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LPS Activated Macrophages Induced Hepatocyte Pyroptosis via P2X7R Activation of NLRP3 in Mice

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ABSTRACT

Background: Pyroptosis is a programmed cell death related to caspase-1, accompanied by the secretion of pro-inflammatory cytokines.

Objectives: To explore the effects of LPS on the P2X7R/NLRP3 pathway in macrophages, and hepatocytes pyroptosis in mice.

Methods: LPS was used to establish an animal model of the acute liver injury. The macrophage RAW264.7 was induced by LPS to establish a cell model. The P2X7R inhibitor A438079 and agonist BZATP were added. RAW264.7 was co-cultured with AML-12 cells. Pyroptosis and the ratio of CD11b+CD86+/CD11b+CD206+ were analyzed by flow cytometry. ELISA, WB, and qRT-PCR were applied to analyze factors involved in the P2X7R/NLRP3 pathway.

Results: LPS induced liver damage in mice, promoted cell pyroptosis and increased the levels of IL-18, IL-1β, ALT, AST, and TBIL. P2X7R, GSDMD, and GSDMD-N expressions also increased in the LPS group. LPS induced macrophage activation in vivo. NLRP3, ASC, P2X7R, and caspase-1 expressions in vitro promoted. ELISA confirmed that the IL-1β and IL-18 levels repressed in the BZATP (P2X7R agonist) group, while the trend was opposite in the A438079 (P2X7R inhibitor) group. LPS activated the P2X7R/NLRP3 pathway in macrophages. After RAW264.7 was co-cultured with AML-12 cells, the pyroptosis of AML-12 cells promoted but the proliferation decreased in the BZATP group. GSDMD and GSDMD-N expressions promoted in the BZATP group, while the trend was opposite in the A438079 group.

Conclusion: LPS activated macrophages via P2X7R activation of NLRP3 and induced hepatocyte pyroptosis, which provided novel potential targets for the liver injury treatment.

Keywords: Pyroptosis, Macrophages, P2X7R, NLRP3, Liver injury

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INTRODUCTION

Liver injury reflects the important mechanism of inflammation and disease progression in various acute and chronic liver diseases (1), and cell pyroptosis plays a vital role in the liver disease (2). Pyroptosis is a caspase-1-dependent programmed cell death (PCD), characterized by cell swelling, plasma membrane rapid rupture, and pro-inflammatory cell contents release (3). Lipopolysaccharide (LPS) is the most common drug that causes liver damage (4-6). This model is widely used to explore liver damage pathogenesis and develop new liver protective agents. In liver injury, the LPS also mediates pyrolysis (7), but its mechanism of action remains unclear.

The LPS is an endogenous intestinal bacterial endotoxin that could promote liver injury by inducing resident liver macrophages (called Kupffer cells) activation (8, 9). Studies have shown that the LPS increases liver inflammation in mice by macrophages polarization into M1 type (4). The LPS induces macrophages polarization into M1 type, thereby increasing the expression of M1 cytokines/chemokines (For example, the IL-6, IL-1β, TNF-α, CCL4, and CXCL11) (10). The activation of caspase-11 atypical inflammasomes by the LPS differs from the activation of typical inflammasomes, which provides a new paradigm for macrophage-mediated inflammatory response (11). Nucleotide-binding oligomerization domain-like receptor protein 3 (NLRP3) is one of the most studied inflammasomes, composed of the NLRP3 sensors, caspase-1 enzyme, and apoptosis-associated speck-like protein (12, 13). Purinergic receptor P2X7 (P2X7R) is the adenosine triphosphate (ATP)-gated cation channel that participated in inflammatory and autoimmune processes by the NLPR3 inflammasome activation and the IL-1β release, in addition to the lymphocyte proliferation and apoptosis (14). Leucodin has been reported to inhibit P2X7R expression, Toll-like Receptor 4 (TLR4) , and NLRP3 in LPS/ ATP-stimulated macrophages (9). Other studies have shown that after the LPS treatment, the NLRP3 and P2X7R protein and molecular levels of primary human monocyte-derived macrophages (MDM) significantly increased (15). But the signaling mechanism leading to the NLRP3 inflammasome activation is still unclear.

It has been reported that the activation of the P2X7R may cause M1-type macrophages polarization and inhibit M2-type macrophages polarization, which implies that P2X7R may have pro-inflammatory effects (16). The P2X7R antagonist A74003 blocks the ATP-dependent release of the IL-1β, which may inhibit the IL-1β, NLRP3, and TNF-α expression in microglia and weaken the inflammatory response (17). The P2X7R deficiency can also reduce the accumulation of lipids in alcoholic liver steatosis and inhibit liver damage (18). A previous study found that the P2X7R-mediated NLRP3 inflammasome activation was associated with the IL-1β production in hepatic stellate cells (HSC), which may contribute to extracellular matrix (ECM) deposition, and suggested that blocking P2X7R-NLRP3 inflammasome axis was the potential therapeutic target for liver fibrosis (19). Therefore, blocking the P2X7R/NLRP3 signaling pathway may inhibit tissue or cell injury, but whether or not it plays a role in the hepatocyte pyroptosis process remains unknown.

