CXCR2i group than in the BAL fluid of the LPS + RvD1 group ($P < 0.05$) (Fig. 3C), but there was no significant difference in neutrophil numbers between the LPS + RvD1 + CXCR2i group and the LPS + CXCR2i group ($P > 0.05$) (Fig. 3C).

The CXCR2 expression on lung tissue homogenates and BAL fluid neutrophils were significantly higher in LPS group than in the normal, untreated lungs ($P < 0.05$) (Fig. 3D, E). However, there was no significant difference between the LPS group and the LPS + RvD1 group ($P > 0.05$) (Fig. 3D, E).

3.4. RvD1 attenuated LPS-induced neutrophil infiltration via downregulation of CXCL2 expression on resident alveolar macrophages without altering TLR4 expression

In addition to macrophages, other cells, such as lung epithelial cells and endothelial cells also express CXCL2 during the course of lung injury. Therefore, F4/80 was used to label macrophages, and we found CXCL2 was mainly expressed on $F4/80^{+}$ cells (> 70%) (Fig. 4A). Next, we examined the mean fluorescence intensity (MFI) for CXCL2 in cells by flow cytometry. As shown in Fig. 4B, CXCL2 was mostly expressed on resident alveolar macrophages. The MFI for CXCL2 in the LPS group was higher than that in the CTR group ($P < 0.05$); moreover, the LPS-induced upregulation of CXCL2 was also substantially inhibited by RvD1 ($P < 0.05$) (Fig. 4B, D). In order to eliminate the effect of RvD1 on pathogen recognition, the expression of TLR4 on cell surface was also detected by flow cytometry. There was no significant difference in the expression of TLR4 on the cells of these groups ($P > 0.05$) (Fig. 4C).

3.5. RvD1 inhibited LPS-stimulated CXCL2 expression on resident alveolar macrophages through ALX/FPR2

The sorted resident alveolar macrophages were cultured to measure the CXCL2 level. As shown in Fig. 5A, CXCL2 mRNA expression on the resident cells and protein levels of CXCL2 in the supernate were significantly decreased in the LPS + RvD1 group compared with the LPS group ($P < 0.05$) (Fig. 5A, B). However, this decrease in the CXCL2 mRNA expression and protein levels was abrogated by BOC-2, which inhibits the RvD1 receptor ALX/FPR2 ($P < 0.05$) (Fig. 5A, B). However, there was no significantly different between the LPS and LPS + BOC-2 groups ($P > 0.05$).

3.6. The protective effect of RvD1 on lung tissues was abrogated by an ALX/FPR2 inhibitor

The protective effect of RvD1 against morphologic changes in lung tissues was abrogated by BOC-2, an ALX/FPR2 inhibitor (Fig. 6A). The ALI score in the LPS + RvD1 + BOC-2 group was higher than that in the LPS + RvD1 group ($P < 0.05$) (Fig. 6B). Moreover, the TNF-α protein concentration and number of BAL neutrophils were higher in lung tissue homogenate from the LPS + RvD1 + BOC-2 group than in that from the LPS + RvD1 group ($P < 0.05$) (Fig. 6C).

4. Discussion

In the present study, we investigated the impact of RvD1 on the pulmonary inflammatory response, neutrophil influx into the lung, and lung tissue damage in a murine model of LPS-induced ALI. RvD1 effectively protected mice against LPS-induced lung injury and significantly ameliorated lung pathological changes, decreased lung TNF-α expression, and attenuated pulmonary neutrophil infiltration. We further demonstrated that RvD1 significantly inhibited the LPS-stimulated overexpression and release of CXCL2 on resident alveolar

macrophages. Moreover, BOC-2 blocked the LPS-induced increase in CXCL2 expression on resident alveolar macrophages.

