Journal of Gastroenterology and Hepatology

JGHF

doi:10.1111/jgh.15493

HEPATOLOGY

Menstrual blood-derived mesenchymal stem cells attenuate inflammation and improve the mortality of acute liver failure combining with A2AR agonist in mice

Dazhi Chen,* Ruichao Zeng,[†,‡,§] Guigen Teng,* Chao Cai,[¶] Tongtong Pan,[¶] Hanxiao Tu,[¶] Hongwei Lin,[¶] Qingjing Du,[¶] Huahong Wang* and Yongping Chen[¶]

*Department of Gastroenterology, Peking University First Hospital, Beijing, [†]Department of Oncological Surgery, The First Affiliated Hospital of Wenzhou Medical University, [¶]Department of Infectious Diseases, Wenzhou Key Laboratory of Hepatology, The First Affiliated Hospital of Wenzhou Medical University, Hepatology Institute of Wenzhou Medical University, Wenzhou, China; [‡]Precision and Systems Biomedicine, QIMR Berghofer Medical Research Institute, Brisbane[§]Faculty of Medicine, The University of Queensland, Woolloongabba, Queensland, Australia

Key words
Acute liver failure, Inflammation, Menstrual blood-derived mesenchymal stem cells.

Accepted for publication 8 March 2021.

Correspondence
Huahong Wang, Department of Gastroenterology, Peking University First Hospital, Beijing, China.
Email: wwwanghuahong@163.com

Yongping Chen, Department of Infectious Diseases, Wenzhou Key Laboratory of Hepatology, The First Affiliated Hospital of Wenzhou Medical University, Hepatology Institute of Wenzhou Medical University, Wenzhou, China.
Email: 13505777281@163.com

Dazhi Chen and Ruichao Zeng contributed equally to this work.

Declaration of conflict of interest: The authors declare that they have no conflict of interest.
Financial support: This study is supported by the Scientific Research Seed Fund of Peking University First Hospital (BMU2020PYB005), Major Program of Natural Science Foundation of Zhejiang Province (LD21H030002) and Major Scientific and Technological Special Project in the Thirteen Five-year Plan, China (2017ZX10203201-002-003).

Abstract

Background and Aim: Acute liver failure (ALF) poses a serious public health issue. The menstrual blood-derived mesenchymal stem cells (MenSCs) have been applied to cure various liver-related diseases. However, the efficacy and mechanism are far from clear. This study aims to explore the efficacy and potential mechanism of MenSCs to cure ALF.
Methods: We investigate the potential mechanism of MenSCs on the ALF *in vitro* and *in vivo*. A2A adenosine receptor (A2AR) activation was investigated as the potential reinforcer for MenSCs treatment. Lipid polysaccharide/D-galactosamine (D-GalN) was employed to induce ALF. Diverse techniques were used to measure the inflammatory cytokines and key signaling molecules. Hematoxylin–eosin stain and aminotransaminases were applied to evaluate the liver injury. Flow cytometry was employed to assess the T cells.
Results: The MenSCs can decrease the lipid polysaccharide-induced inflammatory cytokine elevation and related signaling molecules in ALF, including TLR4, phosphorylated-NF-kBp65 (p-NF-kBp65), PI3K, and p-AKT, p-mTOR and p-IKK *in vitro*. Moreover, MenSCs also can significantly reverse the liver injury, inflammatory cytokines elevation and related signaling molecules increase, and Treg/Th17 ratio decrease *in vivo*. In addition, MenSCs plus A2AR agonist can enhance the above changes.
Conclusions: The MenSCs can attenuate the ALF-induced liver injury via inhibition of TLR4-mediated PI3K/Akt/mTOR/IKK signaling. Then, this inhibits the p-NF-κBp65 translocate into nuclear, which causes a decrease of inflammatory cytokines release. Moreover, A2AR agonist can play a synergic role with MenSCs and enhance the above-mentioned effects.

Introduction

Acute liver failure (ALF) was one of the severe liver diseases featuring coagulopathy, progressive jaundice and hepatic encephalopathy, which has a rapid progression and high mortality.[1–3] However, if early diagnosis and reasonable interventions can be provided, the prognosis can be significantly improved. For example, the mortality rate of ALF was as high as 80%, whereas the 2-year survival rate of patients receiving liver transplantation was significantly improved, reaching 92.4%.[4,5] However, transplantation is difficult to be widely used due to severe shortage of donor liver, high medical costs, and rapid disease progression.[6,7] Thus, it is important to find other effective alternatives.

The activation of the immune system and its cascade-like response to inflammation may play a key role in the progression of ALF, and macrophages are one of the important ingredients of this progress.[8] The liver has the largest population of macrophages in the body, Kupffer cells. In acute liver injury, inflammation activates Kupffer cells in the liver, which results in the release of reactive oxygen species (ROS) and the secretion of a series of cytokines. These cytokines can recruit other potentially cytotoxic inflammatory cells.

