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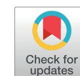
Muscarinic M1 and M2 receptor subtypes play opposite roles in LPS-induced septic shock



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ABSTRACT

Background: To compare pharmacologic effects of pirenzepine and AF-DX116, a selective competitive antagonist for M1 and M2 subtype muscarinic cholinergic receptors (mAChRs), respectively, with atropine, a non-selective competitive antagonist for mAChRs, on Lipopolysaccharide (LPS).
Methods: Male C57BL/6 mice were used to establish models of LPS-induced experimental endotoxemia. Mice were intraperitoneally injected 10 min prior to LPS injection with control (saline), atropine, pirenzepine and AF-DX116, respectively. Overall survival time was estimated using Kaplan-Meier plots. Inflammatory cytokine tumor necrosis factor-α (TNF-α) was monitored at various intervals after LPS injection and individual reagent administration. Pathological alternations in lungs and liver were analyzed.
Results: Pirenzepine and atropine pretreatment improved survival rate of LPS-induced septic shock; in contrast, AF-DX116 accelerated death from sepsis. Moreover, TNF-α plasma level was decreased in response to pirenzepine or atropine, whereas increased in response to AF-DX116. Pirenzepine and atropine relieved whereas AF-DX116 accelerated LPS-induced pulmonary and hepatic injury. Pirenzepine reduced proportion of M1 subtype of macrophages, while AF-DX116 promoted polarization of macrophages to M1 subtype. Pirenzepine pretreatment reduced while AF-DX116 enhanced expression of SOCS3 at mRNA level.
Conclusions: The administration of pirenzepine and atropine may have beneficial effects on septic shock.
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Introduction

Sepsis remains a serious cause of morbidity and mortality especially in intensive care unit (ICU), which is defined as life-threatening multi-organ dysfunction resulted from dysregulated host responses to infection [1].

Great efforts have been put to explore new therapeutic strategies for sepsis [1–3]. The pathogenesis of sepsis involves inflammation, as well as dysfunctional immune and central nervous systems [4–6]. Microbial infection initiates sepsis. A variety of broad-spectrum antibiotics have been developed to attack microorganisms entering bloodstream, but fail to reduce the mortality of sepsis. Antibiotics could not reverse multi-organ damage mediated by dysregulated host response [7]. Cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) together with uncontrolled inflammatory response contribute to pathogenesis of sepsis. These factors cause directly injury to different tissues and organs, such as liver, lung and kidney [8,9]. Unfortunately, none of the clinical trials based on anti-inflammatory strategies, for instances, endotoxin antibody, anti-TNF-α antibody, and IL-1 receptor antagonist [10], have resulted in significantly improved survival of patients with sepsis.

The vagus nerve and acetylcholine can reduce the levels of inflammatory factors (such as TNF-α) in peripheral circulation and mortality of animals in septic models by binding to corresponding receptors. This was called Choleric Anti-inflammation Pathway (CAP) [10–12]. Through activating CAP, nervous, endocrine and immune systems jointly regulate inflammatory responses in sepsis, providing a new avenue for treatment. Cholinergic receptors are classified into nicotinic cholinergic receptors (nAchRs) and muscarinic cholinergic receptors (mAchRs). Activation of cholinergic receptors apparently inhibits the expression of cytokines by post-transcriptional modification [13–15]. The α7 type nAchR, which is expressed on macrophages and activated by acetylcholine, could suppress the secretion of inflammatory cytokines such as IL-1β and TNF-α, while promote the synthesis of anti-inflammatory cytokine IL-10 [16–18]. In contrast, nicotine, a

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main activator of nAchRs, cannot be used as a drug due to toxicity. Muscarinic receptors are classified into five subtypes, expressed on different organs and cells with different functions [19,20]. Atropine as a non-selective mAChR antagonist is proposed to have beneficial effects on septic shock [21].

Macrophage is an important component of natural immune system. Macrophages that encourage inflammation and tissues injury are called M1 macrophages, whereas those that decrease inflammation and encourage tissue repair are called M2 macrophages [22–25]. Suppressors of cytokine signaling (SOCS) consist eight SOCS family proteins in mammals, which regulate the intracellular signaling networks. As reported, SOCS1 and SOCS3 were expressed on the macrophages [26,27], and they can be induced rapidly in macrophages.

In this study, we aim to demonstrate if selective M1 (pirenzepine) or M2 (AF-DX116) subtype mAChR antagonist can exert therapeutic effects and if they play the same or opposite roles on septic shock.