In this research, we primarily wanted to investigate the LPS effects on the P2X7R/NLRP3 signaling pathway, macrophages, and hepatocytes. It is found that the LPS activated macrophages via P2X7R activation of the NLRP3 and induced hepatocyte pyroptosis. In the process of hepatocyte pyroptosis, blocking the P2X7R/NLRP3 signaling pathway may inhibit tissue or cell damage, which might provide novel potential therapeutic targets for liver injury.

MATERIAL AND METHODS

In Vivo Experiments

The Acute Liver Injury Model

Twenty C57BL/6 male mice (eight-week-

old) were randomly divided into sham and the LPS groups, with 10 mice in each group. The LPS (5 mg/kg) was injected intraperitoneally for each 0.1 mL/10g for 6 h to induce an acute liver injury model induced by endotoxemia in mice (20, 21), and the sham group was intraperitoneally injected with the same amount of normal saline. All animal experiments have been approved in advance by the Medical Ethics Committee of Hunan People's Hospital (First Affiliated Hospital of Hunann Normal University) and were following the National Institutes of Health guidelines on the care and use of animals.

In Vitro Experiments

Cell Culture and Treatment

The macrophage line RAW264.7 was cultured at 2×10^6/mL. The experiment was randomly divided into the control, the LPS, A438079 (P2X7R inhibitor), and the BZATP (P2X7R agonist) groups. A438079 and the BzATP groups were added with 10 μM A438079 and 100 μM BzATP respectively. After 2 h of stimulation (22), they were stimulated with 200 μM LPS for 8 h together with the LPS model group, and in the control group, the LPS was replaced by the same amount of normal saline. The method of co-culture between RAW264.7 and AML-12 cells was as follows: the macrophage line RAW264.7 was cultured in Transwell lower compartment at 2×10^6/dish. The experiment was randomly divided into 5 groups: the control, the LPS, A438079 (P2X7R inhibitor), the BZATP (P2X7R agonist), and the blank group (adding the LPS but not cultured RAW264.7). The A438079 group and the BzATP group were added with 10 μM A438079 and 100 μM BzATP, respectively. After 2 h of stimulation, they were stimulated with 200 μM LPS for 8 h together with the blank and the LPS model group. The liver cell line AML-12 was cultured into Transwell upper chamber (1×10^6/dish). After 6 h, AML-12 cells were harvested for detection.

Ex Vivo Experiments

HE Staining

The slices were roasted at 60°C for 12 h, and dewaxed in water, stained with hematoxylin for 1-5 min, stained with eosin for 1s, and dehydrated with gradient alcohol (95-100%). After removal, they were placed in xylene for 10 min, sealed with neutral resin, and observed under the microscope.

Flow Cytometry Assay

Firstly, cells were collected. Then they were washed with PBS once. Then suspended cells were added with 500 μL Binding Buffer, mixed with 5 μL Annexin V-FITC (# KGA108, KeygenBio, Nanjing), and mixed with 5μL Propidium Iodide. Flow cytometry (A00-1-1102, Beckman, USA) was applied after 15 min in a dark reaction at room temperature.

The liver tissue was digested, each containing about 1×10^6 cells. Cells were resuspended with 200 μL PBS, incubated in the dark for 30 min with antibodies against CD86 (#12-0862-82, eBioscience), CD206 (#12-2061-82, eBioscience), iNOS (#53-5920-82, eBioscience), Arg-1 (#53-3697-82, eBioscience) and CD11b (#11-0112-41, eBioscience). The cells were resuspended with 200 μL PBS and filtered with nylon net. The double-positive rates of $CD11b^+CD86^+$ and $CD11b^+CD206^+$, CD86, CD206, iNOS and Arg-1 expressions were analyzed by the flow cytometry (A00-1-1102, Beckman, USA).

ELISA

The serum of mice and cell culture supernatant were taken to detect serum IL-1β and IL-18 levels. IL-1β (CSB-E08054m, CUSABIO) and IL-18 (CSB-E04609m, CUSABIO) the ELISA quantitative kit detected IL-1β and IL-18 levels according to the instructions. Bio-Tek microplate reader (MB-530, Heales, China) measured OD value, IL-1β, and the IL-18 concentrations were calculated according to the standard curve.

Liver Function Indexes Detection

The serum of mice was taken to detect the serum liver function indexes alanine transaminase (ALT), aspartate transaminase (AST), and total bilirubin (TBIL) levels. ALT (C009-2-1), AST (C010-2-1), and TBIL (C019-1-1) commercial kits were all purchased from Nanjing Jiancheng Bioengineering Institute and applied to detect ALT, AST, and TBIL levels according to the instructions.