Throughout the process, macrophages play an important role in the liver injury. When ALF occurs, intestinal and liver axis disorders, intestinal mucosal damage, and intestinal flora imbalance may lead to immune imbalance and endotoxemia in patients. The main component of endotoxin is lipopolysaccharide (LPS).[9] The LPS can travel to liver and activate Toll-like receptor 4 (TLR4), which actives the downstream intracellular NF-κB signaling, thereby releasing a large number of inflammatory cytokines.[10,11]

So far, there are numerous studies indicating the therapeutic effect of mesenchymal stem cells (MSCs) on liver diseases, including ALF.[12] For example, MSCs may increase expression of heme oxygenase-1 to play anti-inflammatory effect in treatment of ALF.[13] In addition, the human umbilical cord MSCs-derived exosomes are found to ameliorate IL-6-induced ALF through miR-455-3p.[14] Menstrual blood-derived stem cells (MenSCs) were first extracted and identified from human menstrual blood by Cui *et al.*[15] Studies have found that it has characteristics similar to other MSCs, such as multi-lineage differentiation ability. Compared with other tissue-derived MSCs, MenSCs have higher proliferation capacity.[16,17] In addition, MenSCs are obviously easier to access compared with other MSCs, such as bone marrow MSCs (BMMSCs). Thus, it received increasingly attention in the treatment of various diseases, including ALF. For example, exosomes derived from MenSCs is found to alleviate the fulminant hepatic failure.[18] However, the efficacy and mechanism of MenSCs in treatment of ALF is still largely unclear. On the other hand, the effects of MSCs transplantation are often unsatisfactory due to complicated *in vivo* environment. Activation of A2A adenosine receptor (A2AR) was found to play an anti-inflammatory and immune regulation role in diverse of diseases.[19,20] Moreover, A2AR activation was found to enhance the curative effect of BMMSCs transplantation on hepatic fibrosis.[21] Therefore, the present study explores the efficacy and potential mechanism of MenSCs to treat ALF. In addition, the potential role of A2AR on improving the therapeutic effect of MenSCs is also investigated.

Materials and methods

Cell culture. RAW264.7 was purchased from the Shanghai Institute of Cell Sciences, the Chinese Academy of Sciences. It was cultured in DMEM (Irvine Scientific) with a mixture of 1% penicillin and streptomycin, and 10% FBS (Irvin Scientific). MenSCs were donated from Xiang's laboratory.[22] Cells from two to eight passages were cultured in Chang's cell complete medium (Irvin Scientific).

Co-culture model of MenSCs and RAW264.7 cells in vitro. We applied a Transwell insert (diameter: 24 mm, filter pore size: 0.4 um, Corning) to construct co-culture model. The insert was placed in six-well plates, and then RAW264.7 cells were seeded in the lower well of chamber with the number of 1×10^6. Then, MenSCs were placed on the upper layer of the chamber, and the number was about 1×10^5. The model was incubated in 37°C with 5% CO_2 in complete medium. We set four different groups: RAW264.7 (control), RAW264.7 + 100 ng/mL LPS (LPS group), MenSCs + RAW264.7 + 100 ng/mL LPS (LPS + MenSCs group), and TAK242 100 ng/mL (TLR4 inhibitor) + RAW264.7 + 100 ng/mL LPS group (LPS + TLR4 inhibitor group). Except for the control group, the other three groups were all added with LPS in the lower wells and incubated for 24 h. For LPS + TLR4 inhibitor group, RAW264.7 cells were pretreated with TLR4 inhibitor (Merck) for 30 min. After 24 h of incubation, the supernatant in the lower well was collected, centrifuged at 5000*g* for 3 min at 4°C to remove the insoluble material, and rest was stored at −80°C until use. The experiments were from duplicate wells for each experimental group and repeated three times.

Reverse transcriptase semi-quantitative polymerase chain reaction for mRNA measurement. Cell pellet was collected and total RNA was extracted using the traditional Trizol (Irvin Scientific) method. Reverse transcription was performed using the iScript Reverse Transcription Kit (Bio-Rad, USA) according to the instructions, and then submitted to subsequent semi-quantitative polymerase chain reaction.

Primers were designed using the BLAST tool and purchased from Invitrogen (Shanghai, China) (Table S1) [Correction added on 15 April 2021, after first online publication: Table 1 citation has been corrected to Table S1]. For semi-quantitative polymerase chain reaction, SYBR Green Supermix kit (Bio-Rad, USA) was used. GAPDH was used as an internal reference. The polymerase chain reaction was carried out with the condition as following: 95°C for 30 s as initial denaturing follow by 35 cycles of denaturing at 95°C for 15 s, annealing at 60°C for 60 s, and elongating at 72°C for 2 min. After the reaction was completed, the cycle threshold (CT) values of the internal reference GAPDH and the target gene were recorded, and the relative mRNA expression was calculated using the $2^{-\Delta\Delta}$ CT method.

Enzyme-linked immunoassay for the detection of IL-6, IL-1β, and TNF-α. The concentrations of cytokines IL-6, IL-1β, and TNF-α were determined by an enzyme-linked immunoassay kit (BD Biosciences, San Diego, CA, USA) with antibodies against IL-6, IL-1β, and TNF-α, respectively, according to the manufacturer's instructions.