Material and methods

Animal models of endotoxic shock

Male C57BL/6 mice (20–23 g, 10-12-week-old) were purchased from Laboratory Animal Center of Peking University Health Science Center. Animal studies were approved by Institute Animal Care and Utilization Committee of Peking University Health Science Center. Animals were housed in a 12-h light/dark cycle for 5 days prior to experiments. Food and water were available *ad libitum* unless animals were on a food restriction protocol, in temperature at 21 °C and humidity of $50 \pm 10\%$. Mice were fasted for 24 h prior to experiments. LPS extracted from *E. coli* serotype 026: B6 (Sigma-Aldrich, St. Louis, MO, USA) was intraperitoneally injected at a dose of 75 or 150 mg/kg. Animals had *ad libitum* access to standard chow and water after LPS injection. All experiments were performed in physiological laboratory of Peking University Health Science Center.

Treatment protocol

To induce septic shock, animals were injected with saline (control) or LPS and randomly divided into sham or experimental groups as follows. (1) control: The mice received only comparable volume of saline instead of LPS or experimental drugs; (2) LPS: The mice were injected with LPS at a dose of 75 mg/kg. (3) Atropine: The mice were injected to non-selective mAChR antagonist atropine (Hefeng Pharmacy, China) at a dose of 0.5 mg/kg, 10 min prior to LPS injection; (4) Pirenzepine: The mice were injected with selective M1 mAChR antagonist pirenzepine (Sigma-Aldrich, St. Louis, MO, USA) at a dose of 0.3 mg/kg, 10 min prior to LPS injection; (5) AF-DX116: The mice were subjected to selective M2 mAChR antagonist AF-DX116 (Tocris, United Kingdom) at a dose of 0.5 mg/kg, 10 min prior to LPS injection. All drugs were injected intraperitoneally.

All animals were monitored vital status for 7 days. Overall survival time and the survival rate of each group were recorded.

Cytokine determination

Mice were sedated. An arterial catheter was inserted into the left common carotid artery and a venous catheter inserted into the right jugular vein. Multi-channel physiology recorder was used to monitor and record blood pressure and heart rate. All mice were administered with atropine, pirenzepine, AF-DX116, or normal saline respectively as described above, 10 min prior to administration of LPS.

Blood samples were collected *via* arterial catheter when systolic pressure was decreased by 20 percent as demonstrated by polygraph, and re-collected at 1, 2, 3, 4 and 5 h afterwards. Plasma samples were obtained by centrifugation and stored at -80 °C for subsequent quantification of tumor necrosis factor-α (TNF-α) using enzyme-linked immunosorbent assay (ELISA) (R&D systems Minneapolis, MN, USA) according to manufacturer's instructions.

Morphological changes

Morphological changes in lung and liver tissues were evaluated. Mice were euthanized at 2 and 12 h after saline, atropine, pirenzepine and AF-DX116 were injected, respectively. Lung and liver tissues were harvested, embedded in paraffin, and sectioned at 4 μm thickness. Hematoxylin and eosin (H&E) staining was performed to describe septic damage to different tissues.

Polymerase chain reaction and quantitative reverse transcription–polymerase chain reaction (qRT–PCR)

Total ribonucleic acid (RNA) was extracted from lung or liver using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol, reversely transcribed and tested with SYBR master mix (Takara, Otsu, Shiga), CFX-96 system (Bio-Rad, Hercules, CA). For real-time polymerase chain reaction (PCR), 2 μg of total RNA was synthesized to first-strand cDNA by reverse transcriptase (Thermo Scientific, Waltham, MA) according to manufacturer's protocol. The expression levels of target genes were analyzed by CFX-96 (Bio-Rad, Hercules, CA) system using SYBR master mix (Takara, Otsu, Shiga).

Gene expression of Suppressor of cytokine signaling (SOCS3) was analyzed by two-step qRT–PCR. RNA (0.2–1 μg) was reverse-transcribed to cDNA in a 20 μl reaction volume (25 °C, 10 min; 50 °C, 30 min; and 85 °C, 5 min) using a QuantiTect Reverse Transcription Kit. The cDNA (2 μl) was amplified using SYBR Green I Mastermix and LightCycler 480 PCR system. The relative expression of gene was determined using the $2^{-\Delta\Delta Ct}$ method, with β-actin as the internal control. The primers for mouse Socs3, and β-actin were as follows:

Socs3 Forward: 5′-CCTGGACTCCTATGAGAAAG-3′; Reverse: 5′-AAGTGGAGCATCATACTGA-3′

β-actin Forward: 5′-CCTCTATGCCAACACAGTGC-3′; Reverse: 5′-CCTGCTTGCTGATCCACATC-3′

Flow cytometry

The liver and lung were cut into small pieces, digested with 1.6 mg/ml Collagenase type Ia solution for 30 min at 37 °C. Cell suspension was filtered, centrifuged, resuspended, and then adjusted to a concentration of 1×10^7 cells/ml. The single-cell suspension was stained for 30 min at 4 °C with PE-cy7 anti-mouse CD45, PerCp5.5 anti-mouse CD11b, or Alexa Fluor488 anti-mouse Ly6c, respectively. After washing twice with PBS, cells were analyzed by flow cytometry using FACS Calibur (BD Biosciences, San Jose, CA, USA).

Statistical analysis

Overall survival was analyzed with Kaplan-Meier plots and log-rank tests. Cytokine was presented as mean ± standard error (SE). General differences in cytokine levels were determined using one-way analysis of variance (ANOVA) with multiple pair-wise comparisons. A $p < 0.05$ (two-tailed) was considered significant. Statistical analysis was performed using GraphPad Prism 5.0 (San Diego, CA, USA).

Results

Pirenzepine improved outcome of sepsis while AF-DX116 accelerated death from endotoxic shock

As shown in Fig. 1A, 60% of mice succumbed within 72 h in response to LPS (75 mg/kg) challenge. All mice died of septic shock induced by a higher dose (150 mg/kg). Both atropine and pirenzepine improved outcome of endotoxic shock (Fig. 1B). Compared to atropine, pirenzepine better prolonged survival ($p < 0.05$) of sepsis induced by LPS at higher dose (150 mg/kg). On the contrary, AF-DX116 accelerated sepsis-induced death (Fig. 1B) ($p < 0.05$). This indicated that M1 and M2 mAChR agonists might play opposite roles in affecting outcome of sepsis.

Pirenzepine modifies inflammatory response by decreasing plasma TNF-α level, attenuating LPS-induced pro-proinflammatory cytokines expression and protecting organs from injury

The release of TNF-α into circulation was determined at different time points after LPS injection as well as atropine, pirenzepine, or AF-DX116 treatment. Plasma levels of TNF-α were significantly decreased (to similar extents) in response to pirenzepine or atropine at 1, 2, 3, 4 and 5 h after LPS induction ($p < 0.05$) (Fig. 2). In contrast, plasma levels of TNF-α were significantly increased in response to AF-DX116 at 1, 2, 3, 4 and 5 h of LPS induction ($p < 0.05$) (Fig. 2).

Atropine and pirenzepine protected multi-organ damage in LPS-induced septic mice. Pathological changes in lung were distinct in response to LPS. Alveoli were filled with exudates and even collapsed. Destroyed or decreased alveoli as well as thickened alveoli septum were common. Significant alleviation of congestion, dropsy, inflammatory effusion of alveoli presented in response to atropine or pirenzepine. Pathological injury was more severe in response to AF-DX116 (Fig. 3A).

Pathological changes in liver were highlighted. Liver lobules were irregular in shape, infiltrated with inflammatory cells. Disordered structures of hepatic lobules, together with congestion in hepatic sinusoid and central vein were obvious upon LPS induction. In response to atropine and M1 cholinergic receptor antagonist pirenzepine, lobule structure was better organized than control, with significant alleviation of congestion, dropsy, and inflammatory effusion. Liver sinuses were lightly dilated. Pathological injury was more severe in response to AF-DX116, with necrosis, dilation and stasis (Fig. 3B).