Western Blot (WB)

RIPA lysis buffer (#P0013B, Beyotime) extracted total protein from liver tissues and cells. The protein of each group was quantified according to the BCA protein determination kit. The protein was mixed with SDS-PAGE loading buffer (#MB2479, meilunbio). Proteins were adsorbed on PVDF membrane by gel electrophoresis and sealed with 5% skim milk solution at room temperature for 2 h, incubated with diluted primary antibodies including P2X7R (1: 1000, ab259942, abcam), GSDMD-N (1: 1000, ab215203, abcam), GSDMD (1: 1000, ab219800, abcam), CD86 (1: 1000, ab239075, abcam), iNOS (1: 1000, ab178945, abcam), CD206 (1: 1000, ab64693, abcam), Arg-1 (1: 1000, ab91279, abcam), NLRP3 (1: 1000, ab263899, abcam), ASC (1: 1000, #67824, CST), caspase-1 (1: 1000, #24232, CST) and β-actin (1: 1000, 66009-1-Ig, proteintech). Then secondary antibodies were incubated. Chemiscope6100 system (Clinx Co., Ltd, Shanghai, China) was applied to measure protein bands. β-actin was acted as an internal reference.

Quantitative Real-time PCR (*qRT-PCR)*

P2X7R, ASC, Caspase-1, NLRP3, and GSDMD expressions in cells were measured. In brief, the total RNA was extracted by the Trizol method, and was reverse transcripted into cDNAs using cDNA reverse transcription kit (#CW2569, Beijing Comwin Biotech, China). Ultra Sybr Mixture (#CW2601, Beijing Comwin Biotech, China) was applied to measure mRNA expression on fluorescence quantitative RCR (QuantStudio1, Thermo, USA). β-actin as internal genes and the $2^{-\Delta\Delta Ct}$ method was applied. Table 1 shows primer sequences.

Cell Counting Kit 8 (CCK-8) Assay

CCK8 detected AML-12 cells proliferation in the control group, the LPS group, A438079 group, the BZATP group, and the blank group. The cells of different groups were inoculated into 96-well plates (1×10^4 cells/well) and incubated in an incubator of 100 μL per well at 37°C and 5% CO_2. 10 μL CCK8 (#NU679, DOJINDO, Japan) was added for 24 h after culture. The absorbance value at 450 nm was measured by the Bio-Tek enzyme plate (MB-530, Heales, China) after further incubation at 37°C and 5% CO_2 for 4 h.

Statistical Analysis

GraphPad 8.0 software was applied for

Table 1. The primers used in this study.

Primer ID	5'-3'
P2X7R-F	AAAGGCCAAGAAGTTCCAACC
P2X7R-R	TCTTAGACGCCTTAAAATGGCA
NLRP3-F	CCTCTTTGGCCTTGTAAACCAG
NLRP3-R	TGGCTTTCACTTCAATCCACT
ASC-F	CAGAGTACAGCCAGAACAGGACACT
ASC-R	AAGCATCCAGCACTCCGTCCAC
caspase-1-F	ACAAGGCACGGGACCTATG
caspase-1-R	TCCCAGTCAGTCCTGGAAATG
GSDMD-F	TTCCAGTGCCTCCATGAATGTGT
GSDMD-R	ATCCCCACGACTCCGAAGCTG
β-actin-F	ACATCCGTAAAGACCTCTATGCC
β-actin-R	TACTCCTGCTTGCTGATCCAC

the statistical analysis, and the data were expressed as the mean ± standard deviation (SD). Student's t-test or One-way ANOVA was performed to compare two or more groups. $P<0.05$ was considered statistically significant.

RESULTS

The LPS Promoted P2X7R Expression and Induced Hepatocyte Pyroptosis

HE staining was used to detect the liver tissue injury. As shown in Figure 1A, the LPS group had a liver injury. Flow cytometry results showed that the LPS promoted cell pyroptosis (Figure 1B). The ELISA detection of inflammatory cytokines showed the LPS increased inflammatory cytokines IL-18, IL-1β expressions (Figure 1C). Biochemical detection showed the LPS increased liver function indexes ALT, AST, and TBIL expressions (Figure 1D). Finally, WB measured P2X7R, GSDMD, and GSDMD-N in the liver. Our results showed P2X7R, GSDMD, and GSDMD-N expressions in the liver of the LPS group increased compared

