The Role of p38 MAPK Signaling Pathway in Macrophage Pyroptosis and Murine Acute Lung Injury

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Abstract

Acute lung injury and acute respiratory distress syndrome (ALI/ARDS) is characterized by the uncontrolled progressive lung inflammation. Macrophage plays a key role in the pathogenesis of ALI/ARDS. Macrophage pyroptosis is a process of cell death with release of pro-inflammatory cytokines IL-1beta and IL-18. We hypothesize that macrophage pyroptosis may partially account for the uncontrolled lung inflammation of ALI/ARDS. In this study, we observed greater macrophage pyroptosis in the LPS-treated macrophages and ALI/ARDS mouse model. The expression of NLRP3, IL-1beta and cleavage of Caspase-1 were significantly elevated after LPS treatment, accompanied with more activation of p38 MAPK signaling in vitro and in vivo. However, blocking p38 MAPK signaling through inhibitor SB203580 significantly suppressed the acute lung injury and excessive lung inflammation in vivo, consistent with the reduced expression of NLRP3 inflammasome, IL-1beta and cleavage of Caspase-1. SB203580 significantly decreased the population of Caspase-1+propidium iodide (PI)+ pyroptotic cells and expression of NLRP3/IL-1beta in the treated rat NR8383 macrophage cell lines. However, we observed more population of Annexin V+PI-apoptotic cells after blocking p38 MAPK signaling. The results indicated that blockade of p38 MAPK signaling skewed macrophage cell death from pro-inflammatory pyroptosis towards non-inflammatory apoptosis. The effects may contribute to the attenuated acute lung injury and excessive inflammation in the SB203580-treated mice. The results provide a novel therapeutic strategy for the treatment of the uncontrolled lung inflammation among patients with ALI/ARDS.

Keywords: ALI/ARDS; Alveolar macrophages; p38 MAPK signaling; Pyroptosis; NLRP3 inflammasome

Introduction

Acute Lung Injury (ALI) and the more severe form Acute Respiratory Distress Syndrome (ARDS) are acute progressing hypoxic respiratory failure. It is characterized by a severe inflammatory process resulting in diffuse alveolar damage, influx of neutrophils, macrophages and a protein rich exudate in the alveolar spaces. Each year there are 190,600 cases of acute lung injury in the United States, which are associated with 74,500 deaths and 3.6 million hospital days [1]. The underlying immunological mechanisms are still not well defined. The uncontrolled excessive lung inflammation may account for the high mortality rate. Patients are usually treated with mechanical ventilation and immunosuppressants, but the therapies does not prolong the survival rate in patients and animal models [2,3]. Altered alveolar macrophage (AM) activation and death are considered as major player in the progression of the uncontrolled acute lung inflammation among patients with ALI/ARDS [4-6].

Macrophages are heterogeneous cell components and play distinct roles in the different stage of diseases [7,8]. After exposure to pathogens, macrophages are activated and release a variety of cytokines and other mediators. At later stage of diseases, the activated macrophages undergo non-inflammatory apoptosis and are finally cleared by monocytes, an important pathological process in inflammation resolution and tissue remodeling. However, it is clinically reported that a high percent of patients with severe sepsis and ALI/ARDS fail to recover from the acute lung inflammation, accompanied with persistent existence of the uncontrolled lung edema and excessive host response to pathogen [9,10]. According to the previous reports [11,12], we hypothesize that the uncontrolled lung inflammation may be caused by both insufficient clearance of dead cells and release of pro-inflammatory cytokines during immune cell death. Recent study indicated that macrophages can undergo inflammatory programmed cell death, called pyroptosis under a certain pathological circumstance [11]. The pyroptotic cells are identified as Caspase-1 and p38 MAPK expression is upregulated and form protein complex, called NLRP3 inflammasome and participates in NLRP3 inflammasome activation. It was reported that ASC deficiency caused defect of NLRP3 inflammasome assembly and protect against liver ischemia/reperfusion (IR) damage in animal models though suppression of Caspase-1/IL-1beta signaling [15]. Other studies also showed that NLRP3 inflammasome can be activated by neutrophil-derived extracellular histones and C5a, ultimately caused excessive tissue inflammation [16,17].