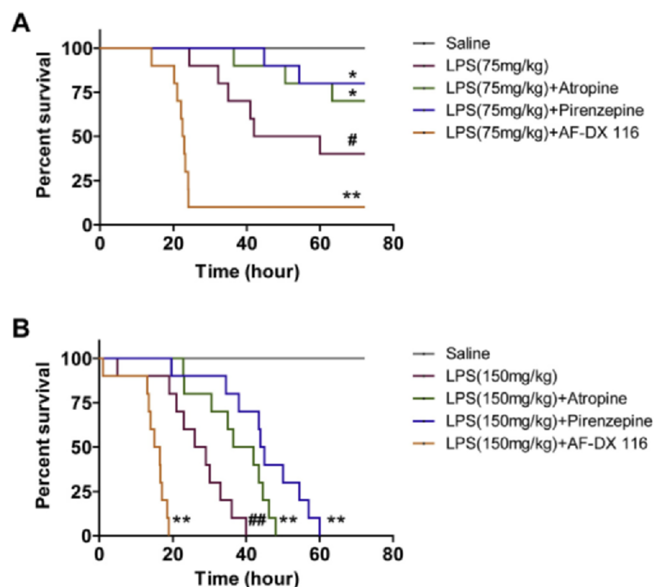

Fig. 1. Antagonism of M1 rather than M2 mAChR prevented lethality in septic mice. C57BL/6 male mice (n = 10/group) were intraperitoneally injected with saline, atropine sulfate (0.5 mg/kg), pirenzepine (0.3 mg/kg) or AF-DX116 (0.5 mg/kg), respectively, 10 min prior to LPS administration at dose of (A) 75 mg/kg or (B) 150 mg/kg. #, $p < 0.05$, ##, $p < 0.01$ (compared to saline control); *, $p < 0.05$, **, $p < 0.01$ (compared to LPS).

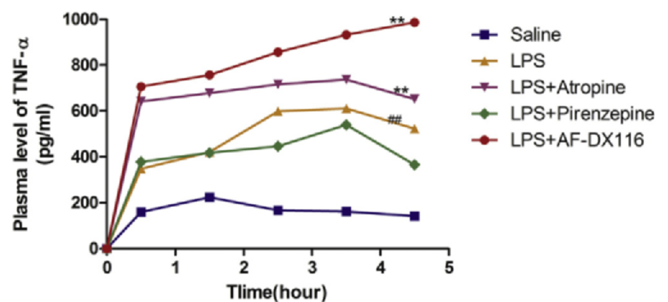

Fig. 2. M1 antagonist pirenzepine and non-selective M antagonist atropine modify inflammatory response by decreasing TNF-α level in plasma. Blood samples were collected after 1, 2, 3, 4 and 5 h, respectively, post-LPS injection and analyzed for TNF-α levels in plasma. Values are expressed as mean ± standard deviation (SD). ##, $p < 0.01$, compared to saline control; **, $p < 0.01$ compared to LPS.

Expression levels of proinflammatory cytokines might be proportional to severity of sepsis. LPS significantly enhanced expression of TNF-α, IL-1β and IL-6 in lung and liver (Fig. 4A, B), which were decreased by pirenzepine or atropine (Fig. 4A, B). In addition, LPS upregulated expression of macrophage marker F4/80 in lung, which was attenuated by atropine or pirenzepine (Fig. 4A). Moreover, AF-DX116 reduced pulmonary expression of IL-1β and IL-6 at mRNA levels (Fig. 4A), without affecting the expression of proinflammatory cytokines or F4/80 in liver (Fig. 4B)

Pirenzepine inhibited whereas AF-DX116 promoted polarization of macrophages to M1 phenotype as mediated by LPS in septic mice

To explore molecular mechanisms underlying distinct roles of M1 and M2 mAChR agonists in sepsis, macrophage markers were detected in lung and liver at 2 and 12 h, respectively, after LPS induction. $CD11b^{+}Ly6C^{hi}$ (M1) and $CD11b^{+}Ly6C^{low}$ (M2) macrophages were examined using flow cytometry. The number of $Ly6c^{hi}$ M1 was decreased by pirenzepine but increased by AF-DX116 in lungs at 12 h ($p < 0.05$, Fig. 5A, B). Pirenzepine decreased but AF-DX116 increased $Ly6c^{hi}$ expression in liver at 2 and 12 h ($p < 0.05$, Fig. 5C, D). Pirenzepine increased but AF-DX116 decreased $Ly6C^{low}$ expression in lung at 2 and 12 h ($p < 0.05$, Fig. 5E, F). Pirenzepine increased $Ly6C^{low}$ expression in liver at 12 h, while AF-DX116 decreased $Ly6C^{low}$ expression in liver at 2 h ($p < 0.05$, Fig. 5G, H).

To explore molecular mechanisms underlying polarization of macrophages in sepsis after blocking M1 or M2 mAChRs, mRNA levels of SOCS3 were measured in lung and liver tissue. Pirenzepine downregulated but AF-DX116 upregulated expression of SOCS3 in both lung and liver tissue at 2 and 12 h after LPS injection ($p < 0.05$, Fig. 6A–D). Blockade of M1 mAChR attenuated while blockade of M2 mAChR promoted SOCS3 gene expression.