Protein extraction from cells and tissues. Cells were collected in a microfuge tube and were extracted with RIPA buffer (Promega) following the standard protocols. Cell lysate was stored in a refrigerator at −80°C until use. Tissue protein extraction is similar to cell protein extract except tissue was grinded in lysate.

For nuclei protein extraction, after cell pellet or grinded tissue were mixed with at least two times volume of cell lysate (10-mM Tris pH 7.5, 25-mM NaF, 1-mM EDTA, 1× protein inhibitor, 0.5-mM AEBSF). Place the tube in the ice for 20 min, and then the crude nuclei were isolated by passing through a 23G injection needle accompanied with centrifugation. Subsequently, the crude cell nucleus was precipitated by sucrose method, and then the precipitation was resuspended in another lysate (20% Glycerol, 20-mM Hepes, pH 7.9, 420-mM NaCl, 1.5-mM MgCl2, 0.2-mM EDTA) with centrifugation. The supernatant containing nucleoprotein was carefully collected. After concentration measurement by BCA kit (Pierce Company), the protein was stored at −80°C until use.

Western blot detection of TLR4 and related proteins. The standard protocols were employed, and we used different primary antibodies against different proteins: anti-TLR4

polyclonal antibody (Abcam, UK), anti-Akt monoclonal antibody (Thermo Fisher, USA), anti-phospho-Akt monoclonal antibody, anti-phospho-NF-κB p65 monoclonal antibody, anti-IKK polyclonal antibody, anti-phospho-IKK monoclonal antibody, anti-mTOR polyclonal antibody, anti-phospho-mTOR monoclonal antibody, anti-PI3K monoclonal antibody, anti-histone H3 monoclonal antibody, and anti-GAPDH monoclonal antibody (USA CST company). The bands were exposed to films, and the quantification and analysis of the bands were performed using the Quantity One image analysis system.

Establishment ALF mouse model with different interventions. Male C57B/6 mice weighing 18.0–22.0 g about 6–8 weeks old were purchased from Shanghai Experimental Animal Centre (Shanghai). All mice were housed in a temperature-controlled and humidity-controlled room under specific-pathogen-free (SPF) conditions. Animal experiments were approved by the Ethics Committee of Zhejiang University.

Eighty-four C57BL/6 mice were randomly divided into six groups: control group, ALF model group, MenSCs transplantation group, TLR4 inhibitor group, MenSCs transplantation + A2AR agonist (CGS21680) group, and A2AR agonist (CGS21680) group. There were 14 mice in each group, of which eight were used to establish survival curves. Except mice in the control group that were treated with phosphate-buffered saline (PBS), mice in other groups were intraperitoneally injected with 800 mg/kg D-galactosamine (D-GalN) and 50 μg/kg LPS to induce ALF model. The other interventions were treated according to different groups. Specifically, TLR4 groups accepted 3 mg/kg TLR4 inhibitor injection 1 h before induction of ALF; 2 mg/kg A2AR agonist, CGS21680 (R&D Systems), was injected 1 h after induction of ALF for A2AR agonist alone and A2AR agonist plus MenSCs transplantation groups; and 300 μl of a 1.0×10^7/ml of freshly isolated MenSCs cell suspension was injected 1 h after induction of ALF for all MenSCs-related groups (Table 1). All treatments were through tail vein.

After the sacrifice, blood was drawn from the sub-hepatic vena cava. Serum was extracted from blood and was stored at −80°C until use. Moreover, a portion of the liver tissue samples was immediately placed in liquid nitrogen for proteins and RNA extraction; another part of the liver tissue was used for hematoxylin–eosin staining.

Liver histopathological hematoxylin–eosin staining and quantitative determination of ALT and AST in serum. According to standard protocols, liver sections were stained with hematoxylin and eosin solutions and mounted.[23] Then placed under a light microscope to observe histological changes. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) kits (Nanjing Jiancheng Biotechnology Research Institute) were used to quantify serum ALT and AST levels according to the manufacturer's instructions. The amounts of ALT and AST were determined according to a standard curve.

Flow cytometry. The fresh spleen was removed from mice and spleen lymphocytes were separated with Lymphocyte Separation Medium Kit (TBDscience, Tianjin, China). After obtaining spleen-derived lymphocytes, Treg and Th17 cells were analyzed by flow cytometry. To analyze Treg cells, the isolated lymphocytes were incubated with anti-mouse CD4 FITC (Thermo Fisher Scientific) and CD25 APC (Thermo Fisher Scientific) antibodies, and then stained with anti-mouse Foxp3 PE antibody (Thermo Fisher Scientific). To analyze Th17 cells, the isolated lymphocytes were first incubated with anti-mouse CD4 FITC and then incubated with anti-mouse IL-17 PE (Thermo Fisher Scientific). Cells were analyzed using a BD FACS Calibur flow cytometer (BD Bioscience, CA, USA). The percentage of cells was analyzed by FLOWJO software (USA).

Statistical analysis. All experimental data were expressed as mean ± standard deviation. Continues data were compared by independent Student *t* test. The comparisons between multiple groups were performed by one-way ANOVA, and pairwise comparisons between groups were run by Tukey HSD method with SPSS 19.0. Survival analysis was calculated by Kaplan–Meier survival curve with log–rank test. $P < 0.05$ was considered statistically significant. Graphs were drawn using GRAPHPAD Prism (version 5.0 for Windows).