A body of evidences confirmed that p38 Mitogen-activated protein kinases (MAPKs) signaling participated in the progression of ALI/ARDS [18,19], p38 MAPK regulates cell growth, proliferation, differentiation, migration, apoptosis [20,21]. In animals with LPS-induced acute lung injury, p38 MAPK expression is upregulated [19]. However, there is limited information about the role of p38 signaling pathw...

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in macrophage pyroptosis and uncontrolled lung inflammation among ALI/ARDS mouse model. In this study, we treated macrophage cell lines and ALI/ARDS mouse model with p38 MAPK inhibitor SB203580 to block p38 MAPK signaling prior to LPS treatment. Our results revealed that blockade of p38 MAPK signaling with SB203580 suppressed macrophage pyroptosis and LPS-induced acute lung injury though negatively regulation of NLRP3 inflammasome activation.

Materials and Methods

Cell culture and treatments

Murine macrophage cell lines, RAW264.7 and NR8383 cells (ATCC) were cultured in RPMI1640 and F-12K medium (Life Science) respectively and supplied with 10% fetal bovine serum (FBS), 2 μM of glutamine, 100 U/mL of penicillin and 100 μg/mL of streptomycin. NR8383 cells were treated with 1 μg/mL LPS (Sigma-Aldrich, St Louis, MO) for 4 h, with or without 1 h pre-treatment of 10 μM MAPK p38 inhibitor SB203580 (Selleck Chemicals). Conditioned media and cells were collected for measurement of protein expression level by ELISA, qRT-PCR and Western blotting analysis.

Animal procedure

8-10 weeks old male wild-type C57BL/6 mice were purchased from Shanghai Biomodel Organism Science & Technology. Animal protocol was approved by the laboratory animal care and use committee at the Medical College of Fudan University, Zhongshan Hospital. The mice were intratracheal treated with 5 mg/kg LPS with or without 1 h intraperitoneal (i.p.) treatment with 20 mg/kg p38 MAPK inhibitor SB203580 (LPS group and SB203580/LPS group). The mice treated with PBS and the same doses of SB203580 were used as controls (PBS and SB203580 groups). Broncho alveolar lavage fluid (BAL) and lung tissues were collected 24 h after LPS treatment.

Wet/dry weight ratio

The wet and dry weight (W) of the left lung tissues were measured prior to and after drying at 65°C for 48 h. Water content was obtained by calculating the W/D weight ratio.

Lung histology

Lung was fixed in 4% paraformaldehyde (PFA) and stained with Hematoxylin and Eosin (H&E) for histopathological examination. The lung histology was viewed under a light photomicroscope and evaluated for pathological changes using a double-blind method. The severity of lung injury was evaluated using a semi-quantitative histological index, including alveolar edema, hemorrhage, alveolar septal thickening and infiltration of polymorphonuclear leukocytes. Each item was divided into four grades from 0 to 3 (0=normal; 1=mild; 2=moderate; 3=severe) and then calculated for a total ALI score.

Western blotting

The collected cells were incubated with 200 μL RIPA lysis buffer supplied with proteinase inhibitor PMSF (Shanghai Beyotime Biotechnology) for 30 min on ice. Protein concentration was determined by BCA protein assay kit (Shanghai Beyotime Biotechnology). 20 μg protein sample was resolved on 10% SDS-PAGE gel. The protein was transferred onto polyvinylidene fluoride membrane blots (Millipore, Bedford, MA, USA) and blots were incubated with blocking buffer (Shanghai Beyotime Biotechnology) for 1 h at room temperature and followed by incubation with indicated primary antibodies against NLRP3 (Novus Biotechnology, Littleton, CO), Caspase-3 (Santa Cruz Biotechnology, CA) and MAPKp38 (Cell Signaling Technology, Danvers, MA) (dilution 1:1000) at 4°C overnight. Antibody anti-mouse beta-actin (Boster Biotechnology, Wuhan, China) was used for internal loading control after blots were incubated with stripping buffer at 37°C for 15 min. Immune reactivity was visualized using the enhanced chemiluminescent reagent (ECL) (Beyotime Institute of Biotechnology). Band intensity was quantitatively analyzed by densitometric analysis on Image J software.