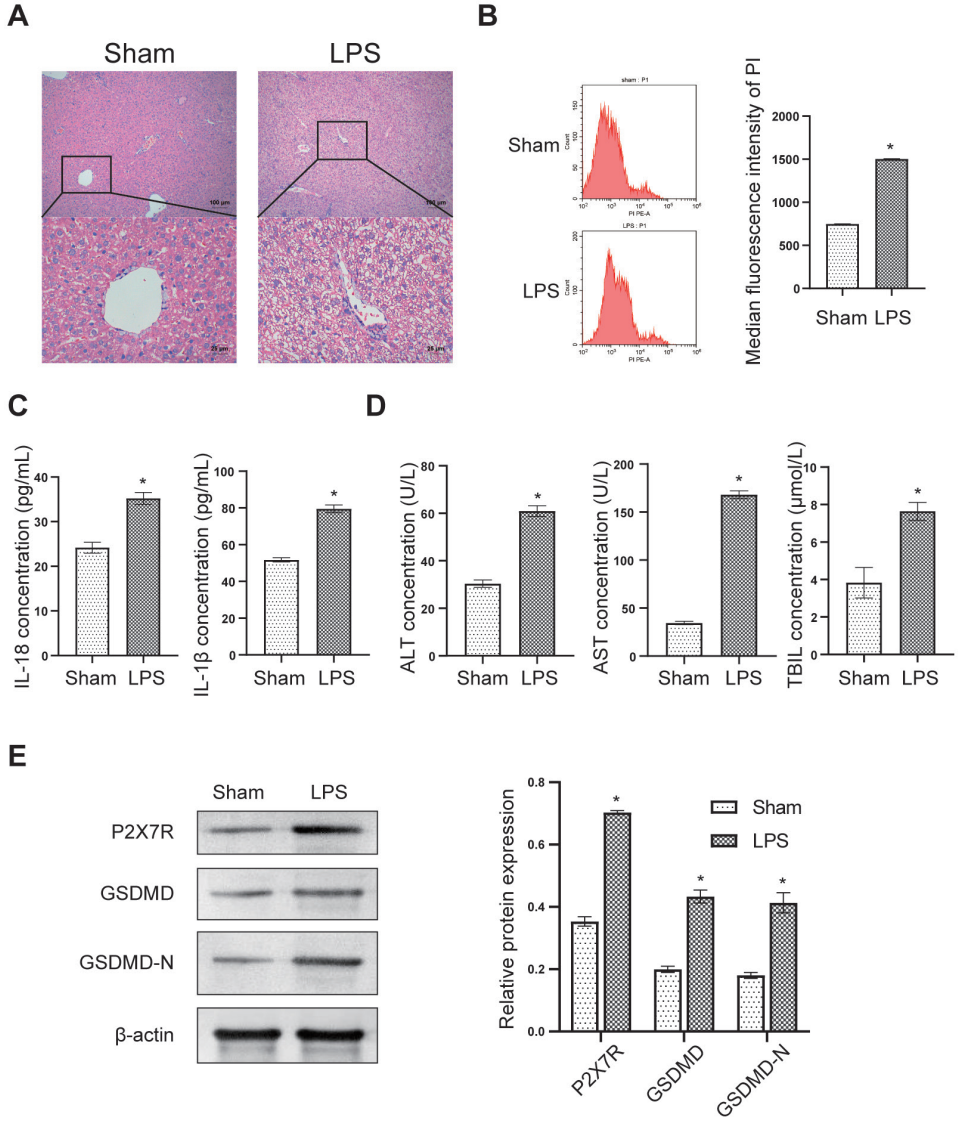

Figure 1. LPS promoted P2X7R expression and induced hepatocyte pyroptosis. (A) The damage of liver tissue in sham and the LPS group. (B) Flow cytometry detected the pyroptosis of liver tissue. (C) The ELISA detected the IL-18 and IL-1β expression. (D) The biochemical experiment detected ALT, AST, and TBIL levels in serum. (E) WB measured P2X7R, GSDMD, and GSDMD-N expressions in the liver. Scale bar = 100 μm (× 100) and Scale bar = 25 μm (× 400) respectively; *P<0.05 vs sham group.

with the sham group (Figure 1E), which implied that the LPS promoted the expression of P2X7R and induced hepatocyte pyroptosis.

LPS Induced Macrophage Activation in a Liver Injury Model

We found that the ratio of $CD11b^+CD86^+$ double positive in the LPS group increased, and iNOS expression also increased, while the ratio of double-positive $CD11b^+CD206^+$ decreased, and the expression of Arg-1 also decreased by the flow cytometry (Figure 2A). As shown in Figure 2B, WB further verified that CD86 and iNOS expression promoted, but CD206 and Arg-1 expression decreased in the LPS group. Flow cytometry and WB results showed that the LPS could induce macrophage activation in the liver injury model.

The LPS Activated the P2X7R/NLRP3 Signaling Pathway in Macrophages

We have confirmed that the LPS promoted P2X7R expression induces hepatocyte pyroptosis and macrophage activation in vivo. Next, we want to verify the mechanism of the in vitro experiments. We first induced macrophage line RAW264.7 with LPS and added P2X7R inhibitor and P2X7R agonist to study the LPS effect on P2X7R/NLRP3 signaling pathway in macrophages. As shown in Figures 3A and 3B, NLRP3, ASC, P2X7R, and Caspase-1 expressions in macrophages were measured by qRT-PCR and WB. NLRP3, ASC, P2X7R, and Caspase-1 expressions in the BZATP group promoted compared with the LPS group, while NLRP3, ASC, P2X7R, and Caspase-1 expression in the A438079 group down-regulated, but the protein expressions in the treatment group were higher than in the control group. This indicated the LPS may activate the P2X7R/NLRP3 signaling pathway, and the LPS-induced hepatocyte pyroptosis may be associated with this pathway activation.