Results

MenSCs attenuated LPS-induced inflammatory cytokines in RAW264.7 cells. As shown in Figure S1, LPS induced a significant increase of IL-6, IL-1β, and TNF-α at both protein and mRNA levels in LPS-treated RAW264.7 cells compared with negative control. MenSCs co-culture significantly attenuated LPS-induced these elevations. In the other hand, when LPS was added into TLR4 inhibitor pretreated RAW264.7 cells, the increase of inflammatory cytokines was also significantly inhibited to some extent.

Table 1 The interventions for various groups

Group	Intervention*			
Control group	PBS	PBS	PBS	PBS
Model group	PBS	LPS/D-GalN	PBS	PBS
MenSCs group	PBS	LPS/D-GalN	PBS	MenSCs transplantation
TLR4 inhibition	TAK242	LPS/D-GalN	PBS	PBS
MenSCs + A2AR agonist group	PBS	LPS/D-GalN	CGS21680	MenSCs transplantation
A2AR agonist group	PBS	LPS/D-GalN	CGS21680	PBS

Note: *All the intervention was added sequentially and PBS was applied as negatives.

MenSCs reduced LPS-induced TLR4 mediated PI3K/Akt/mTOR/IKK expression in RAW264.7 cells. The result indicated that TLR4 and nuclear phosphorylated NF-κBp65 (p-NF-κBp65) significantly increased in LPS-induced inflammatory status cells (Fig. 1). MenSCs significantly reduced LPS-induced elevation of TLR4 and nuclear p-NF-κBp65. Similar results were observed in pretreatment of TLR4 inhibitor. Then, as shown in Figure 2, LPS treatment significantly increased PI3K and phosphorylated Akt (p-Akt), p-mTOR and p-IKK proteins, whereas those proteins were significantly decreased in the MenSCs co-culture group, and similar results were observed in the TLR4 inhibitor pretreatment group.

The effect of MenSCs on survival and liver injury in ALF mouse model. The Kaplan–Meier plot indicated a significant difference of overall survival rate among those six groups (Fig. S2, $P < 0.001$). The survival analysis showed mice in ALF

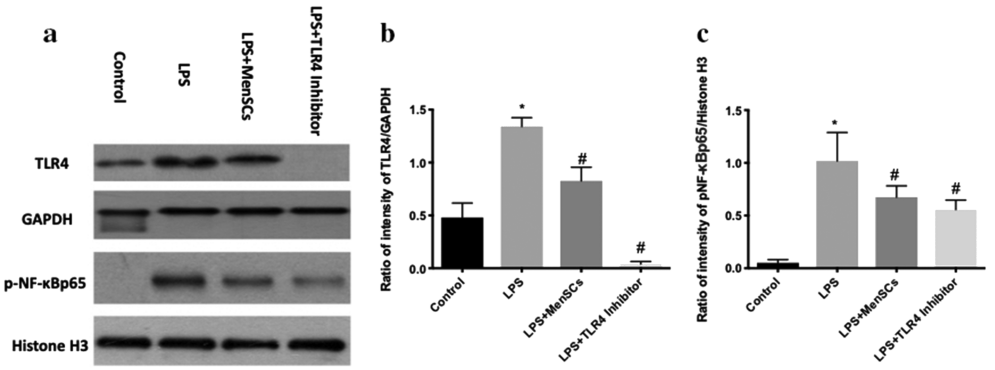

Figure 1 Effect of menstrual blood-derived mesenchymal stem cells (MenSCs) on TLR4 and p-NF-κBp65 protein expression in RAW264.7 cells. (a) Typical image of Western blot. (b) Relative intensity of TLR4 protein. (c) Relative intensity of p-NF-kB-p65 protein. Data are expressed as mean ± SD. * Indicates $P < 0.05$ between the control group and the lipopolysaccharide (LPS) model group; # indicates $P < 0.05$ between the LPS model group and the other treatment groups.