Quantitative real-time reverse transcriptase PCR (qRT-PCR)

cDNA was synthesized from 1 μg total RNA with ReverTra Ace qPCR RT Master Mix kit. qRT-PCR was performed using SYBR green PCR Master Mix-Plus (Toyobo Co. Ltd, Osaka, Japan). All primers were synthesized by the Shanghai BioSune Biotechnology Co. Ltd and primer sequences were listed in Table 1. Real-time PCR reaction was performed on 7500 real time PCR systems (AB Applied Biosystems) under condition of 95°C 1 min and followed by 40 cycles of 95°C 15 s; 60°C 30 s, 72°C 30 s. The expression level was quantified using 2-ΔΔCt method, relative to internal control beta-actin.

ELISA assay for cytokines

IL-1beta and IL-6 expression levels were measured by ELISA assay using IL-1beta and IL-6 ELISA kits (R&D systems Inc, Minneapolis, MN) according to the manufacturer's instructions.

Immunohistochemical staining

The treated RAW264.7 and NR8383 cells were incubated with fixing buffer and blocked with blocking buffer for 30 min (Shanghai Beyotime Biotechnology). Then cells were incubated with primary antibodies against rabbit anti-NLRP3 and mouse anti-Caspase-1 for 2 h. Second antibodies Cy3-conjugated anti-rabbit and FITC-conjugated anti-mouse IgG were incubated for 1 h following incubation with first antibody and washing. DAPI was used for staining nuclei. Finally, the stained cells were visualized under Olympus microscope.

### Table 1: Primers used for detection of gene expression.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense (5’-3’)</th>
<th>Antisense (5’-3’)</th>
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<tbody>
<tr>
<td>Rat IL-1beta</td>
<td>5’-AAAAATGCCCTGCTGCTGCT-3’</td>
<td>5’-TCGGTTGCTGCTGCTGCTG-3’</td>
</tr>
<tr>
<td>Rat IL-6</td>
<td>5’-AGTTGCGCTTGGAGACTGA-3’</td>
<td>5’-ACTGGTCTGTGGGTGTTG-3’</td>
</tr>
<tr>
<td>Rat beta-acin</td>
<td>5’-TAGCACAAGTCGGAGGACAAT-3’</td>
<td>5’-GGGGTTGATAGGCTCTAAA-3’</td>
</tr>
<tr>
<td>Mouse IL-1beta</td>
<td>5’-AGAGCCTCCAGGACAGGCGAT-3’</td>
<td>5’-AGTTGCTACTGCTCAGTC-3’</td>
</tr>
<tr>
<td>Mouse TNF- alpha</td>
<td>5’-AGGCCATGGACTCTAAAGAC-3’</td>
<td>5’-GTTGGAGGGAGCAGTGT-3’</td>
</tr>
<tr>
<td>Mouse IL-6</td>
<td>5’-CAGCTCGGCTTGGGACTC-3’</td>
<td>5’-GGTCTGTTGAGGGTATTCC-3’</td>
</tr>
<tr>
<td>Mouse NLRP3</td>
<td>5’-CAGGCTGCTGCTGCTGCTGCTG-3’</td>
<td>5’-CAGCAGCTCGTGCTGCTGCTGCTG-3’</td>
</tr>
<tr>
<td>Mouse TLR2</td>
<td>5’-GTGGCTGCTGCTGCTGCTGCTGCTG-3’</td>
<td>5’-GCTTTGGCTGCTGCTGCTGCTGCTG-3’</td>
</tr>
<tr>
<td>Mouse TLR4</td>
<td>5’-TGGCATCTGCTGCTGCTGCTGCTGCTG-3’</td>
<td>5’-TGGTCCTGCTGCTGCTGCTGCTGCTGCTG-3’</td>
</tr>
</tbody>
</table>
Flow cytometry analysis