Discussion

Inflammation is a major component of pathological conditions such as infection, sepsis, injury and ischemia. Inflammatory response is regulated by complex interaction of cytokines, hormones, prostaglandins, as well as nervous and endocrine systems. In particular, parasympathetic nervous system innervated by vagus nerve exerts anti-inflammatory effects. Thus, the release

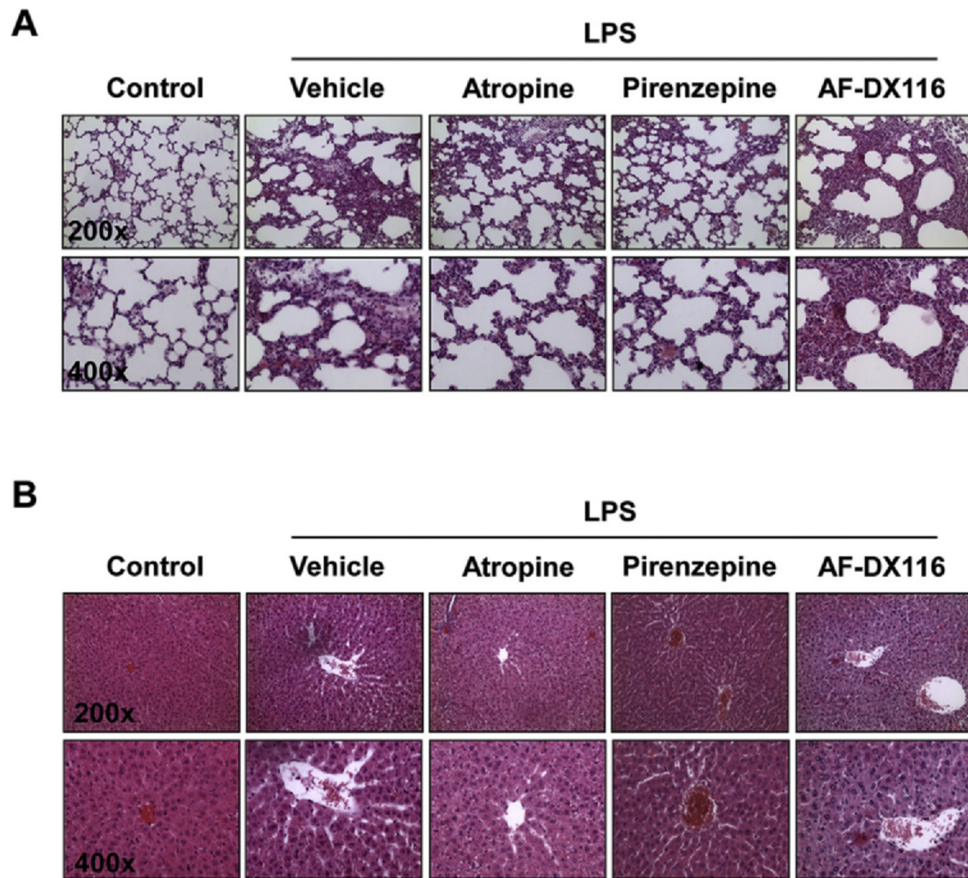

Fig. 3. M1 but not M2 mAChR inhibition protected multi-organ injury in LPS-induced septic mice. Representative histopathology of lung and liver tissues. C57BL/6 mice were injected with pirenzepine (0.3 mg/kg) or AF-DX116 (0.5 mg/kg), 30 min prior to LPS challenge (75 mg/kg). 12 h after LPS injection, mice were sacrificed. Sections of (A) lung and (B) liver tissues were stained with HE (n = 10 per group). HE: hematoxylin and eosin.