Neutrophils are frequently the first inflammatory cells to enter an inflamed or infected tissue site [21,22]. However, the exuberant infiltration and accumulation of neutrophils in the lung is one of the most important pathological hallmarks of ALI [23–25]. Although an appropriate influx of neutrophils to sites of inflammation and infection is essential to clear microbial pathogens and debris, the excessive and persistent sequestration of neutrophils may cause additional injury by their release of several toxic mediators, including reactive oxygen species (ROS), proteases and proinflammatory cytokines [24,26,27]. Neutrophil depletion in an LPS-induced ALI model was reported to ameliorate lung injury via reducing endothelial-epithelial damage and capillary-alveolar permeability [28,29]. In the present study, the aerosol inhalation of LPS in mice led to tissue damage and increased TNF-α levels and neutrophil infiltration in the lung. Treatment with RvD1 significantly attenuated the LPS-induced increase in TNF-α levels and the influx of neutrophils into the lung, thus ameliorating lung injury, which is consistent with the results of our previous report, in which RvD1 had a potential protective effect on LPS-induced lung injury [30].

Next, we investigated the underlying mechanisms by which RvD1 attenuates LPS-induced neutrophil infiltration and accumulation in the lung. Previous studies have suggested that macrophages play an important role in the initiation of neutrophil infiltration in peritoneal and dermal inflammation [31,32]. In rat models of peritonitis, resident peritoneal macrophages were also suggested to orchestrat neutrophil recruitment [33–35]. In the present study, resident alveolar macrophages ($F4/80^+Ly6C^-CD11c^+Siglec\ F^+CD11b^-$) in the BAL fluid were depleted by clodronate liposomes. The number of neutrophils was decreased in the absence of resident alveolar macrophages, indicating that resident alveolar macrophages are directly correlated with neutrophil influx. Treatment with RvD1 did not reduce neutrophil infiltration after the depletion of resident alveolar macrophages following LPS-induced lung injury, indicating that the inhibition of neutrophil infiltration by RvD1 is dependent on resident alveolar macrophages.

The chemoattractant CXCL2 plays an important role in neutrophil recruitment, and BAL fluid obtained from ARDS patients is highly chemotactic for human neutrophils [36,37]. In the present study, RvD1 treatment significantly inhibited the LPS-stimulated upregulation of CXCL2 mRNA expression in lung tissue homogenates and the CXCL2 concentration in BAL fluid, however, there was no effect of RvD1 on the CXCR2 mRNA expression in lung tissue homogenates and the MIF of CXCL2 on BAL fluid neutrophils, suggesting that treatment with RvD1 inhibits CXCL2 expression and release, but not CXCR2. In addition, the inhibitory effects of RvD1 on neutrophils were abrogated by a CXCR2 inhibitor in vivo, indicating that RvD1 inhibits neutrophil infiltration through CXCL2.

Macrophages are a potent source of chemokines and cytokines [38]. CXCL2 was the first major chemokine shown to be produced by LPS-treated macrophages. We investigated CXCL2 expression on cells in Single-cell suspensions from lung homogenates and found that CXCL2 was mainly expressed on resident alveolar macrophages. Treatment with RvD1 decreased CXCL2 expression, suggesting that RvD1 can inhibit CXCL2 expression on resident alveolar macrophages to inhibit neutrophil infiltration.

ALX/FPR2 was the first receptor identified as a GPCR for RvD1 with demonstrated cell type specific signaling pathways. RvD1 potently regulates human PMN recruitment and lipid mediator biosynthesis enhances the clearance of zymosan via binding to ALX/FPR2 [19]. In this study, the inhibitory effects of RvD1 on CXCL2 mRNA expression and protein level in resident alveolar macrophages, and the number of BAL neutrophils were abrogated by an ALX/FPR2 antagonist (BOC-2), indicating that RvD1 binds with ALX. Furthermore, BOC-2, the in vivo anti-inflammatory effects of which were recently identified via medical chemistry screening [39], also abolished the beneficial effects of RvD1 on lung injury and TNF-α levels in vivo. This is consistent with a previous report where RvD1 attenuated LPS-induced ALI in mice with protective mechanisms that might be related to its selective reaction with ALX/FPR2 [40]. Taken together, these results suggest that the RvD1 response is ALX/FPR2 dependent.

In conclusion, these data demonstrate that RvD1 strongly attenuates lung injury and neutrophil infiltration partially through the inhibition of CXCL2 expression and release on resident alveolar macrophages via activating ALX/FPR2 in LPS-induced ALI without affecting normal lung tissues. Our findings reveal a novel mechanism for neutrophil infiltration and that RvD1 may be a new therapeutic target for ALI/ARDS.

