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ORIGINAL ARTICLE

PP2ACα of Alveolar Macrophages Is a Novel Protective Factor for LPS-Induced Acute Respiratory Distress Syndrome

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Abstract— Protein phosphatase 2A (PP2A) is one main serine/threonine phosphatase in eukaryotes, and its activation changes have been linked to modulation of numerous pathological processes, such as cancer, inflammation, fibrosis, and neurodegenerative diseases. Acute respiratory distress syndrome (ARDS), the major cause of respiratory failure, remains with limited therapies available up to now. Alveolar macrophages (AMs) are essential to innate immunity and host defense, participating in the pathogenesis of ARDS. As a result, AMs are considered as a potential therapeutic target for ARDS. In our study, we firstly found that PP2A activity was significantly decreased in the lipopolysaccharide (LPS)-stimulated AMs. Furthermore, adoptive transfer of AMs with enhanced PP2A enzyme activity that was improved by C2-ceramide prior to LPS exposure alleviated acute lung inflammation. Conversely, AM-specific ablation of PP2ACα exacerbated inflammatory responses to LPS. Mechanistically, PP2ACα negatively regulates LPS-induced cytokine secretion of AMs by NF-κB and MAPK pathways. Together, these findings provide the evidence to guide the development of novel therapeutic options targeting PP2ACα for ARDS/acute lung injury.

KEY WORDS: PP2ACα; Alveolar macrophages; C2-ceramide; Acute respiratory distress syndrome.

INTRODUCTION

Acute respiratory distress syndrome (ARDS), a more relentless form of acute lung injury (ALI), characterized by severe hypoxemia, overwhelming pulmonary inflammation, and pulmonary edema, results in significant morbidity and mortality [1]. Despite long-term research and numerous clinical studies, mechanical ventilation is the only treatment to reduce the mortality [2, 3]. As a result, it is urgently necessary to better understand the pathophysiology and develop new therapeutic interventions for ARDS/ALI.

Alveolar macrophages (AMs) reside in alveolar space and account for about 90% of the leukocytes under physiological condition [4], which form the first defense line against invaders and coordinate the penetration of leukocyte in innate immunity [5]. Recent studies support the importance of AMs in initiating the lung inflammation and triggering neutrophil infiltration into alveolar space during ARDS/ALI by secreting proinflammatory cytokines [6–10]. This suggests that a therapeutic option targeting AMs would be valuable for treating ARDS [11, 12].

Lipopolysaccharide (LPS)-induced ALI of mouse model is able to replicate the key pathologic processes of ARDS, including macrophage activity, loss of vascular integrity, infiltration of neutrophils, and permeation of protein-rich fluid to the airspaces of pulmonary [10, 13,

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14]. During ALI, LPS is recognized by TLR4 located on AMs, which activates MyD88/NF-κB and MyD88/MAPK pathways, leading to the accumulation of classical (M1) activation macrophages, pro-inflammatory cytokine production, and finally resulting in sepsis and septic shock [15–18].

The activation of these pathways *via* phosphorylation is compromised through dephosphorylation mediated by phosphatases to keep a dynamic regulatory process [19]. PP2A, as a primary serine/threonine phosphatase, is a heterotrimeric complex including a scaffold subunit (also known as A or PR65 subunit), a catalytic subunit (PP2ACα), and a variable regulatory subunit (B subunit) [20]. Previous studies have reported blocked lung inflammation for pharmacological activation of PP2A [21]. However, the target cells remain to be explored for the potential nonspecific effects of the agonists. Firstly, our study found that PP2A activity was significantly decreased in the LPS-stimulated AMs, which suggested an involvement of PP2ACα in AM-mediated inflammatory response. Then, clodronate liposome was administrated to deplete AMs, and adoptive transfer of AMs treated with an agonist C2-ceramide which enhances PP2A activity showed a striking protection from LPS-induced ALI in mice. Further experiments suggested loss of PP2ACα in AMs exacerbated lung inflammatory responses to LPS. Experiments *in vitro* proved that PP2ACα prevented LPS-induced cytokine secretion of AMs through negatively regulating NF-κB p65, JNK, and Erk activation. Our results highlight the importance of PP2ACα in AMs during ARDS/ALI and suggest a potential clinically relevant therapeutic option for ARDS/ALI.

MATERIALS AND METHODS

PP2A Activity Determination

PP2A phosphatase activity was determined according to previous studies [22, 23]. Firstly, the catalytic subunit of PP2A (PP2ACα) in AMs was immunoprecipitated, and the phosphatase activity was measured using a specific PP2A assay kit (Millipore, Billerica, USA). All procedures were performed following the manufacturer's recommendations.

Isolation and Culture of AMs

To isolate and purify AMs from the bronchoalveolar lavage fluid (BALF), cell pellets were resuspended in complete medium (DMEM/F12 with 10% heat-inactivated fetal bovine serum, 50 U/ml penicillin, and 50 U/ml streptomycin) and cultured for 2 h. Nonadherent cells were discarded, and the adherent cells were collected for FACS analysis. About 90% adherent cells are AMs as described previously [24]. Then, LPS (100 ng/ml) was used to stimulate AMs to detect the cytokine secretion or the activation of relevant signaling pathways.

AM Depletion and Adoptive Transfer

To deplete AMs, 200 μl of clodronate liposome (Liposoma, Amsterdam, The Netherlands) was intratracheally (i.t.) injected 2 days before AM adoptive transfer [24–26]. For adoptive transfer assay, AMs isolated from C57B6/J mice (6–8 weeks old) were pretreated with 100 μM C2-ceramide (Sigma-Aldrich) or DMSO for 12 h and then 1×10^6 AMs in 20 μl of PBS were i.t. injected into AM-depleted mice at 12 h before LPS (Sigma-Aldrich, St. Louis, USA) administration. After 24 h, these mice were sacrificed for analysis of ALI.