Activation of P2X7R/NLRP3 Signaling Pathway Activated Macrophages

We have previously verified that the LPS

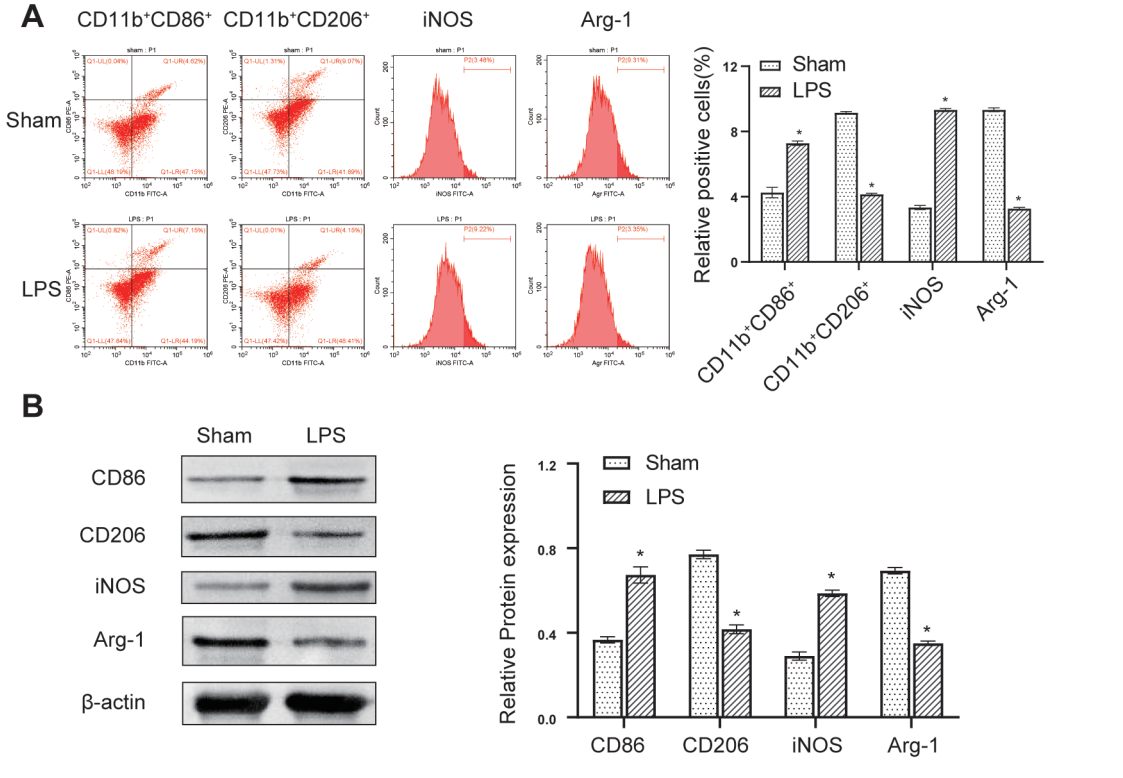

Figure 2. The LPS induced macrophage activation in liver injury model. (A) Flow cytometry detected a double positive ratio of $CD11b^+CD86^+$, $CD11b^+CD206^+$, expression of iNOS, , and Arg-1. (B) CD86, CD206, iNOS and Arg-1 expressions were examined by WB. *P<0.05 vs sham group.

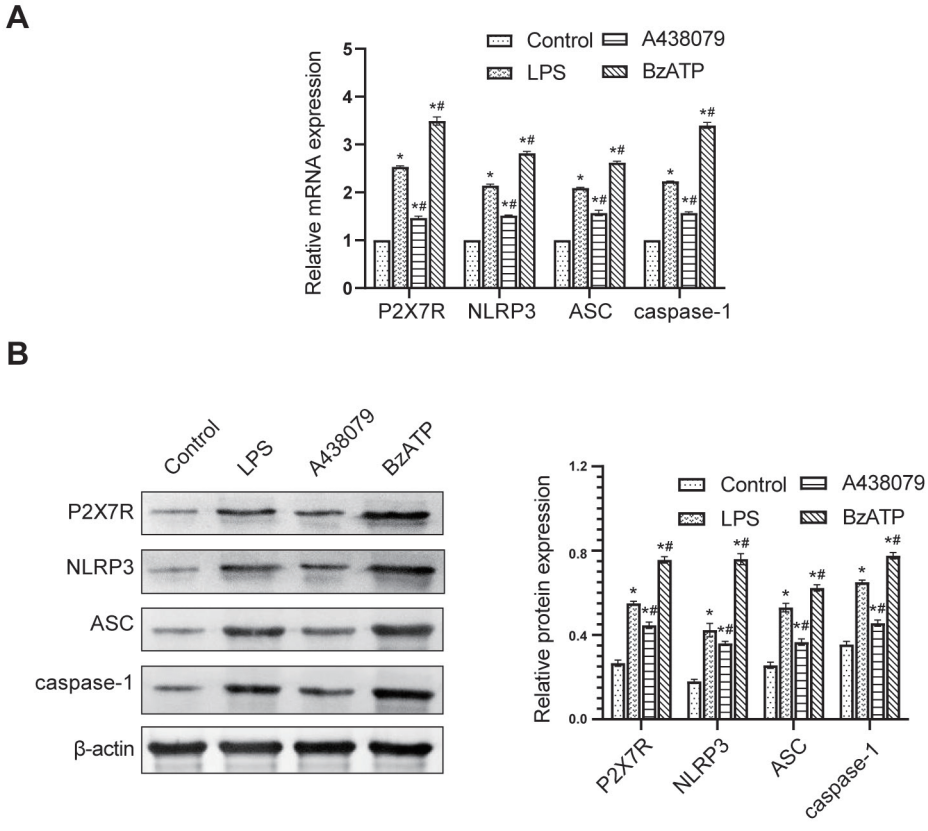

Figure 3. The LPS activated P2X7R/NLRP3 signaling pathway in macrophages. (A) P2X7R, NLRP3, ASC, and caspase-1 levels in macrophages of the control, LPS, A438079 and the BZATP group were tested by qRT-PCR. (B) WB detected ASC, P2X7R, NLRP3, and caspase-1 expressions in macrophages. *P<0.05 vs the control group; # P<0.05 vs the LPS group.