Flow cytometry was performed according to manufacturer’s instruction to analyze apoptotic and pyroptotic macrophages in vivo and in vitro (BD Pharmingen, San Jose, CA). Briefly, the cells were washed with PBS for 2 times and followed by staining with antibodies against F4/80 and CD11b to identify F4/80+CD11b+ macrophages. Then the cells were incubated with 5 μl FITC-Annexin V and 5 μl PI in dark room temperature for 20 min. Finally, the cells were fixed in 4% paraformaldehyde. Apoptotic macrophages were identified as AnnexinV+ PI-cells after gating on F4/80+CD11b+ cell types. To analyze pyroptotic cells, the cell surface marker stained cells were incubated with 5 μl FITC-conjugated Annexin V (FL1 channel), 5 μl PI (FL2 channel) and 5 μl FLICA 660-YVAD-FMK far-red Caspase-1 reagent (FL4 channel) (Edina, MN) in dark room temperature for 20 min, and then washed with PBS and fixed with 4% paraformaldehyde for 10 min. The pyroptotic cells were identified as Annexin V+Caspase-1+ cells or Caspase-1+PI+ phenotypes. Analysis was performed on a FACS can cytometer (Becton Dickinson, Mountain View, CA) and data were analyzed on Flow Jo software (Tree Star, San Carlos, CA).

Statistical analysis

All experimental values were presented as mean ± standard error in each group. Statistical comparison between groups was examined by student’s t test. Differences in values were considered significant if p value is<0.05.

Results

Intraperitoneal instillation of p38 MAPK inhibitor SB203580 suppressed murine acute lung injury and inflammation. To establish an acute lung injury mouse model, we injected intratracheal (i.t.) to 8-10 weeks old male mice with 5 mg/kg LPS. The mice injected with PBS as controls. A massive inflammatory infiltrates, perivascular edema and severe alveolar space destruction were observed as compared to the PBS-treated group 24 h after LPS instillation (Figure 1A). Meanwhile, LPS treatment significantly induced more total BAL protein content (Figure 1C) and cell counts (Figure 1D). The weight loss (Figure 1E) and lung wet/dry ratio (Figure 1F) were also significantly elevated after i.t. LPS instillation. However, pre-instillation of p38 MAPK inhibitor SB203580 significantly reversed the severity of acute lung injury, as compared to the mice treated with LPS alone (Figures 1A and 1B). The beneficial effects were consistent with the reduced total BAL protein content (Figure 1C), cell counts (Figure 1D), weight loss (Figure 1E) and lung wet/dry ratio in the group of mice treated with both SB203580 and LPS (Figure 1F).

Blockade of p38 MAPK signaling attenuated the expression of pro-inflammatory cytokines and NLRP3 in the inflamed lung tissues.

To determine whether the reduced acute lung injury and inflammation in the SB203580-treated mice was supported by the decreased expression of pro-inflammatory cytokines, we measured IL-1beta, TNF-alpha and IL-6 expression levels in BAL and lung tissues by ELISA and qRT-PCR analysis. Supporting the observation above, we found that the expression of IL-1beta (Figure 2A), TNF-alpha (Figure 2B) and IL-6 (Figure 2C) were greatly elevated in mRNA and protein levels in the mice treated with LPS. However, blockade of p38 MAPK signaling by p38 MAPK inhibitor SB203580 significantly attenuated the expression of IL-1beta, TNF-alpha and IL-6.

NLRP3 and Caspase-1 are important components of NLRP3 inflammasome and mediators. To further investigate the effects of p38 MAPK signaling blockade on NLRP3 and Caspase-1 expression, the alveolar macrophages were collected in BAL. Annexin V+ cells and Caspase-1+ expression were analyzed by flow cytometry analysis 1 and 2 days after LPS i.t. instillation, we observed the increased populations of Annexin V+Caspase-1+apoptotic cells and Annexin V+Caspase-1+pyroptotic cells 1 day after LPS treatment at a time-dependent manner (Figures 3A and 3B). The increased pyroptosis after LPS treatment was associated with the elevated expression levels of NLRP3, pro-Caspase-1 (Figure 3C) and TLR2 (Figure 3D) as analyzed by Western blot analysis.
However, pre-SB203580 treatment significantly reversed LPS-induced up-regulation of NLRP3, cleaved Caspase-1 and TLR2, indicating the beneficial role of p38 MAPK signaling blockade in reducing alveolar macrophage pyroptosis.