References

[1] U. Hamid, A. Krasnodembskaya, M. Fitzgerald, M. Shyamsundar, A. Kissenpfennig, C. Scott, Aspirin reduces lipopolysaccharide-induced pulmonary inflammation in human models of ARDS, Thorax 72 (2017) 971–980.

[2] L.B. Ware, M.A. Matthay, The acute respiratory distress syndrome, N. Engl. J. Med. 342 (2000) 1334–1349.

[3] N.R. Aggarwal, K. Tsushima, Y. Eto, A. Tripathi, P. Mandke, J.R. Mock, B.T. Garibaldi, B.D. Singer, V.K. Sidhaye, M.R. Horton, L.S. King, F.R. D'Alessio, Immunological priming requires regulatory T cells and IL-10-producing macrophages to accelerate resolution from severe lung inflammation, J. Immunol. 192 (2014) 4453–4464.

[4] S. Yona, K.W. Kim, Y. Wolf, A. Mildner, D. Varol, M. Breker, D. Strauss-Ayali, S. Viukov, M. Guilliams, A. Misharin, D.A. Hume, H. Perlman, B. Malissen, E. Zelzer, S. Jung, Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis, Immunity 38 (2013) 79–91.

[5] D. Hashimoto, A. Chow, C. Noizat, P. Teo, M.B. Beasley, M. Leboeuf, C.D. Becker, P. See, J. Price, D. Lucas, M. Greter, A. Mortha, S.W. Boyer, E.C. Forsberg, M. Tanaka, N. van Rooijen, A. García-Sastre, E.R. Stanley, F. Ginhoux, P.S. Frenette, M. Merad, Tissue-resident macrophages self-maintain locally throughout adult life with minimal contribution from circulating monocytes, Immunity 38 (2013) 792–804.

[6] K.J. Mould, L. Barthel, M.P. Mohning, S.M. Thomas, A.L. McCubbrey, T. Danhorn, S.M. Leach, T.E. Fingerlin, B.P. O'Connor, J.A. Reisz, A. D'Alessandro, D.L. Bratton, C.V. Jakubzick, W.J. Janssen, Cell origin dictates programming of resident versus recruited macrophages during acute lung injury, Am. J. Respir. Cell Mol. Biol. 57 (2017) 294–306.

[7] Z. Zasłona, S. Przybranowski, C. Wilke, N. van Rooijen, S. Teitz-Tennenbaum, J.J. Osterholzer, J.E. Wilkinson, B.B. Moore, M. Peters-Golden, Resident alveolar macrophages suppress, whereas recruited monocytes promote, allergic lung inflammation in murine models of asthma, J. Immunol. 193 (2014) 4245–4253.

[8] X. Huang, H. Xiu, S. Zhang, G. Zhang, The role of macrophages in the pathogenesis of ALI/ARDS, Mediat. Inflamm. 13 (2018) 1264913.

[9] J.A. Frank, C.M. Wray, D.F. McAuley, R. Schwendener, M.A. Matthay, Alveolar macrophages contribute to alveolar barrier dysfunction in ventilator-induced lung injury, Am. J. Physiol. Lung Cell. Mol. Physiol. 291 (2006) 1191–1198.

[10] E. Broug-Holub, G.B. Toews, J.F. van Iwaarden, R.M. Strieter, S.L. Kunkel, R. Paine 3rd., T.J. Standiford, Alveolar macrophages are required for protective pulmonary defenses in murine Klebsiella pneumonia: elimination of alveolar macrophages increases neutrophil recruitment but decreasesbacterial clearance and survival, Infect. Immun. 65 (1997) 1139–1146.

[11] T. Narasaraju, E. Yang, R.P. Samy, H.H. Ng, W.P. Poh, A.A. Liew, M.C. Phoon, N. van Rooijen, V.T. Chow, Excessive neutrophils and neutrophil extracellular traps contribute to acute lung injury of influenza pneumonitis, Am. J. Pathol. 179 (2011) 199–210.

[12] F. Blengio, F. Raggi, D. Pierobon, P. Cappello, A. Eva, M. Giovarelli, L. Varesio, M.C. Bosco, The hypoxic environment reprograms the cytokine/chemokine expression profile of human mature dendritic cells, Immunobiology 218 (2013) 76–89.