could activate the P2X7R/NLRP3 pathway in macrophages. To further explore the activating P2X7R/NLRP3 signaling pathway effect on macrophages, we first detected IL-1β and IL-18 expressions in the LPS group, the A438079 group, and the BZATP group by the ELISA. Compared with the LPS group, IL-1β and IL-18 expressions in the BZATP group promoted, while the IL-1β and IL-18 expressions in the A438079 group down-regulated, indicating the P2X7R expression in macrophages could promote inflammation (Figure 4A). Flow cytometry and WB detected CD86, CD206, iNOS, and Arg-1 expressions and found that compared with the LPS group, CD86 and iNOS expressions in BzATP group promoted, but CD206 and Arg-1 expressions down-regulated. CD86 and iNOS expressions down-regulated, but CD206 and Arg-1 expressions down-regulated in the A438079 group (Figure 4B and 4C). The above results indicated that P2X7R expression in macrophages could promote inflammation, and the LPS may promote macrophages polarization into M1 type via P2X7R/NLRP3 pathway, leading to the inflammatory response.

The LPS Activated Macrophages Via P2X7R/NLRP3 Pathway to Induce Hepatocyte Pyroptosis

To investigate the effect of the LPS activation of macrophages via the P2X7R/NLRP3 signaling pathway on hepatocytes, we co-cultured RAW264.7 with AML-12. Flow cytometry was applied to measure the AML-12 cells pyroptosis in the control group, the LPS group, the A438079 group, the BZATP group, and the blank group. As shown in Figure 5A, the AML-12 cells pyroptosis in the BZATP group promoted compared with the LPS group, but AML-12 cells pyroptosis