LPS treatment increased expression of NLRP3 and Caspase-1 in macrophages

To further confirm the effects of LPS in inducing macrophage pyroptosis and the role of p38 MAPK signaling in this process, we investigated the expression levels of NLRP3, cleaved Caspase-1, and TLR2 in macrophages treated with LPS and/or p38 MAPK inhibitor SB203580. Our results showed that LPS treatment significantly induced the expression of NLRP3, cleaved Caspase-1, and TLR2, while pre-treatment with SB203580 significantly suppressed these expressions. These findings suggest that p38 MAPK signaling plays a crucial role in mediating LPS-induced macrophage pyroptosis.

Figure 2: p38 MAPK inhibitor SB203580 suppressed IL-1beta, TNF-alpha and IL-6 expression in mice after i.t. instillation of LPS; 24 h after SB203580 i.p. and LPS i.t. treatment, the lung and BAL fluids were collected; IL-1beta (A), TNF-alpha (B) and IL-6 (C) mRNA transcripts in lung tissues were analyzed by qRT-PCR analysis; The IL-1beta (D), TNF-alpha (E) and IL-6 (F) protein levels in BAL were analyzed by ELISA analysis; Data was presented as mean ± standard error; **p<0.01, ***p<0.001 vs. PBS group; #p<0.05, ##p<0.001 vs. LPS group, n=5 mice per group.

Figure 3: p38 MAPK inhibitor SB203580 suppressed Caspase-1, NLRP3 and TLR2 expression in mice after i.t. instillation of LPS; (A) LPS i.t. treatment increased expression of Caspase-1 and Annexin V in alveolar macrophages of mice 24 h after treatment; BAL was collected and Caspase-1 expression and Annexin V were analyzed by flow cytometry analysis; F4/80+CD11b+ cell phenotypes were identified as alveolar macrophages (AMs); Annexin V+Caspase-1+ cells were gated on F4/80+CD11b+ macrophages and identified as pyroptotic AMs; One representative data was shown; Data was presented as mean ± standard error; *p< 0.05, ** p<0.01 vs. the untreated 0 group, n=3 per group; The cleaved Caspase-1 (C), NF-kappa B and TLR2 (D) were analyzed by Western blot analysis after LPS i.t. treatment; One representative data was shown; NLRP3 (E) and TLR2 (F) mRNA transcripts were analyzed by qRT-PCR analysis; ** p<0.01, ***p<0.001 vs. the PBS-treated control group, n=5 per group.
pyroptosis, we treated mouse macrophage cell line, RAW264.7 cells with 1 µg/ml LPS for 4, 6, 12, and 24 h. The NLRP3 expression was analyzed by immunofluorescence staining. As a result, we observed that NLRP3-expressing cells were increased 6 h after LPS treatment that was gradually increased from 10.1 ± 1.1% of the untreated cells to maximal 20.2 ± 1.7% of the cells treated for 24 h (p<0.01) (Figures 4A and 4B). In addition, we observed the increasing number of NLRP3+ and cleaved Caspase-1+ cells by immunofluorescence staining after LPS treatment, indicating the role of LPS in NLRP3 inflammasome formation and activation (Figure 4C).

**Blockade of p38 MAPK signaling reversed LPS-induced expression of NLRP3 and Caspase-1 in macrophages**

Pyroptotic cells were characterized by high expression of the cleaved Caspase-1 and NLRP3 with positive PI staining. Our further analysis indicated that LPS resulted in 1.7-fold increases of Caspase-1+PI+ cells. However, blockade of p38 MAPK signaling reversed LPS-induced increases of Caspase-1+PI+ cell population (Figures 5A and 5C). In addition, LPS increased Annexin V+PI- apoptotic cell population and blocking p38 MAPK signaling by SB203580 further increased the apoptotic cell population (Figure 5B and 5D). Thus, SB203580 reduced cell pyroptosis, but increased cell apoptosis. LPS induced the uncontrolled lung inflammation possibly though inducing more cell pyroptosis and but few cell apoptosis via activation of p38 MAPK signaling pathway.