[13] S. Subat, K. Mogushi, M. Yasen, T. Kohda, Y. Ishikawa, H. Tanaka, Identification of genes and pathways, including the CXCL2 axis, altered by DNA methylation in hepatocellular carcinoma, J. Cancer Res. Clin. Oncol. 145 (2019) 675–684.

[14] K. Miura, J. Matsuo, M.A. Rahman, Y. Kumagai, X. Li, Y. Rikihisa, Ehrlichia chaffeensis induces monocyte inflammatory responses through MyD88, ERK, and NF-κB but not through TRIF, interleukin-1 receptor 1 (IL-1R1)/IL-18R1, or toll-like receptors, Infect. Immun. 79 (2011) 4947–4956.

[15] K. Haraguchi, A. Kawamoto, K. Isami, S. Maeda, A. Kusano, K. Asakura, H. Shirakawa, Y. Mori, T. Nakagawa, S. Kaneko, TRPM2 contributes to inflammatory and neuropathic pain through the aggravation of pronociceptive inflammatory responses in mice, J. Neurosci. 32 (2012) 3931–3941.

[16] Z. Liao, J. Dong, W. Wu, T. Yang, T. Wang, L. Guo, L. Chen, D. Xu, F. Wen, Resolvin D1 attenuates inflammation in lipopolysaccharide induced acute lung injury through a process involving the PPARg/NF-kB pathway, Respir. Res. 13 (2012) 110.

[17] A.F. Bento, R.F. Claudino, R.C. Dutra, R. Marcon, J.B. Calixto, Omega-3 fatty acid-derived mediators 17(R)-hydroxy docosahexaenoic acid,aspirin-triggered resolvin D1 and resolvin D2 prevent experimental colitis in mice, J. Immunol. 187 (2011) 1957–1969.

[18] B. Wang, X. Gong, J.Y. Wan, L. Zhang, Z. Zhang, H.Z. Li, S. Min, Resolvin D1 protects mice from LPS-induced acute lung injury, Pulm. Pharmacol. Ther. 24