Animals and LPS Challenge

PP2ACα$^{f/f}$ and *LysMcre* mice were respectively generous gifts from Dr. Xiang Gao (Nanjing University, Nanjing, China) and Dr. Ximei-Wu (Zhejiang University, Hangzhou, China). C57B6/J mice were bought from Laboratory Animal Research Center of Zhejiang Chinese Medical University. *PP2ACα* conditional knockout mice (*LysMcrePP2ACα*$^{f/f}$, C57B6/J) were generated by crossing *PP2ACα*$^{f/f}$ mice with *LysMcre* mice. To confirm the mice's genotypes, tail DNA samples were subjected to PCR amplifications on the *LysMcre* and *PP2ACα*$^{f/f}$ genes, using the following primers: *LysMcre* (5′-CCC AGA AAT GCC AGA TTA CG-3′, 5′-CTT GGG CTG CCA GAA TTT CT-3′, and 5′-TTA CAG TCG GCC AGG CTG AC-3′), *PP2ACα*$^{f/f}$ (5′-TAG CCC ATG CCT TTA ATC TCA GAG C-3′ and 5′-CAC TCG TCG TAG AAC CCA TAA ACC). Wild-type and *LysMcrePP2ACα*$^{f/f}$ female littermates (6–8 weeks old) were injected intratracheally with 3 mg/kg or 10 mg/kg LPS. C57B6/J mice (6–8 weeks old)-obliterated AMs were divided randomly into two groups for adoptive transfer of DMSO or C2-ceramide pretreated AMs followed by saline or LPS (3 mg/kg) challenging. All the animal protocols in this study were reviewed and approved by Institutional Animal Care and Use Committee of Zhejiang Chinese Medical University.

BALF Analysis and Cytokine Detection

At 24 h after LPS treatment, mice were euthanized by injection of pentobarbital. The left bronchus was tied, and BALF from each mouse was collected as described previously [27]. All BALF samples were centrifuged at 1000 rpm at 4 °C for 10 min. Cell pellets were resuspended with 100 μl of PBS, and differential cell counts were performed using standard morphological criteria after Wright–Giemsa staining. The BALF supernatant samples were frozen at − 80 °C for chemokine quantification. The Th1 cytokine levels in the BALF supernatant were measured using ELISA kits (IL-1β, TNF-α, IL-6, and CCL2; eBioscience Inc., San Diego, USA) according to the manufacturer's instructions.

Assessment of Lung Inflammation and Histology

Lungs were fixed with an intratracheal injection of 4% paraformaldehyde. The left lung of each mouse was embedded in paraffin according to standard procedures. Sections (5 μm) were mounted on slides for hematoxylin and eosin staining (H&E) to evaluate the inflammation and morphology changes in the lungs.

Quantitative PCR Analysis

Total RNA from AMs was extracted using TRIzol reagent (Invitrogen, Carlsbad, USA) following the manufacturer's recommendations. The concentration of RNA was measured with a NanoDrop spectrophotometer (Thermo Scientific Inc., Bremen, Germany), and reverse-transcription (1 μg RNA) was performed using the ReverTra Ace® qPCR RT Kit (Toyobo, Osaka, Japan). Real-time quantitative PCR was performed using the FastStart Universal SYBR Green Master Kit (Roche, Mannheim, Germany). The q-PCR primer sequences were as follows: *Actin*, sense 5′-GGCTGTATTCCCCT CCATCG-3′, antisense 5′-CCAGTTGGTAACAA TGCCATGT-3′; *il-1β*, sense 5′-GAAATGCCACCTTT TGACAGTG-3′, antisense 5′-TGGATGCTCTCATC AGGACAG-3′; *tnf-α*, sense 5′-CCCTCACACTCAGA TCATCTTCT -3′, antisense 5′-GCTACGACGTGGGC TACAG-3′; *il-6*, sense 5′-TAGTCCTTCCTACC CCAATTTCC-3′, antisense 5′-TTGGTCCTTAGCCA CTCCTTC-3′; and *ccl2*, sense 5′-TTAAAAACCTGGAT CGGAACCAA-3′, antisense 5′-GCATTAGCTTCAGA TTTACGGGT-3′.The mRNA levels were quantified with the $^{\Delta\Delta}$Ct method.

Immunoblotting Analysis

AMs were homogenized in RIPA buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS, supplemented with protease and phosphatase inhibitors). Cell lysates (30 μg) were separated on SDS-PAGE and immunoblotted using antibodies against the following proteins: phospho-NF-κB p65 (Ser536), NF-κB p65, phospho –JNK (Thr183/Tyr185), JNK, phospho-p38 (Thr180/Tyr182), p38, phospho-Erk (Thr202/Tyr204), Erk (Cell Signaling Technology, Danvers, MA, USA), PP2ACα (BD Biosciences, San Diego, USA), and β-actin (Sigma-Aldrich). Scanned immunoblots were representative of three independent experiments. Integrated densities indicated bands were quantified using ImageJ software (U.S. National Institutes of Health, Billerica, USA).

Statistical Analysis

All results were expressed as mean ± SE and assessed for significance by Student's *t* test. A value of $p < 0.05$ was considered statistically significant.

RESULTS

LPS Exposure Decreases PP2A Activity in AMs

Dysregulation of PP2A affects the development of multiple diseases, such as cancer [28, 29], neurodegenerative diseases [30, 31], and inflammation [21, 32]. Macrophages are the major producers of proinflammatory cytokines in response to LPS [33]. To investigate the role of PP2ACα in AMs, we firstly assessed the activity of PP2A in AMs isolated from LPS-challenged mice (Fig. 1a) and in LPS-treated AMs (Fig. 1c). It was evident that the PP2A activity was significantly decreased in AMs with LPS treatment (Fig. 1a and c), while the expression of PP2ACα was unaltered (Fig. 1b and d), which suggested that PP2A activation is involved in LPS-induced AM activation.