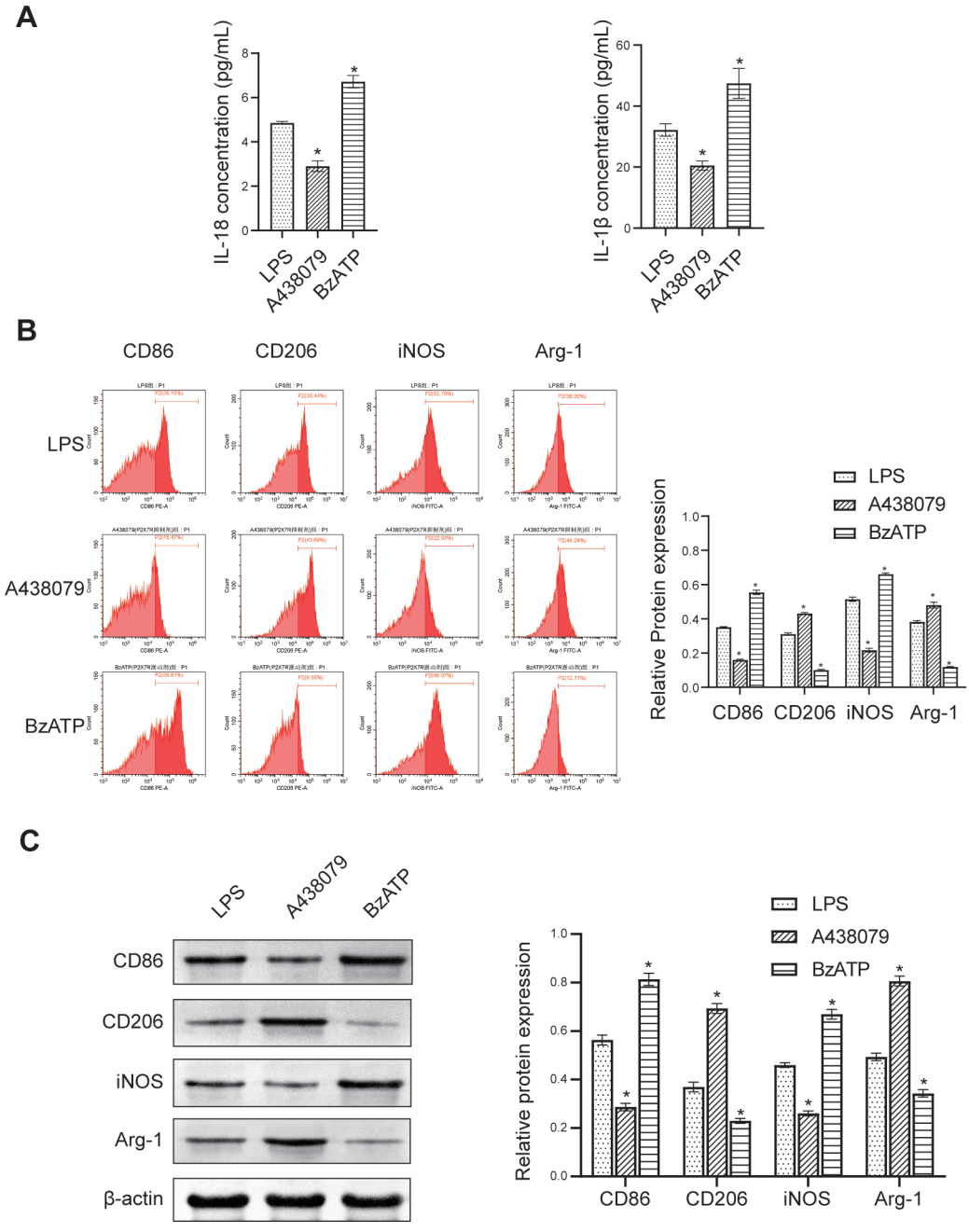

Figure 4. The Activation of P2X7R/NLRP3 signaling pathway activated macrophages. (A) The IL-18 and IL-1β expressions were examined by the ELISA in the LPS, A438079, and BZATP groups. (B) and (C) CD86, CD206, iNOS and Arg-1 expressions were detected by flow cytometry and WB respectively. *P<0.05 vs the LPS group.

in the A438079 group down-regulated, but both were higher than in the control and the blank groups. The CCK8 revealed compared with the LPS group, AML-12 proliferation ability in the BZATP group decreased, while the proliferation ability of AML-12 in the A438079 group increased, but both were lower than the control and the blank groups (Figure 5B). As shown in Figures 5C and 5D, compared with the LPS group, GSDMD and GSDMD-N expressions in the BZATP group promoted, while GSDMD and GSDMD-N expressions in the A438079 group down-regulated, but both were higher than in the control and the blank groups. These results suggested the P2X7R/NLRP3 signaling pathway promoted macrophages polarization into M1 type during the LPS-

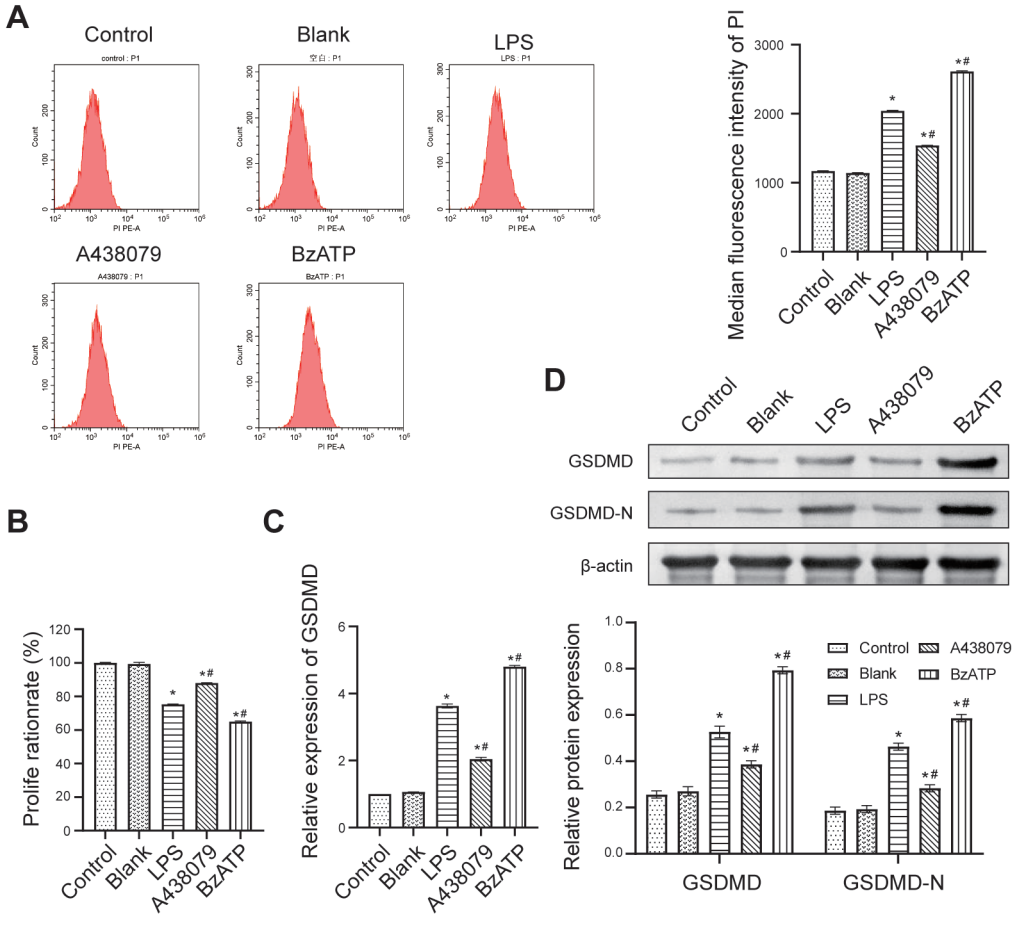

Figure 5. The LPS activated macrophages through P2X7R/NLRP3 signaling pathway to induce hepatocyte pyroptosis. (A) Flow cytometry detected the pyroptosis of AML-12 in the control, the LPS, A438079, BzATP, and the blank group. (b) CCK8 detected the proliferation of AML-12 cells. (c) GSDMD mRNA expression in AML-12. (d) WB detected GSDMD and GSDMD-N expressions in AML-12 cells. *P<0.05 vs the control and the blank group; # P<0.05 vs the LPS group.

induced hepatocyte pyroptosis, and the LPS-induced hepatocyte pyroptosis may be related to the polarization of macrophages.