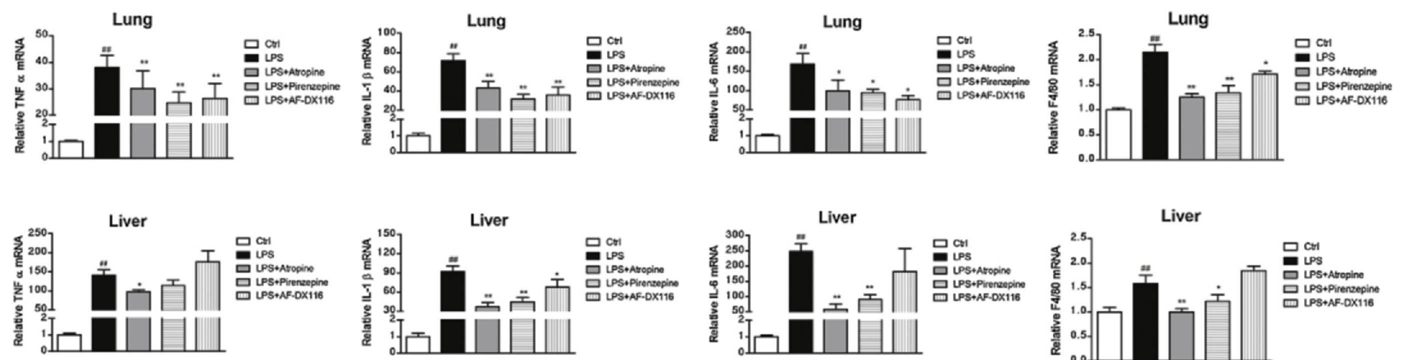

Fig. 4. Effects of M1 or M2 mAChR inhibition on LPS-induced neutrophil infiltration and TNF-α expression. The expression of TNF-α, IL-1β, IL-6 and F4/80 mRNA levels in lung and liver differs greatly by treatment protocols. ##, $p < 0.01$, compared to saline control; *, $p < 0.05$, **, $p < 0.01$ compared to LPS.

of ACh upon vagus nerve activation has been proposed to provide negative-feedback control of inflammation, defined as cholinergic anti-inflammatory pathway [10–15].

Atropine, a non-selective mAChR antagonist, has been shown to decrease the mortality of septic shock [21]. In addition, selective M1 antagonist pirenzepine prolonged survival of septic mice better than atropine. In contrast, M2 antagonist AF-DX116 accelerated death from endotoxic shock. M1 mAChR might increase the secretion of inflammatory factors, promote sepsis progression and reduce survival of septic mice, which is in sharp contrast to M2 mAChR. Different M receptor subtypes may have distinctive functions in sepsis. These results are consistent with the effects of atropine on inflammatory response.

In this study, selective M1 antagonist pirenzepine attenuated whereas selective M2 antagonist AF-DX116 promoted infiltration of M1 macrophages in lung and liver. Macrophages play critical roles in development and resolution of inflammation. Macrophages are polarized into classically activated M1 and alternatively activated M2. M1 macrophages are activated by LPS or interferon-gamma, with strong propensity to present antigen and cause tissue injury [22]. M2 macrophages are activated by IL-4, with capability of tissue repair and response to parasites [23]. Abnormal polarization of