PP2A Activation in AMs Counters LPS-Induced Lung Inflammation in Mice

Given that LPS exposure decreases PP2A activity of AMs in mice, we postulated that increasing the enzymatic activity of AMs is most likely to counter the proinflammatory response triggered by LPS. To test this hypothesis, clodronate liposome was used to clear local AMs. After 2 days, normal AMs incubating with C2-ceramide for 12 h, an agonist able to specifically raise PP2A activity (Fig. 2a),

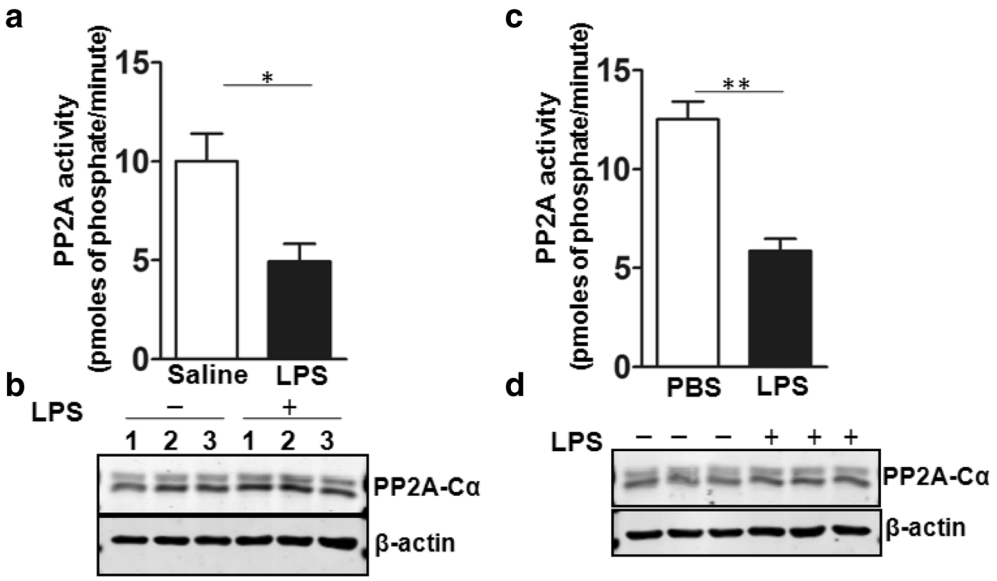

Fig. 1. PP2A activity is declined in LPS-treated AMs. **a** PP2A activities were decreased in the AMs isolated from LPS-challenged mice. $n = 3$/group, $^{*}p < 0.05$. **b** PP2ACα expression levels have no changes in the AMs isolated from LPS-challenged mice. Each protein lane represents one mouse. **c** PP2A activities were attenuated in LPS-stimulated AMs isolated from WT mice. $N = 3$/group, $^{**}p < 0.01$. **d** LPS stimulation has no influence on PP2ACα expression of AMs. Each protein lane represents one mouse.

were administered intratracheally into clodronate liposome-treated mice 12 h prior to LPS challenge (Fig. 2b). Compared with the control, adoptive transfer of C2-ceramide-incubated AMs protected mice from LPS-induced ALI. Firstly, the histological examination of alveolar structure suggested that the lung injuries and inflammation in the ALI mice were dramatically renovated for the adoptive transfer of C2-ceramide-treated AMs (Fig. 2c). Meanwhile, the total number of leukocytes in BALF was determined using cell counting, and a remarkable reduction was found in C2-ceramide-treated group (Fig. 2d). Differential cell counts revealed a notable decrease in the number of macrophages and neutrophils (Fig. 2e). Furthermore, we measured representative Th1 cytokines including IL-1β, TNF-α, IL-6, and CCL2 in the BALF, and results showed that the secretion of these cytokines was also blocked for C2-ceramide treatment (Fig. 2f). Taken together, our findings suggested that the specific enhancement of PP2A activity in AMs alleviated LPS-induced lung inflammation in mice.

Increasing PP2A Activity Attenuates Th1 Cytokine Secretion from LPS-Stimulated AMs with Reduced NF-κB p65, JNK, and Erk Phosphorylation

Next, we further explored the mechanisms underpinning the Th1 cytokine secretion in C2-ceramide treated AMs. AMs were harvested at the indicated time points and then analyzed following LPS stimulation *in vitro*. We found that LPS-induced IL-1β, TNF-α, IL-6, and CCL2 levels in AMs were reduced time-dependently for C2-ceramide incubation (Fig. 3a). Further analysis of the downstream signal pathway response for LPS showed the phosphorylation level of p65, JNK, and Erk was substantially decreased in C2-ceramide-incubated AMs, while phosphorylation of p38 had little change (Fig. 3b and c). These results demonstrated that increasing PP2A activity deters the activation of NF-κB and MAPK signaling pathways and finally results in decreased Th1 cytokine expression. All results above suggest that PP2A activation is able to prevent the LPS-induced inflammatory cytokine secretion of AMs, contributing to the alleviation of ALI.

Loss of PP2ACα in AMs Enhances LPS-Induced Lung Inflammation Responses *in vivo*

PP2ACα is the catalytic subunit directly responsible for enzymatic activity of PP2A. To further determine the physiological role of AM PP2A phosphatase activity in LPS-induced lung inflammation, we generated mice with a conditional PP2ACα deletion in macrophages (LysMcrePP2ACα$^{f/f}$) (Fig. 4A). Conditional disruption of PP2ACα in AMs was confirmed using westernblot analysis (Fig. 4b). LysMcrePP2ACα$^{f/f}$ mice appeared to