DISCUSSION

Cell pyroptosis is an inflammatory process of caspase-1-dependent PCD (23). However, few cases are studied concerning the cell pyro ptosis's role in acute liver injury. We have clarified three key findings in this paper. First, the LPS promoted P2X7R expression and induced hepatocyte pyroptosis, which can induce macrophage activation in the liver injury model. Second, the LPS activated the P2X7R/NLRP3 signaling pathway in macrophages, thereby activating macrophages. Third, the LPS activated macrophages through the P2X7R/NLRP3 signaling pathway to induce hepatocyte pyroptosis.

Many reports have shown that the LPS could induce a variety of organ damages. For example, liver injury (4-6), pulmonary inflammation (24), kidney injury (25), and myocarditis (26). The LPS could cause severe endothelial cell pyroptosis through the Caspase-11 pathway (26). It has also been reported that the NLRP1 and the NLRP3 inflammasomes mediated the cell pyroptosis of the LPS/ ATP-induced knee arthritis (27). GSDMD was a pyroptosis execution protein, which could be cleaved by

inflammatory body activated caspase-1 and the LPS activated caspase-11/4/5 (28). It could induce cell pyroptosis via forming membrane pores and increasing pro-inflammatory mediators secretion (29). We induced acute liver injury with the LPS and found that the LPS increased inflammatory cytokines IL-1β and IL-18 expression, increased P2X7R, GSDMD , and GSDMD-N levels in the liver, and also increased ALT, AST , and TBIL concentrations related to liver cell injury. Flow cytometry and WB further confirmed that the LPS could induce macrophage activation in the liver injury model. These results suggest that the LPS induces hepatocyte pyroptosis and promotes acute liver injury development.

More and more pieces of evidence have shown that the NLRP3 inflammasome activation was an important driver of various acute and chronic liver diseases (29, 30). In the process of cell pyroptosis, caspase-1 was synthesized into an inactive proenzyme, which was activated by inflammasomes and led to pre-IL-1β, and IL-18 maturation (23). IL-1β was the potent inflammatory cytokine mainly produced by macrophages and was the most widely recognized downstream mediator of the NLRP3 inflammasome (31). Menzel et al. found that caspase-1 alleviated liver injury and inflammation (32). Zhao et al. found that the NLRP1 and the NLRP3 activated in the process of pyroptosis could promote IL-1β secretion (33). These findings led us in our current study to investigate whether or not specific NLRP3 activation in hepatocytes led to cell pyroptosis, downstream signaling pathways, and the potential impact of this process on liver injury. In vitro cell model, we measured ASC, P2X7R, and NLRP3, caspase-1, IL-1β and IL-18 levels. We observed these indicators expression promoted in acute liver injury induced by the LPS, especially after adding P2X7R agonist. We found that the LPS may activate the P2X7R/NLRP3 signaling pathway, and the LPS induced hepatocyte pyroptosis might be associated with this pathway activation. Flow cytometry and WB further suggested that the LPS might promote macrophages polarization into M1 type through the P2X7R/NLRP3 signaling pathway, leading to the inflammatory response.

Pyroptosis mainly occurred in Kupffer cells or renal macrophages (34, 35). M1-type macrophages are the main source of pro-inflammatory cytokines (36). The pro-inflammatory response of macrophages was related to the pathophysiology of acute liver injury (37). The LPS could increase liver inflammation in mice by the polarization of macrophages into M1 type (4). We hypothesized that the LPS-induced hepatocyte pyroptosis might be related to macrophage polarization. In this study, macrophage RAW264.7 was co-cultured with hepatocyte AML-12 to investigate the effect of the LPS activation of macrophages via the P2X7R/NLRP2 signaling pathway on hepatocytes. Flow cytometry and CCK8 confirmed that the LPS induction promoted pyroptosis of AML-12 hepatocytes and inhibited the proliferation of hepatocytes. The effect was more obvious when the P2X7R agonist was added. We detected GSDMD and GSDMD-N expressions and found proteins expressions promoted in the LPS-induced acute liver injury, especially after adding the P2X7R agonist. However, the P2X7R inhibitor significantly reduced these proteins expression. Our results showed that in the process of the LPS-induced hepatocyte pyroptosis, the P2X7R/NLRP3 signaling pathway promoted macrophages polarization into M1 type, and blocked the P2X7R/NLRP3 signaling pathway to inhibit tissue or cell damage.

CONCLUSION

In conclusion, our results suggested that the LPS could activate macrophages via P2X7R activation of the NLRP3 and induce hepatocyte pyroptosis in mice. Our study provided a theoretical basis for acute liver injury pathogenesis and helped to enrich new therapeutic strategies for acute liver injury.

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Conflicts of Interest: None declared.

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