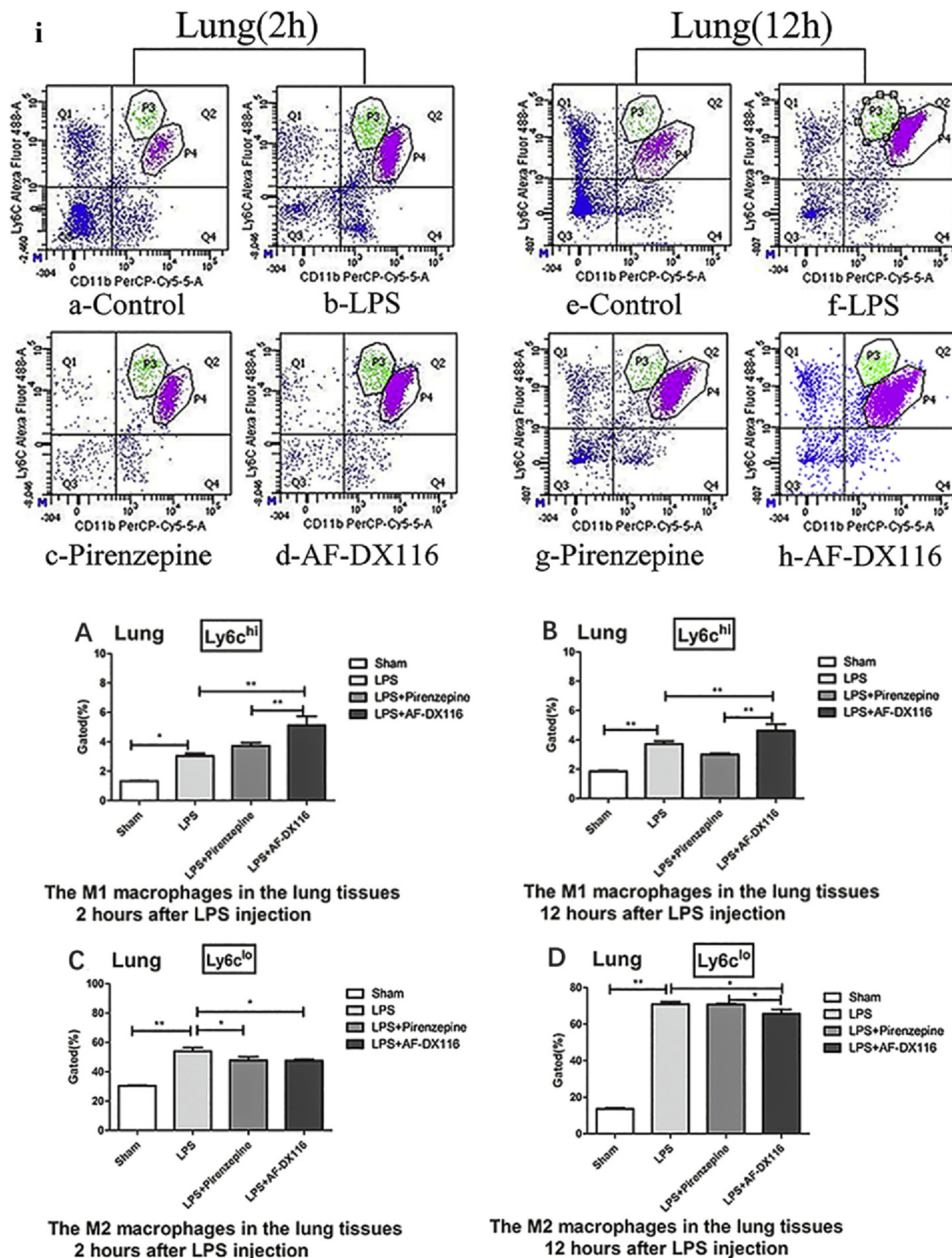

Fig. 5. Polarization of M1 and M2 macrophages in lung and liver tissues as measured by flow cytometry. Fig. 5.1 (A–D) Polarization of M1 macrophages in liver and lung tissues at 2 and 12 h, respectively, after LPS injection. Fig. 5.2 (E–H): Polarization of M2 macrophages in liver and lung tissues at 2 and 12 h, respectively, after LPS injection. *, $p < 0.05$, **, $p < 0.01$.