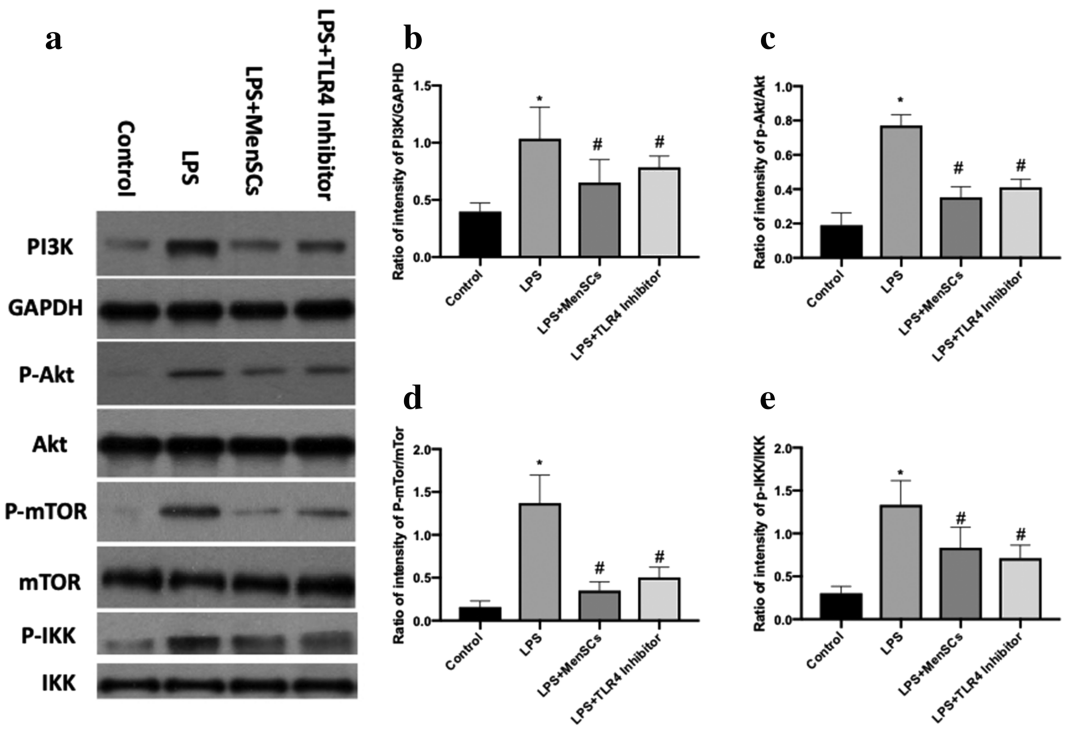

Figure 2 Effect of menstrual blood-derived mesenchymal stem cells (MenSCs) on PI3K/Akt/mTOR/IKK signaling molecules in RAW264.7 cells after treatment of lipopolysaccharide (LPS). (a) Typical graph of the results of Western blot. (b–e) Relative intensity of PI3K, pAKT, pmTOR, and pIKK protein, respectively. Data are expressed as mean ± SD. * Indicates $P < 0.05$ between the control group and the model group; # indicates $P < 0.05$ between the LPS model group and the other treatment groups.

model were died after 7 h. With MenSCs treatment, 20% of mice were alive 16 h after ALF establishment. Moreover, there were 50% of mice still alive after transplantation of MenSCs and injection of A2AR agonist, while with injection of A2AR agonist alone, only 10% mice survived after 16 h with injection of LPS/D-GalN.

Liver injury level was shown in Figure 3. There was a significant destruction of liver lobule of mice in all groups except control. However, destruction of liver lobule in MenSCs plus A2AR agonist group was minimal (Fig. 3a–f). Serum ALT and AST levels were significantly elevated after injection of LPS/D-GalN compared with the control. MenSCs transplantation alone, TLR4 inhibitor, MenSCs plus A2AR agonist, and A2AR agonist alone all significantly attenuated ALT and AST levels to various levels. Moreover, the most significant reduction of ALT and AST was observed in mice of MenSCs plus A2AR agonist group (Fig. 3h,i).

The effect of MenSCs on inflammatory cytokines and TLR4 mediated PI3K/Akt/mTOR/IKK pathway in ALF model. As shown in Figure 4, the serum and mRNA expression of IL-6, IL-1β, and TNF-α were significantly increased after injection of LPS/D-GalN compared with that in control. All the groups with different interventions significantly decreased these changes in ALF model, while the most significant reduction was observed in the treatment of MenSCs plus A2AR agonist. Moreover, elevation of TLR4 and nuclear p-NF-kB-p65 were observed in the ALF mice (Fig. 5a,b), and significant reduction in various levels were observed in all other intervention groups. The most significant changes were identified in MenSCs plus A2AR agonist treatment group. In the meantime, TLR4 mediated pathway-related proteins, including PI3K, p-Akt, p-mTOR, and p-IKK were also significantly elevated in the ALF model, whereas all treated groups could attenuate those changes with the most