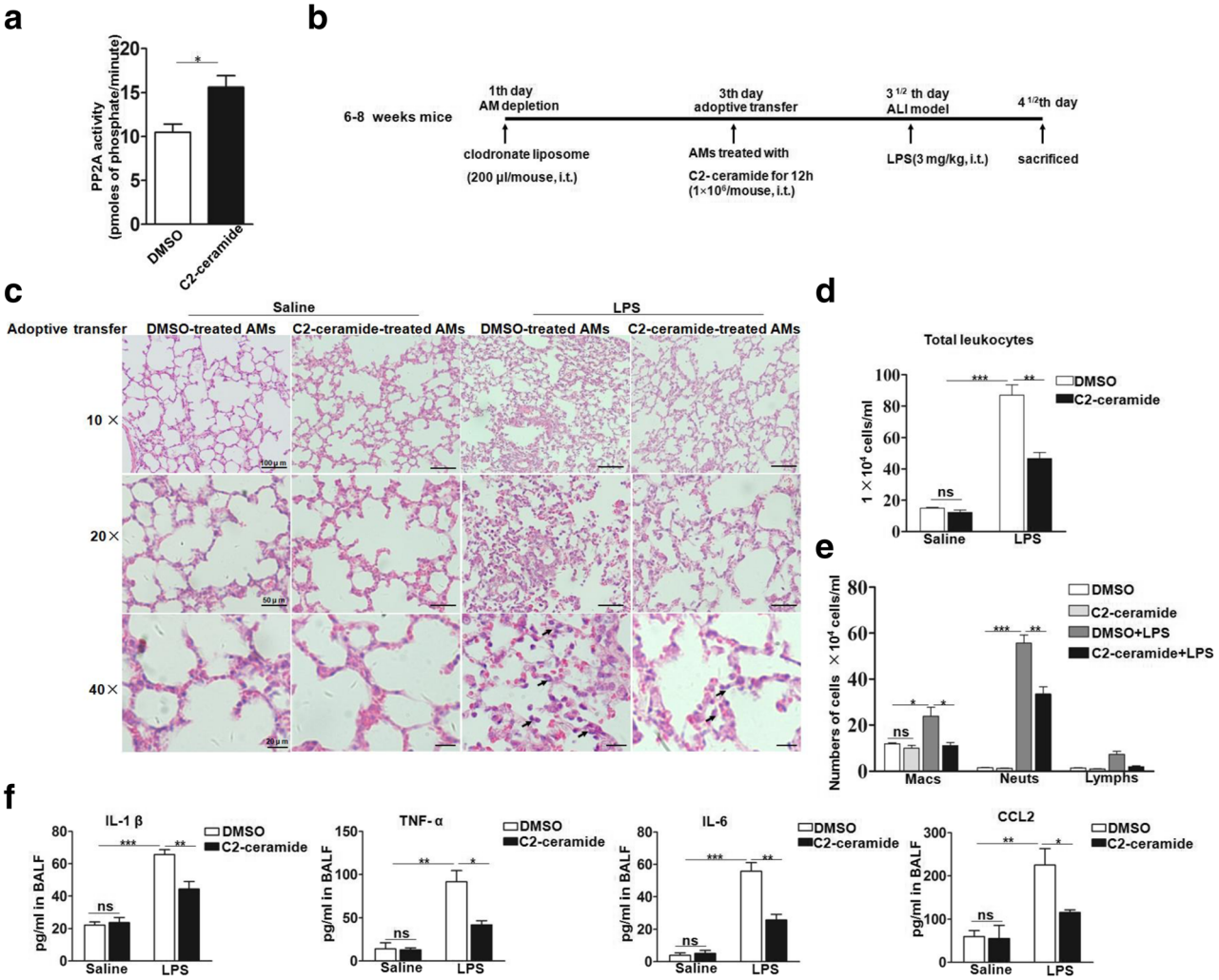

Fig. 2. Adoptive transfer of AMs pretreated with C2-ceramide relieves LPS-induced lung inflammation. **a** C2-ceramide enhanced PP2A activity in AMs. $N = 3$,$^{*}p < 0.05$. **b** Adoptive transfer of AMs and LPS challenge were performed following indicated scheme. **c** H&E staining showing that the adoptive transfer of C2-ceramide-pretreated AMs attenuated lung inflammation in response to LPS. Arrows indicated leukocyte infiltration. Scale bar = 100/50/20 μm, $n = 4$ or 6/group. **d–e** The adoptive transfer of AMs pretreated with C2-ceramide blocked LPS-induced leukocyte infiltration in BALF. $N = 4$ or 6/group, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. **f** ELISA results showing that the increase of LPS-induced Th1 cytokines (IL-1β, TNF-α, IL-6, and CCL2) in BALF was reduced for the adoptive transfer of AMs treated with C2-ceramide. $N = 4$/group, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

be normal, with weights and lifespan similar to littermate controls. Histological evaluation also revealed no signs of inflammation or pathological changes in the LysMcrePP2ACα$^{f/f}$ lungs (Fig. 4c–e). On this basis, we firstly examined the effect of PP2ACα deficiency in LPS-exposed lung injury. Histological analysis of the lungs showed that the loss of PP2ACα in AMs exacerbated LPS-induced lung inflammation (Fig. 5a). Meanwhile, The total number of leukocytes in BALF was determined using cell counting, and a remarkable increase was found in LysMcrePP2ACα$^{f/f}$ mice (Fig. 5b), and differential cell counts also revealed a notable improvement in the number of macrophages and neutrophils while slight change in lymphocytes in LysMcrePP2ACα$^{f/f}$ mice compared with PP2ACα$^{f/f}$ mice (Fig. 5c). Consistent with these results, Th1 responses were augmented; remarkable increases of secreted IL-1β, TNF-α, IL-6, and CCL2 were observed in BALF of LPS-exposed LysMcrePP2ACα$^{f/f}$ mice compared with control mice (Fig. 5d). Furthermore, age- and sex-matched LysMcrePP2ACα$^{f/f}$ mice and PP2ACα$^{f/f}$