Additional analysis by Western blot revealed the similar results, by which blockade of p38 MAPK signaling suppressed LPS-induced p-38 MAPK activation (Figure 6A) and inhibited the LPS-induced up-regulation of NLRP3 and TLR2 proteins (Figure 6B), consistent with the results analyzed by flow cytometry (Figure 5). However, we observed more cleaved Caspase-1 in the cells treated by both SB203580 and LPS (Figure 6C), that was in line with the results analyzed by flow cytometry, showing more Annexin V+PI- apoptotic cell population after SB203580 treatment (Figures 5B and 5D).
Figure 5: p38 MAPK inhibitor SB203580 suppressed Caspase-1 expression, but increased Annexin V+ cell population in macrophages. Rat macrophage cell lines NR8383 were stimulated with 1 µg/ml LPS for 4 h with/without 10 μM SB203580 pretreatment; The Caspase-1+, Annexin V+ and PI+ cells were measured by flow cytometry; Caspase-1+PI+ cell population was identified as pyroptotic macrophages and Annexin V+PI- cell population was identified as apoptotic macrophages; (A) LPS increased Caspase-1 expression that was reversed by SB203580; (B) LPS increased Annexin V+Pleasing apoptotic cells, p38 MAPK inhibitor SB203580 exaggerated the LPS-induced increases in Annexin V+ apoptotic macrophages; (C) Quantitative analysis of Caspase-1+PI+ pyroptotic cells; (D) Quantitative analysis of Annexin V+PI+ apoptotic cells; Data was presented as mean ± standard error **p<0.01 vs. the untreated control group; #p < 0.05, ###p < 0.001 vs. the LPS-treated group, n=3 per group.

Figure 6: p38 MAPK inhibitor SB203580 suppressed expression of NLRP3 and cleavage of Caspase-1 in macrophages; Rat macrophage cell lines NR8383 were stimulated with 1µg/ml LPS for 4 h with/without 10 μM SB203580; The expression and phosphorylation of p38 MAPK (p-p38MAPK); (A) NLRP3 and TLR2/TLR4; (B) pro-and cleaved-Caspase-1; (C) were analyzed by Western blot analysis; Beta-actin protein was detected as an internal protein loading control; One representative blot data was shown; Quantitative analysis for p-p38MAPK (A, lower panel), TLR2 and NLRP3 (B, lower panel) and the cleaved-Caspase-1 (C, lower panel) was performed and data was presented as mean ± standard error of densitometric density relative to beta-actin; *p<0.05, **p<0.01 vs. the untreated control; #p<0.05 vs. the LPS treated group alone; n=3 per sample.
Further analysis of cytokines in the supernatants and cell lysates of the treated cells indicated that LPS elevated the expression of IL-1β (Figures 7A and 7C) and IL-6 (Figures 7B and 7D) at mRNA and protein levels in vitro. However, the elevated expression of IL-1β and IL-6 were significantly suppressed by SB203580 treatment (p < 0.05), further supporting the effects of p38 MAPK blockade in reducing macrophage pyroptosis in vitro and in vivo.

Discussion

Our previous study revealed that LPS can up-regulate the expression of Toll-like receptor 4 (TLR4), myeloid differentiation protein 2 (MD-2), TNF-alpha, IL-6 and IL-1β in rat alveolar macrophage cell lines NR8383 [22,23]. Because IL-1β is mainly released from the activated macrophages [13]. We hypothesize that LPS may induce release of IL-1β though macrophage pyroptosis that is partially responsible for the uncontrolled lung inflammation in ALI/ARDS. To address this issue, in this study we for the first time investigated the effects of LPS on macrophage pyroptosis and lung inflammation and in vivo. The results in vivo indicated that LPS i.t. treatment significantly increased excessive lung inflammation, neutrophil infiltration, in association with increased Caspase-1 cleavage and active IL-1beta release. The similar result was also observed in RAW264.7 and NR8383 macrophage cell lines, in which NLRP3 and the cleaved Caspase-1 were up-regulated and co-localized within intracellular NLRP3 inflammasome after LPS treatment. Thus, not only LPS induced excessive lung inflammation though increasing macrophage and neutrophil recruitment, but also LPS caused a pyroptosis-biased macrophage death and subsequent IL-1β release.