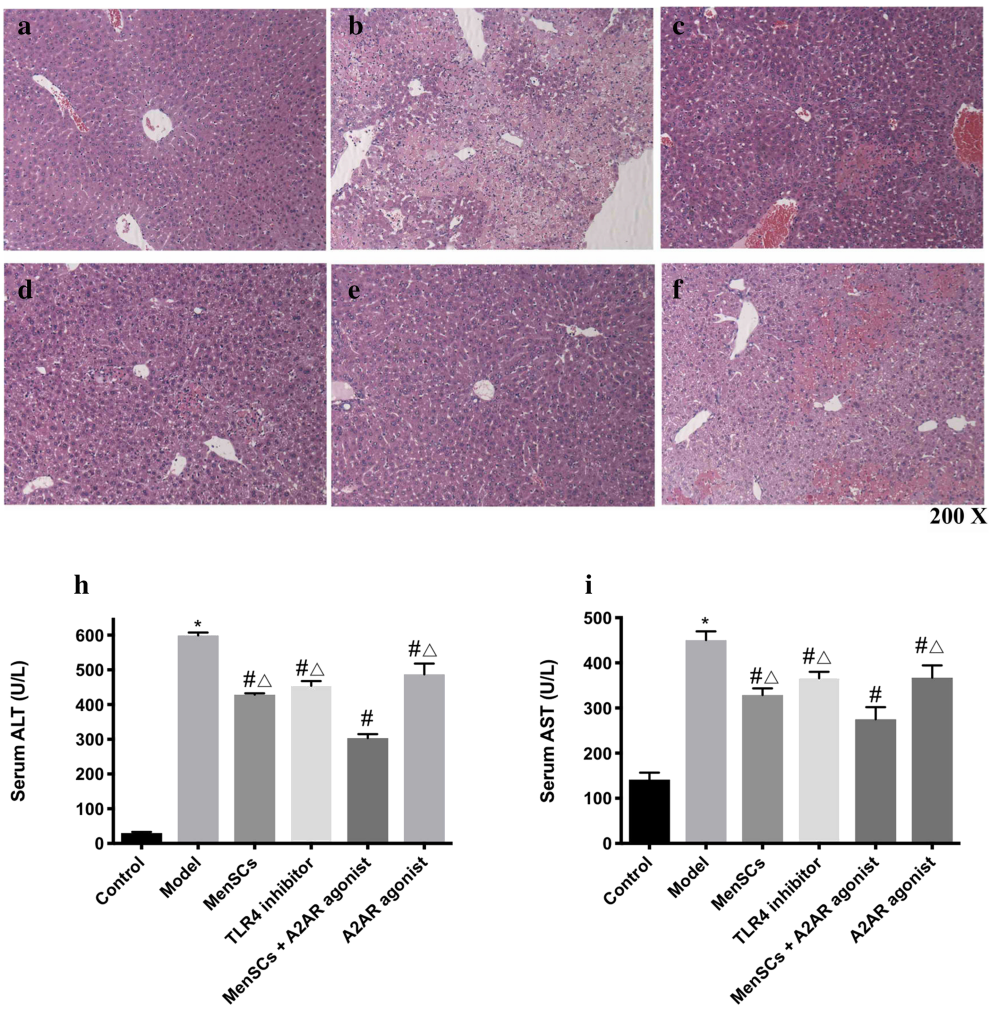

Figure 3 Histological evaluation of liver sections by hematoxylin and eosin staining and liver transaminase expression in serum in acute liver failure (ALF) mice model. (a–f) Liver histological image from the control group, model group, menstrual blood-derived mesenchymal stem cells (MenSCs) group, TLR4 inhibitor group, MenSCs + A2AR agonist group, and A2AR agonist alone group, respectively. (h,i) Serum expression of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in different groups. Data are expressed as mean ± SD. * Indicates $P < 0.05$ between control group and ALF model group; # indicates $P < 0.05$ between ALF model group and other treatment groups; △ indicates $P < 0.05$ between MenSCs + A2AR agonist group and other groups.

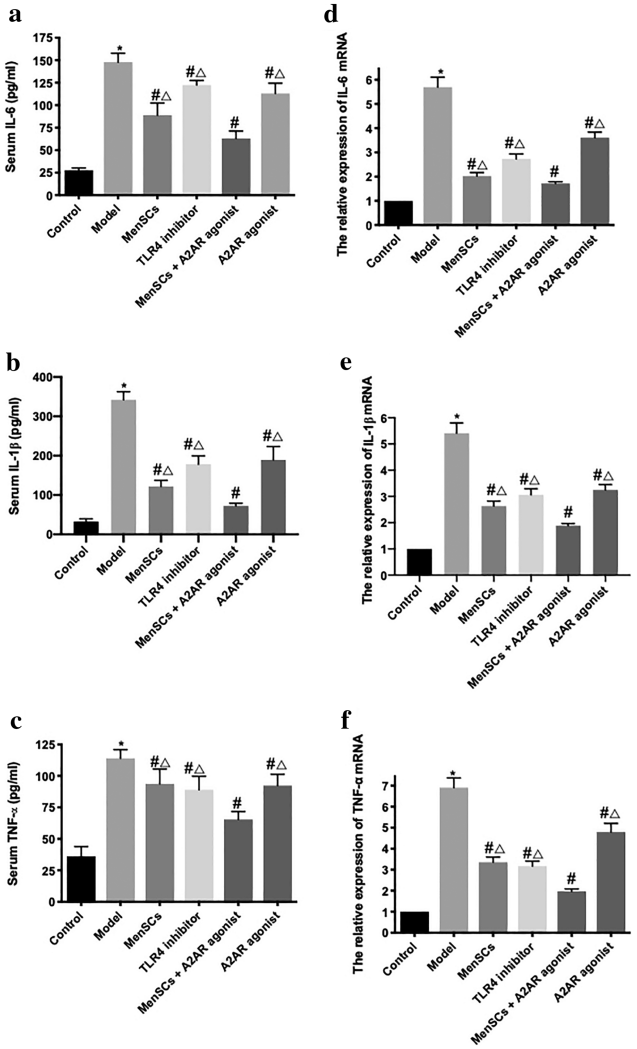

Figure 4 Serum IL-6, IL-1β, and TNF-α and corresponding mRNA expression in various mice groups. (a,d) IL-6 in serum and mRNA, respectively. (b,e) The amount of IL-1B in serum and mRNA, respectively. (c,f) The levels of TNF-α in serum and mRNA, respectively. Data are expressed as mean ± SD. $^{*}P < 0.05$ between control group and acute liver failure (ALF) model group; $\#P < 0.05$ between ALF model group and other treatment groups; $^{\triangle}$ $P < 0.05$ between menstrual blood-derived mesenchymal stem cells (MenSCs) + A2AR agonist group and other groups.

significance observed in MenSCs plus A2AR agonist group (Fig. 5c,d).