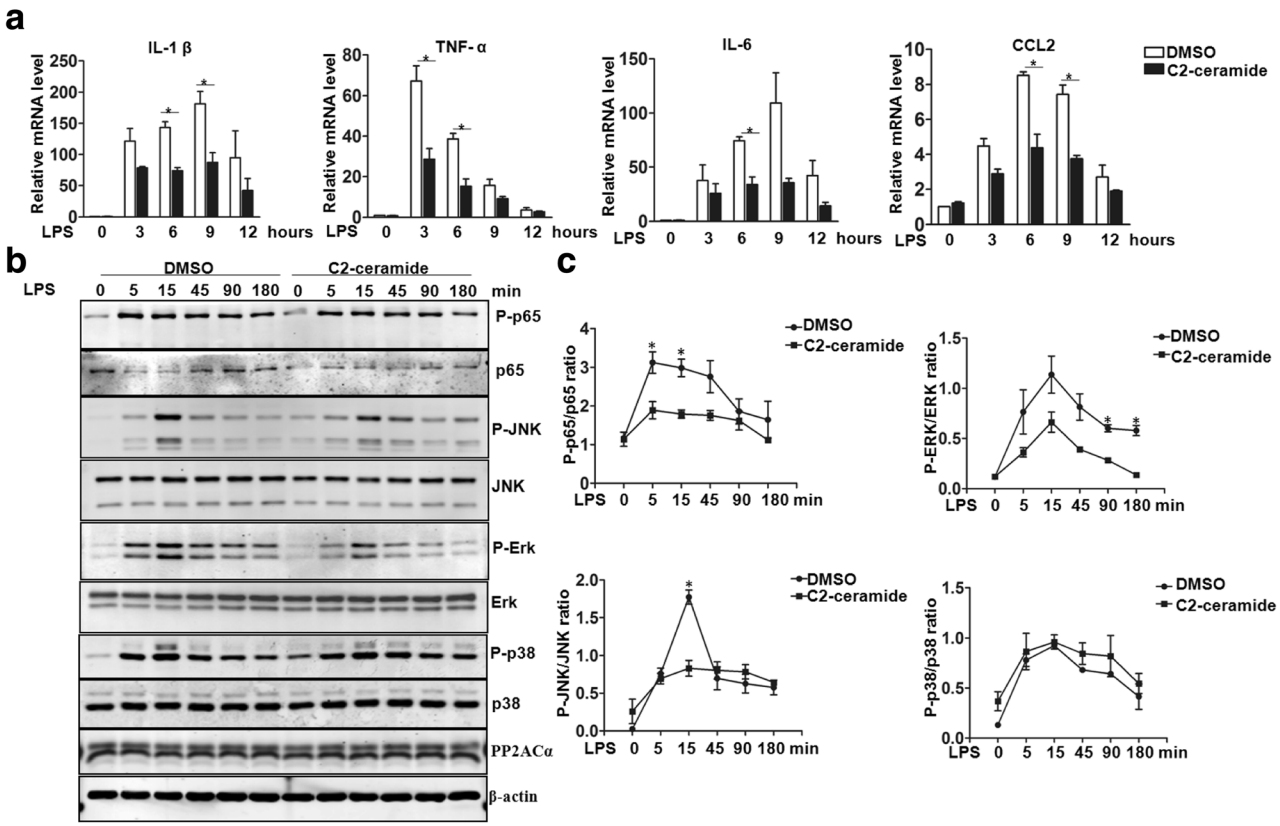

Fig. 3. PP2A activation pretreated with C2-ceramide prevents Th1 cytokine secretion by LPS-stimulated AMs with decreased NF-κB p65, JNK, and Erk phosphorylation. **a** Analysis of IL-1β, TNF-α, IL-6, and CCL2 mRNA expression level by Q-PCR. AMs were stimulated with LPS (100 ng/ml) for 0, 3, 6, 9, 12 h. $n = 3$/group, $^*p < 0.05$. **b** Activation of PP2A inhibited the phosphorylation of NF-κB p65, JNK, and Erk in response to LPS stimulation, while the phosphorylation of p38 was little changed. AMs were stimulated with LPS (100 ng/ml) for 0, 5, 15, 45, 90, and 180 min. β-actin was used as loading control. Data are representative of three independent experiments. **c** Densitometry analysis of the indicated band intensities about NF-κB p65, JNK, Erk, and p38 phosphorylation. $N = 3$, $^*p < 0.05$.

mice were challenged intratracheally with 10 mg/kg LPS, and survival was monitored for 3 days. The results showed that the mortality in LysMcrePP2ACα$^{f/f}$ mice was 70%, while control mice was 20% (Fig. 5e). This data indicated that PP2ACα-knockout mice were substantially more sensitive for LPS challenge. Collectively, this evidence clearly defines the negative regulation role of PP2ACα in LPS-induced lung inflammatory response.

PP2ACα Ablation in AMs Improves the Expression of LPS-Induced Cytokines with Augmented Phosphorylation of NF-κB p65, JNK, and Erk

To verify our finding further, AMs were isolated from PP2ACα-deficient mice or control mice and stimulated with LPS for indicated time points. Q-PCR assay showed that loss of PP2ACα in AMs resulted in improved Th-1 cytokine expression including IL-1β, TNF-α, IL-6, and CCL2 (Fig. 6a). Consistent with this, we also noticed that PP2ACα knockdown causes a time-dependent increase in p65, Erk, and JNK phosphorylation levels, and p38 phosphorylation show little change (Fig. 6b–c). Altogether, we demonstrated that PP2ACα negatively mediates LPS-induced Th-1 inflammatory factor expression *via* NF-κB and MAPK pathways and finally regulates ALI process.

DISCUSSION

ARDS/ALI is a devastating disorder of acute respiratory failure, and lung infection is frequently the underlying cause of ARDS/ALI. As a major cause of morbidity and mortality in patients in ICU, few effective therapies are currently available for the inflammatory response [34, 35].