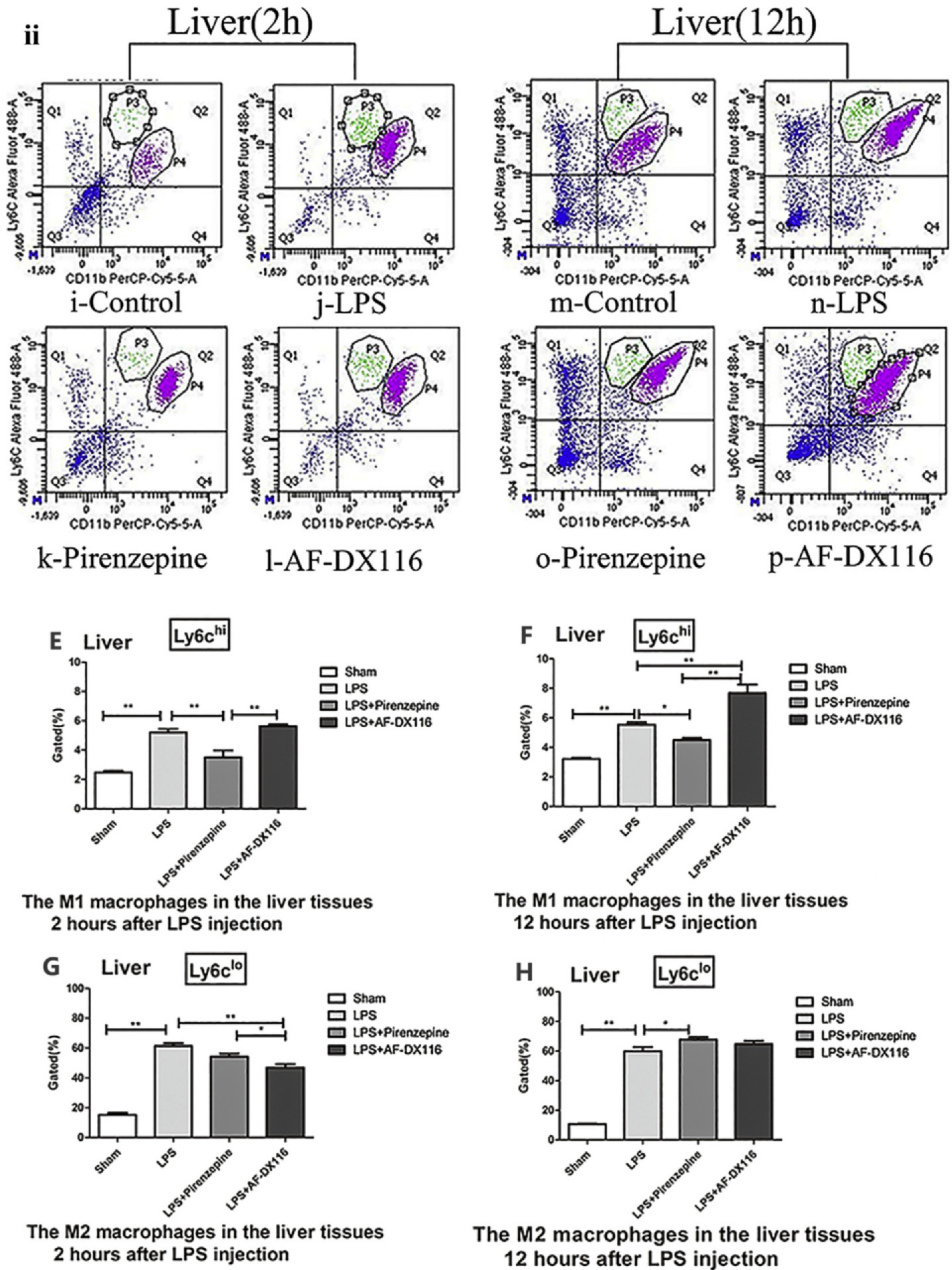

Fig. 5. (Continued)

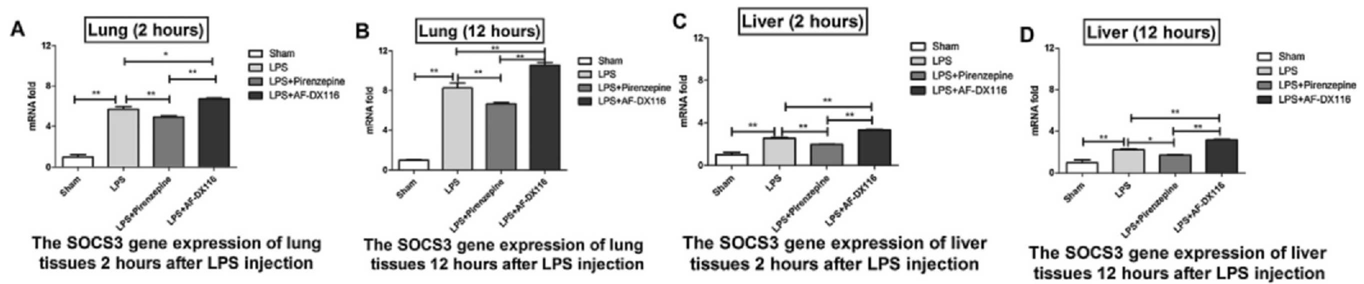

Fig. 6. The mRNA levels of SCOS3 gene in lung and liver tissues. *, $p < 0.05$, **, $p < 0.01$.

macrophages is involved in various diseases. Asthma was associated with M2 macrophages [24]. M1 macrophages increased lung injury. Anti-TNF-α antibody reduced infiltration of inflammatory cells in trachea [25]. M2 polarization could attenuate kidney injury in rhabdomyolysis. Polarization of macrophages determines inflammatory response. Excessive activation of M1 induces injury in tissues and organs due to high levels of pro-inflammatory factors. M1 mAChR promotes but M2 mAChR inhibits M1 polarization induced by LPS. This indicates different roles of M1 and M2 antagonists in predicting prognosis of septic mice.

Suppressors of cytokine signaling (SOCS) family proteins regulate various intracellular signaling pathways in mammals. As reported, SOCS1 and SOCS3 were induced rapidly on macrophages [26,27]. Majority of macrophages infiltrating inflamed glomeruli in experimental nephritis could upregulate expression of SOCS3, which is necessary for M1-polarized phenotype [28]. Upon LPS induction, myeloid-restricted Socs3 deletion (Socs3 Lyz2cre) mice had longer survival time and lower levels of TNF-α and IL-6 (mainly secreted by M1 macrophages) [29]. In our experiment, we confirmed the relationship between macrophage polarization and SOCS3 mRNA expression. Pirenzepine attenuated but AF-DX116 enhanced the expression of SOCS3 in lung and liver tissues. Thus, SOCS3 promotes polarization of macrophages to M1 subtype.

Here, we found that mAChRs plays an important role in regulating polarization of macrophages, providing a promising approach for inflammation and sepsis treatment. However, only M receptor agonists were used in animal models. In the near future, knockout or transgenic murine models will be employed to investigate molecular mechanisms underlying M receptors in sepsis, and to explore new therapeutic strategies for sepsis.

Conflict of interest

All authors declare no conflicts of interest.

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