The effect of MenSCs on the Treg and Th17 cells.

The spleen was isolated from the mice in different treatment groups to further analyze immune system involvement in these treatments. There was a significant decrease in Treg cells but an increase in Th17 cells in the ALF model group (Fig. S3). All the treatments groups respectively significantly increased Treg cells and significantly decreased Th17 cells. Among these interventions, the treatment of MenSCs and A2AR agonist had the most significant regulatory effect.

Discussion

With *in vitro* assays, we found that MenSCs co-culture with RAW264.7 cells significantly attenuate the increases in inflammatory cytokines, which is consistent with other investigations.[24] Our *in vitro* findings indicated that MenSCs may attenuate the LPS-induced inflammation via regulation of TLR4 mediated PI3K/Akt/mTOR/IKK pathway. A previous study has found that BMMSCs can inhibit the TLR4/NF-κB pathway and then attenuate the inflammatory effects on human umbilical vein endothelial cells and alveolar macrophages.[25] Another study demonstrated that MenSC-derived exosome may alleviate the ALF through anti-apoptotic effect, which partially supported our findings from another perspective.[18]

With establishment of the promising underlying mechanisms of MenSC-induced improvement of ALF *in vitro*, we further established an ALF mouse model to explore whether the above mechanism available *in vivo*. Previous studies have indicated that the MSCs treatment is often unsatisfactory due to internal environmental disorders, inflammatory reactions, and endotoxemia.[26] Therefore, how to improve the curative effect of MSCs transplantation has become another hot issue for liver diseases treatment. A study indicated that a combination of A2AR agonist and BMMSCs can enhance the therapeutic effect of MSCs transplantation on hepatic fibrosis.[21] A2AR is expressed on a variety of cell surfaces, including liver cells, macrophages, and other ALF-related immune cells. Activation of A2AR plays an important role in anti-inflammatory and immune regulation in a variety of diseases.[19,20] Therefore, in the current study, an A2AR agonist and MenSCs were simultaneously as well as alone employed for the treatment of ALF. The results showed that mice received combination of MenSCs, and A2AR agonist revealed the best outcomes compared with others, such as liver injury and OS. Therefore, A2AR agonist may enhance the curative effect of MenSCs transplantation on ALF.

The activation of the immune system and its cascade-like response to inflammation may play a key role in the ALF progression, and the liver damage caused by mainly T lymphocytes.[27] Specifically, Th17 cells can express corresponding pro-inflammatory factors such as IL-17, IL-6, and TNF-α and play an important role in immune activation and pro-inflammatory response.[28,29] In contrast, Treg cells exhibit negative immunomodulatory and anti-inflammatory effects, and they express anti-inflammatory cytokines such as TNF-β and IL-10.[30] Correspondingly, an important mechanism of MSCs for ALF treatment is the regulation of Th17 and Treg cells.[8] In our findings, ALF showed a significant Treg/Th17 ratio imbalance, which was closely related to their prognosis, so rebuilding a suitable Treg/Th17 ratio may ease the progress.[31,32] In our findings, the Treg/Th17 ratio in MenSCs group was close to the control group, indicating that ALF significantly improved. In addition, A2AR agonists can enhance the regulatory effect of MenSCs on ALF.

The potential mechanism of synergic effect of A2AR activation and MenSCs in ALF treatment is still unclear. The previous study found that the immune imbalance and excessive inflammatory response in the ALF may inhibit MSCs and even cause their apoptosis.[26] A2AR activation can improve the internal environment by regulating immune cells, such as causing local neutrophil reduction, and inhibiting inflammation in ALF mice.[33]