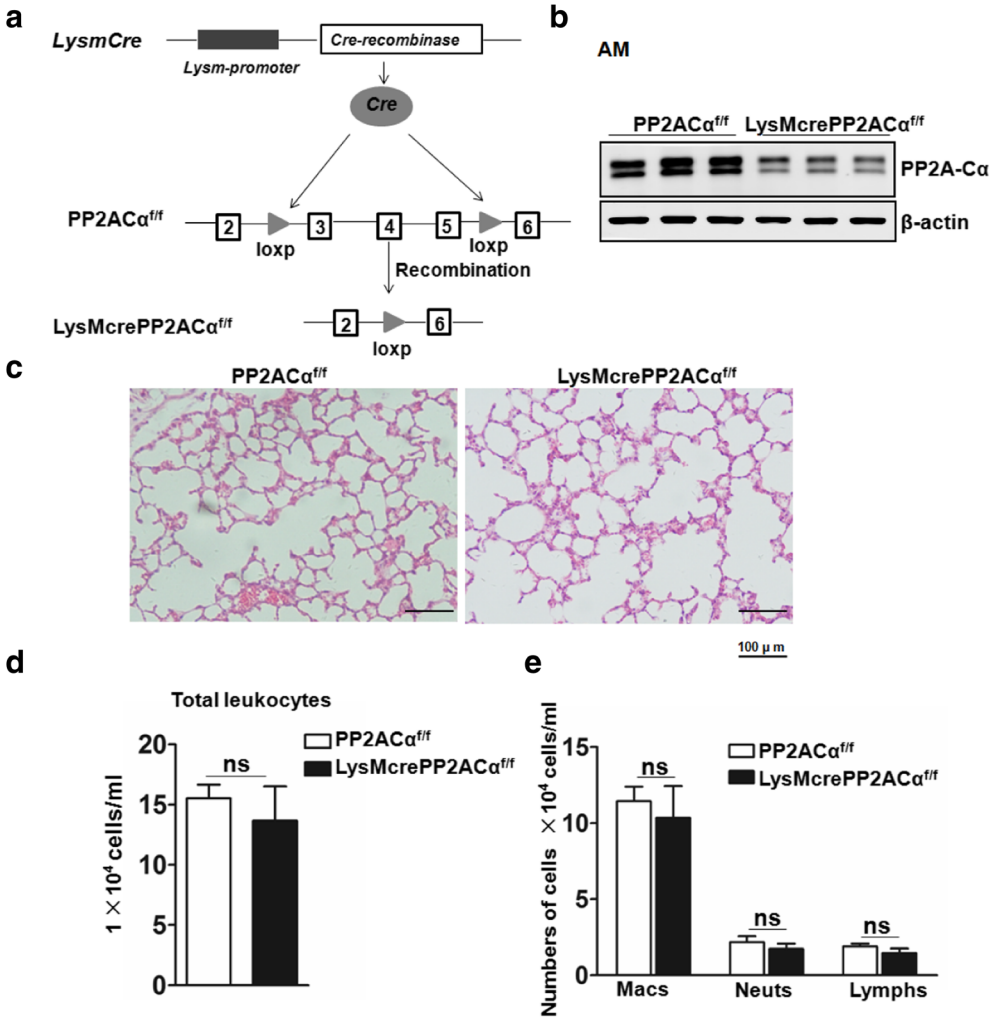

Fig. 4. Generation of PP2ACα conditional knockout in AMs. **a** Schematic map of the generation of macrophage-specific knockout mice. The LysM promoter was used to express the Cre recombinase, removing the floxed exon 3, 4, and 5 of PP2ACα gene, resulting in specific deletion of PP2ACα in macrophages. **b** Immunoblotting analysis of PP2ACα levels in purified AMs. Each protein lane represents one mouse and data are representative of three independent experiments. **c** HE-stained lung sections of LysMcrePP2ACα$^{f/f}$ mice were normal compared to controls. $N = 4$/group; scale bar = 100 μm. **d–e** LysMcrePP2ACα$^{f/f}$ mice had normal leukocyte numbers, including total and differential leukocyte counts in BALF. $N = 4$/group.

As a result, there is an urgent need to explore new potential targets for the treatment of this disease. In this study, we defined a protective role of AM PP2ACα in regulating LPS-induced ALI. Firstly, PP2A activity was found to observably declined in the AMs separated from LPS-treated mice, as well as in LPS-incubated directly AMs. Then adoptive transfer of C2-ceramide-incubated AMs in which PP2A enzyme activity was increased before LPS exposure relieved the lung inflammation. Conversely, AM-specific deletion of PP2ACα exacerbated inflammatory responses to LPS. Mechanistically, PP2ACα negatively regulates LPS-induced cytokine secretion of AMs by NF-κB and MAPK pathways. Therefore, our data collectively suggests accurate cell option of targeting PP2ACα for ARDS/ALI.

As a pivotal member of the serine/threonine phosphatase family, PP2A is proved to involve in pulmonary inflammation, including airway inflammation [36], airway hyperresponsiveness [37], COPD [38], and ALI [21, 39]. Despite previous studies showing increased PP2A activity in cigarette smoke–exposed lung tissues and declined JNK phosphorylation level, we found PP2A activity is decreased in AMs isolated from LPS-treated mice or LPS-explored AMs accompanied with enhanced activities of