Previous study in primary porcine alveolar macrophages showed that PRRSV infection activated NLRP3 and IL-1 beta expression and activation though TLR4/MyD88/NF-kappa B signaling pathway [24]. The results in our study also indicated that LPS treatment activated NLRP3 and IL-1 beta expression and activation. The effects may be mediated though p38 MAPK signaling pathway, because blocking p38 MAPK signaling though SB203580 treatment reversed the LPS-induced up-regulation of TLR2, NLRP3, Caspase-1 and IL-6 in NR8383 cell lines. The suppressed p38 MAPK signaling is correlated with lower Caspase-1+PI+ pyroptotic cell population as analyzed by flow cytometry analysis. In addition, we also observed more population of Annexin V+PI- apoptotic cells in the cells pre-treated with SB203580. The results suggested that p38 MAPK signaling pathway contributed to the macrophage pyroptosis. Inhibition of p38 MAPK signaling pathway may promote shift of macrophage cell death from pro-inflammatory pyroptosis towards non-inflammatory apoptosis process.

Thus, blockade of p38 MAPK signaling has potential to suppress excessive lung inflammation by suppression of the pro-inflammatory macrophage pyroptosis-biased cell death. It encourages us to further investigate the beneficial role of p38 MAPK signal blockade in damping the uncontrolled lung inflammation in ALI/ARDS animal model. Our revealed that i.p. pretreatment with SB203580 significantly suppressed acute lung injury, that was characterized by reduced alveolar edema, hemorrhage, alveolar septal thickening and infiltration of polymorphonuclear leukocytes, as compared to the mice treated with LPS alone. In consistent with the pathological observation, NLRP3 and TLR2 expression and Caspase-1 cleavage were significantly reduced after SB203580 treatment. In consistent with the observation, IL-1beta, TNF-alpha and IL-6, were attenuated, supporting the role of p38 MAPK signaling in the LPS-induced acute lung injury and excessive lung inflammation. However, we did not observe markedly decreases in TLR4 expression and NF-kappaB activation, implying the possible less involvement of TLR4/NF-kappaB signaling in p38 MAPK blockade immunotherapy for ALI/ARDS.

It should be noted that we observed a greater cell apoptosis after the cells were treated with both LPS and p38 MAPK inhibitor than LPS treatment alone. The results were evidenced by a higher Annexin V+PI- cell population and greater cleavage of pro-Caspase-3 in the apoptotic cells after treatment with both p38 MAPK inhibitor and LPS. We speculate that un-controlled lung inflammation in ALI/ARDS is caused by predominant pro-inflammatory macrophage pyroptosis though MAPK p38 signaling pathway; whereas blocking MAPK p38 signaling switch the predominant pro-inflammatory...
macrophage pyroptosis into un-inflammatory macrophage apoptosis death program, with lower production of inflammatory cytokines. The effects ultimately improve lung inflammation resolution and recovery of ALI/ARDS in vivo. Therefore, it is important to maintain an optimal balance between pyroptosis and apoptosis during disease progression. However, contrary to the enhanced macrophage apoptosis by p38 MAPK signaling blockade in macrophages, recent results in endothelial cells showed that lack of MAPK-activated protein kinase (MK)2, one of p38 MAPK’s immediate downstream effectors can reduce translocation of cleaved Caspase-3 into nucleus of endothelial cells in response to LPS and protect mice from pulmonary vascular permeability [25,26]. The distinct effects in two different cell types warrants us to further investigate the role in animal models in the future.

Taken together, we conclude that blocking p38 MAPK signaling ameliorates acute lung injury and lung inflammation, partially though suppressing macrophage pyroptosis (Figure 8). The results provide a rational for ALI/ARDS immunotherapy though modulation of p38 MAPK signaling and macrophage pyroptosis.

Acknowledgements

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Authors’ Contributions

DL performed experiments, experimental design, data assembly, analysis and manuscript writing. ZJ participated in experimental design, data interpretation and manuscript writing. WY. Ren participated in generation of concepts and data interpretation. L. Zhu participated in generation of hypothesis, data interpretation and was responsible for the overall direction of work. All authors read and approved the final manuscript.

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