(2011) 434–441.
[19] S. Krishnamoorthy, A. Recchiuti, N. Chiang, S. Yacoubian, C.H. Lee, R. Yang, N.A. Petasis, C.N. Serhan, Resolvin D1 binds human phagocytes with evidence for proresolving receptors, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 1660–1665.
[20] M. Ueno, T. Maeno, S. Nishimura, F. Ogata, H. Masubuchi, K. Hara, K. Yamaguchi, F. Aoki, T. Suga, R. Nagai, M. Kurabayashi, Alendronate inhalation ameliorates elastase-induced pulmonary emphysema in mice by induction of apoptosis of alveolar macrophage, Nat. Commun. 6 (2015) 6332.
[21] N. Borregaard, Neutrophils, from marrow to microbes, Immunity 33 (2010) 657–670.
[22] C. Nathan, Neutrophils and immunity: challenges and opportunities, Nat. Rev. Immunol. 6 (2006) 173–182.
[23] M.A. Matthay, L.B. Ware, G.A. Zimmerman, Acute respiratory distress syndrome, J. Clin. Invest. 122 (2012) 2731–2740.
[24] E. Abraham, Neutrophils and acute lung injury, Crit. Care Med. 31 (2003) 195–199.
[25] J. Grommes, O. Soehnlein, Contribution of neutrophils to acute lung injury, Mol. Med. 17 (2011) 293–307.
[26] J. Bhattacharya, M.A. Matthay, Regulation and repair of the alveolar-capillary barrier in acute lung injury, Annu. Rev. Physiol. 75 (2013) 593–615.
[27] Y. Mai, K. Kuba, G.G. Neely, R.M. Yaghubian, T. Perkmann, L.G. van, M. Ermolaeva, R. Veldhuizen, Y.H. Leung, H. Wang, H. Liu, Y. Sun, M. Pasparakis, M. Kopf, C. Mech, S. Bavari, J.S. Peiris, A.S. Slutsky, S. Akira, M. Hultqvist, R. Holmdahl, J. Nicholls, C. Jiang, C.J. Binder, J.M. Penninger, Identification of oxidative stress and Toll-like receptor 4 signaling as a key pathway of acute lung injury, Cell 133 (2008) 235–249.
[28] E. Abraham, A. Carmody, R. Shenkar, J. Arcaroli, Neutrophils as early immunologic effectors in hemorrhage- or endotoxemia-induced acute lung injury, Am. J. Physiol. Lung. Cell. Mol. Physiol. 279 (2000) 1137–1145.
[29] M. Chignard, V. Balloy, Neutrophil recruitment and increased permeability during acute lung injury induced by lipopolysaccharide, Am. J. Physiol. Lung. Cell. Mol. Physiol. 279 (2000) 1083–1090.
[30] Q. Wang, X. Zheng, Y. Cheng, Y.L. Zhang, H.X. Wen, Z. Tao, H. Li, Y. Hao, Y. Gao, L.M. Yang, F.G. Smith, C.J. Huang, S.W. Jin, Resolvin D1 stimulates alveolar fluid clearance through alveolar epithelial sodium channel, Na,K-ATPase via ALX/cAMP/PI3K pathway in lipopolysaccharide-induced acute lung injury, J. Immunol. 192 (2014) 3765–3777.
[31] M.N. Ajuebor, A.M. Das, L. Virag, R.J. Flower, C. Szabo, M. Perretti, Role of resident peritoneal macrophages and mast cells in chemokine production and neutrophil migration in acute inflammation: evidence for an inhibitory loop involving endogenous IL-10, J. Immunol. 162 (1999) 1685–1691.
[32] R. Chen, J.A. Fairley, M. Zhao, G.J. Giudice, D. Zillikens, L.A. Diaz, Z. Liu, Macrophages, but not T and B lymphocytes, are critical for subepidermal blister formation in experimental bullous pemphigoid: macrophage-mediated neutrophil infiltration depends on mast cell activation, J. Immunol. 169 (2002) 3987–3992.
[33] G.E. de Souza, S. Ferreira, Blockade by antimacrophage serum of the migration of PMN neutrophils into the inflamed peritoneal cavity, Agents Actions 17 (1985) 97–103.
[34] C. Barja-Fidalgo, C.R. Carlini, J.A. Guimaraes, C.A. Flores, F.Q. Cunha, S.H. Ferreira, Role of resident macrophages in canatoxin-induced in vivo neutrophil migration, Inflammation 16 (1992) 1–12.
[35] G.E. Souza, F.Q. Cunha, R. Mello, S.H. Ferreira, Neutrophil migration induced by inflammatory stimuli is reduced by macrophage depletion, Agents Actions 24 (1988) 377–380.
[36] T.S. Olson, K. Ley, Chemokines and chemokine receptors in leukocyte trafficking, Am. J. Physiol. Regul. Integr. Comp. Physiol. 283 (2002) 7–28.
[37] P.E. Parsons, A.A. Fowler, T.M. Hyers, P.M. Henson, Chemotactic activity in bronchoalveolar lavage fluid from patients with adult respiratory distress syndrome, Am. Rev. Respir. Dis. 132 (1985) 490–493.
[38] J.F. Cailhier, M. Partolina, S. Vuthoori, S. Wu, K. Ko, S. Watson, J. Savill, J. Hughes, R.A. Lang, Conditional macrophage ablation demonstrates that resident macrophages initiate acute peritoneal inflammation, J. Immunol. 174 (2005) 2336–2342.
[39] Q. Wang, Q.Q. Lian, R. Li, B.Y. Ying, Q. He, F. Chen, X. Zheng, Y. Yang, D.R. Wu, S.X. Zheng, C.J. Huang, F.G. Smith, S.W. Jin, Lipoxin A(4) activates alveolar epithelial sodium channel, Na,K-ATPase, and increases alveolar fluid clearance, Am. J. Respir. Cell Mol. Biol. 48 (2013) 610–618.
[40] B. Wang, X. Gong, J.Y. Wan, L. Zhang, Z. Zhang, H.Z. Li, S. Min, Resolvin D1 protects mice from LPS-induced acute lung injury, Pulm. Pharmacol. Ther. 24 (2011) 434–441.