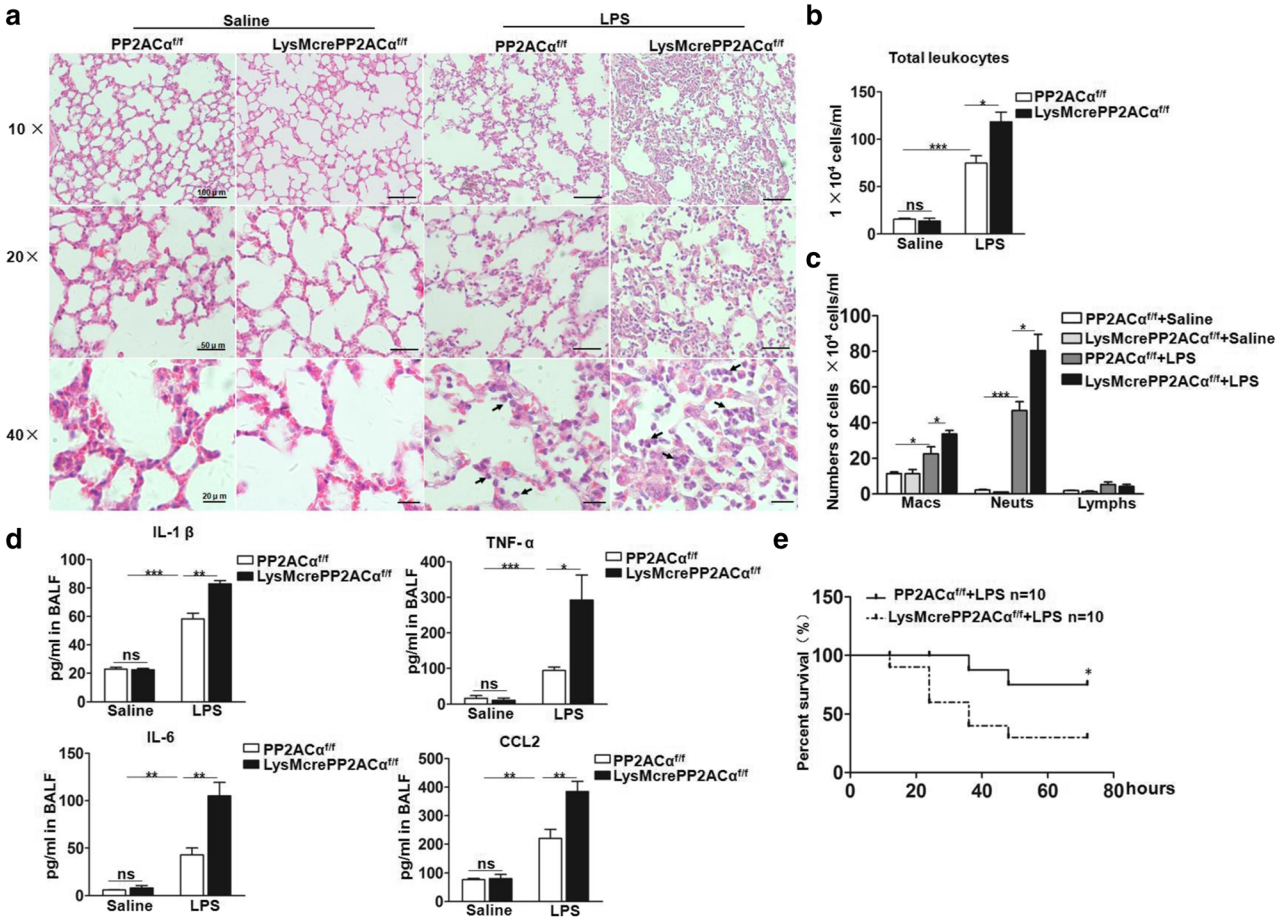

Fig. 5. Loss of PP2ACα in AMs enhances LPS-induced lung inflammation responses *in vivo*. **a** H&E staining showing that PP2ACα deficiency in AMs exacerbated lung inflammation in response to LPS. Arrows indicated leukocyte infiltration. Scale bar = 100/50/20 μm, $n = 4$ or 6/group. **b–c** Total and differential leukocyte counts indicated more leukocyte infiltration in LPS-challenged LysMcrePP2ACα$^{f/f}$ mice compared with PP2ACα$^{f/f}$. $N = 4$ or 6/group, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. **d** ELISA results showing that the secretion of Th1 cytokines (IL-1β, TNF-α, IL-6, and CCL2) in BALF was increased in LPS-treated LysMcrePP2ACα$^{f/f}$ mice compared with control. $N = 4$/group, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. **E** PP2ACα$^{f/f}$ and LysMcrePP2ACα$^{f/f}$ mice were injected intratracheally with 10 mg/kg LPS, and survival was monitored for 3 days. Results showed that LysMcrePP2ACα$^{f/f}$ mice were more sensitive for LPS challenge. $n = 10$/ group, $^*p < 0.05$.

NF-κB and MAPK pathways (Fig. 1a and c, Fig. 3b), which suggested that PP2ACα mediates different lung inflammatory diseases *via* various mechanisms. In addition, a selective adenosine A1 receptor agonist (N6-CPA), which is able to enhance PP2A activity, was used to treat MH-S cells. Results showed that the phosphorylation of JNK was attenuated and the inflammatory cytokine (CXCL1, CXCL2) secretion was inhibited [21]. Besides, improved PP2A activity targeting alveolar epithelial cells, monocytes, and neutrophils also blocked TNF-α-induced cytokine secretion [39]. However, PP2A functions of AMs remained unclear. Here, clodronate liposome was used for AM depletion, and then C2-ceramide-incubated AMs were transferred prior to LPS administration. Results showed suppressed pulmonary inflammation for PP2A activation in AMs, including reduced proinflammatory cytokine secretion and leukocyte infiltration, which is consistent with *in vitro* declined inflammatory factor expression in C2-ceramide-treated AMs stimulated with LPS.

Having established that PP2A activation is able to protect mice from LPS-induced ALI, transgenic mice ablated PP2ACα in AMs were used to confirm our conclusion. We found that loss of PP2ACα in AMs enhanced pulmonary inflammation, including exacerbated cytokine expression in BALF, increased infiltration of various leukocytes, and improved mouse mortality challenged with

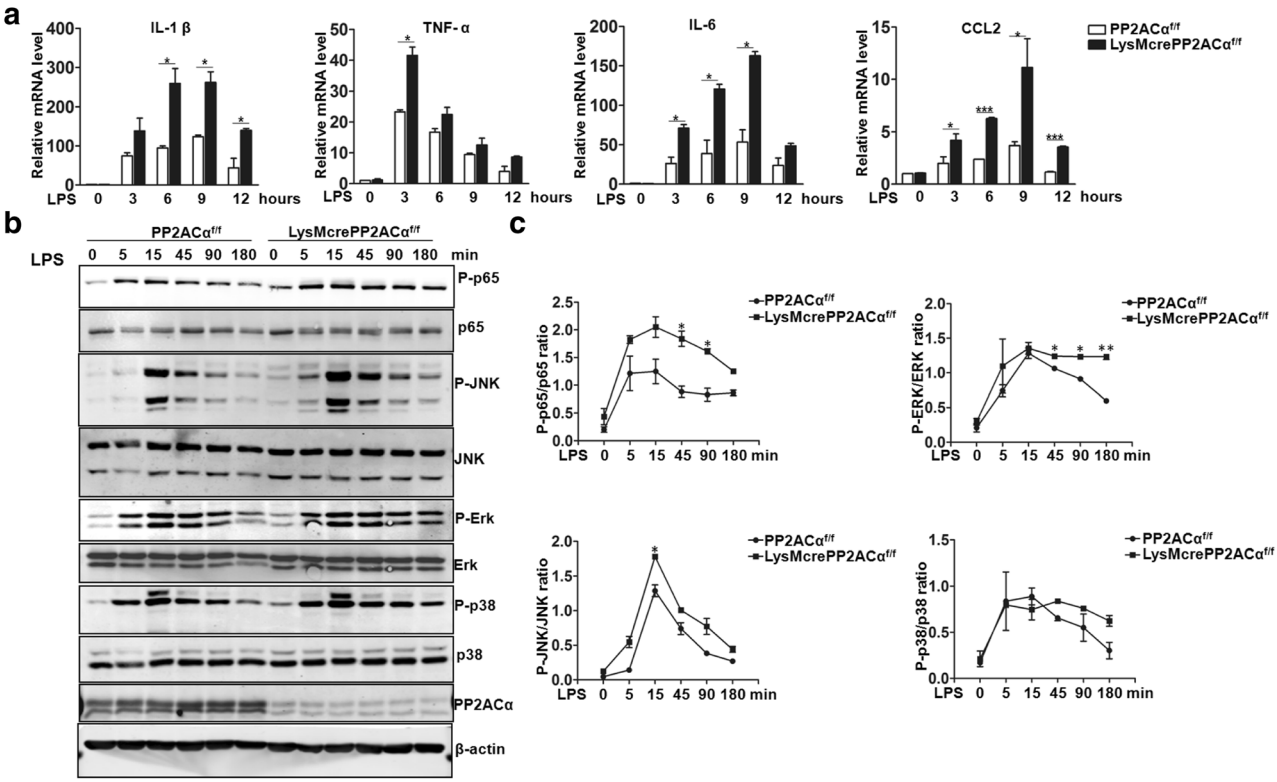

Fig. 6. PP2ACα ablation in AMs improves the expression of LPS-induced cytokines with augmented activation of NF-κB p65, JNK, and Erk. **a** Enhanced expression of IL-1β, TNF-α, IL-6, and CCL2 in PP2ACα-deficient AMs. AMs were stimulated with LPS (100 ng/ ml) for 0, 3, 6, 9, and 12 h. $N = 3$/group, $*p < 0.05$, $***p < 0.001$. **b** Increased activation of NF-κB p65, JNK, and Erk responsing to LPS stimulation in PP2ACα-deficient AMs, while the phosphorylation of p38 was little changed. AMs were stimulated with LPS (100 ng/ml) for 0, 5, 15, 45, 90 and 180 min. β-actin was used as loading control. Data are representative of three independent experiments. **c** Densitometry analysis of the indicated band intensities about NF-κB p65, JNK, Erk, and p38 phosphorylation. $N = 3$, $*p < 0.05$, $**p < 0.01$.

high-dose LPS. In addition, inflammatory factor expression also amplified in PP2ACα deficient AMs incubated with LPS *in vitro*. As a result, we demonstrated that the catalytic subunit of PP2A negatively regulates LPS-induced ALI.

It is well known that proinflammatory cytokine expression is dominated by TLR4/MyD88 signaling, and downstream NF-κB and MAPK pathways were activated for LPS stimulation [40]. PP2A has been demonstrated to directly interact and dephosphorylate NF-κB p65 [41] and MAPKs [42, 43]. Here, we found attenuated phosphorylation p65, JNK, and Erk of MAPK family members for the enhanced PP2A activity mediated by C2-ceramide, accompanied with decreased inflammatory factor expression. At the same time, PP2ACα-deficient AMs showed improved phosphorylation level of p65, JNK, and Erk. Our results provided a mechanistic link between attenuated inflammatory level with blocked NF-κB and MAPK pathways consistent with reported researches [44, 45]. Besides, previous study reported that C2-ceramide inhibited TLR4 pathway by interfering the interaction of LPS and TLR4 in microglia [46], while the mechanisms PP2ACα regulating LPS/TLR4 signaling of AMs remained to be further explored.

In conclusion, our finding highlights the important role of PP2ACα in AM-mediated ALI. Adoptive transfer of AMs with enhanced PP2ACα activity blocks LPS-induced inflammation storm in pulmonary, and loss-of-function experiments using *LysMcrePP2ACα$^{f/f}$* support our hypothesis, which could help guide the development of novel therapeutic options for ARDS/ALI clinically.

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AUTHOR CONTRIBUTIONS

H.Z., L.J., C.P., Y.K., and Y.Z. designed research; H.Z., L.J., and Y.Z. performed experiments, collected and analyzed data. H.Z. and Y.Z. wrote the paper; C.P. and Y.Z. critically revised the manuscript.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors declare that they have no conflict of interest.

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