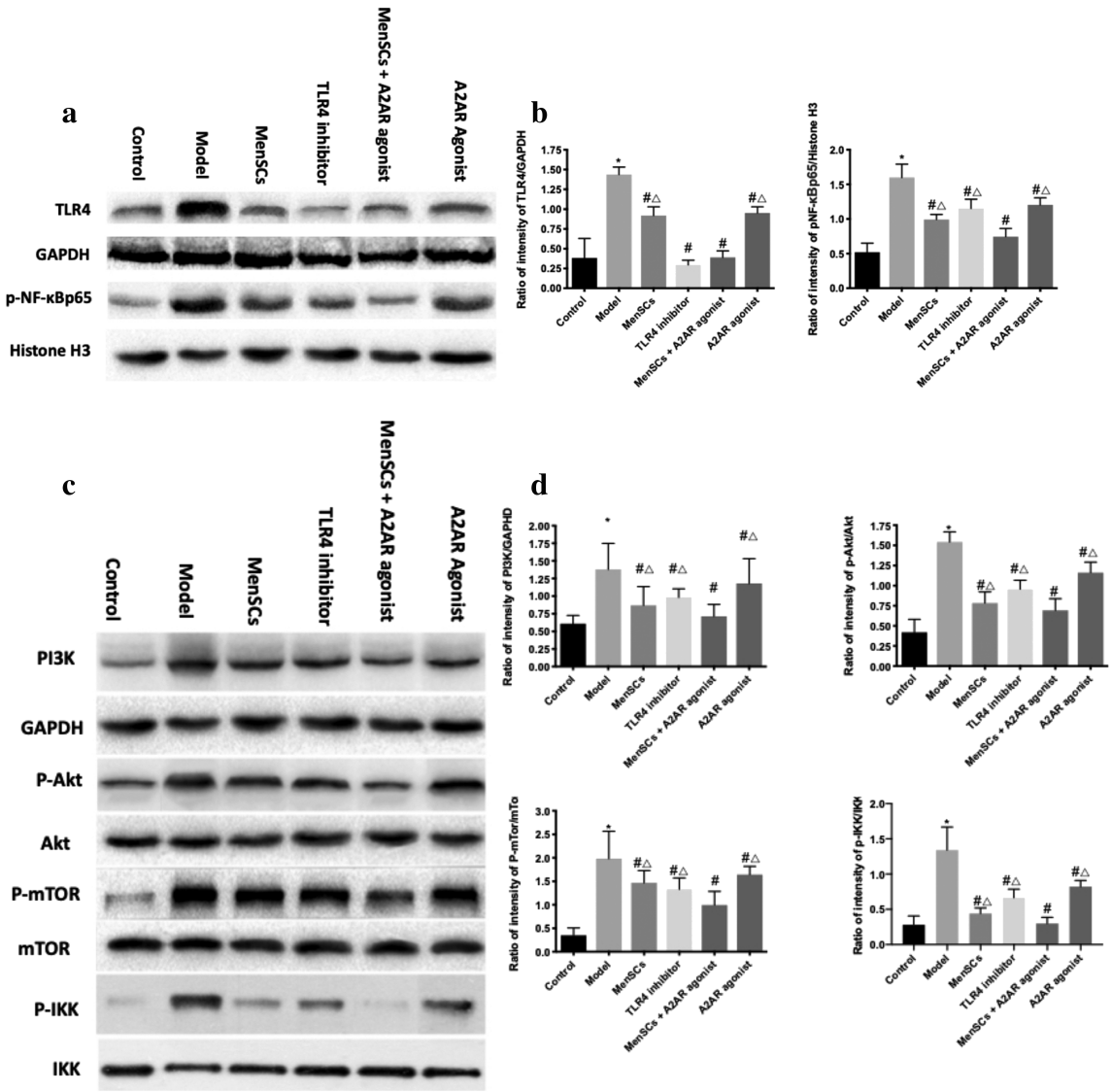

Figure 5 TLR4, p-NF-κBp65, and PI3K/AKT/mTOR/IKK pathway-related proteins expression in various mice groups. (a) typical image of Western blot. (b,c) Relative intensity of TLR4 and p-NF-kB-p65 protein. (c) typical graph of the results of Western blot. (d) Relative intensity of PI3K, pAKT, pmTOR, and pIKK, respectively. Data are expressed as mean ± SD. * Indicates $P < 0.05$ between control group and acute liver failure (ALF) model group; # indicates $P < 0.05$ between ALF model group and other treatment groups; △ indicates $P < 0.05$ between menstrual blood-derived mesenchymal stem cells (MenSCs) transplantation + A2AR agonist group and other groups.

Specifically, A2AR activation can increase the proportion and enhance the negative immune suppression function of Treg.[34] In addition, after A2AR activation, cAMP response element binding protein (CREB) is phosphorylated and has a competitive inhibitory effect with NF-κBp65 in the nuclear because it shares the same co-factor CBP. In this way, it may inhibit cytokine release and inflammatory responses.[35] Besides, A2AR activation also may inhibit the activation of IKK, a key enzyme of NF-κB activation, to attenuate inflammation,[36] or further inhibit NF-κB activity via inhibiting Akt.[37] Additionally, A2AR activation may directly inhibit TLR4 expression.[38] Taken our findings into consideration, we speculated the synergic therapeutic effect of A2AR activation and MenSCs may have through the inhibition of TLR4/NF-κB pathway as well as the regulation of other immune cells, such as Treg and Th17 (Fig. 6). The detailed mechanisms required to further investigate in future.

In summary, this study demonstrates that MenSCs may reduce the expression of p-NF-κBp65 in the nucleus by inhibiting the TLR4-mediated PI3K/Akt/mTOR/IKK pathway on ALF. In addition, MenSCs may regulate Th17 and Treg immune cells and finally achieves the result of relieving ALF. At the same time, our study also found that A2AR activation can enhance the therapeutic effect of MenSCs. Furthermore, our findings demonstrate a potential treatment for ALF in future, that is, the MenSCs-derived exosomes may be infused into ALF patients with A2AR.

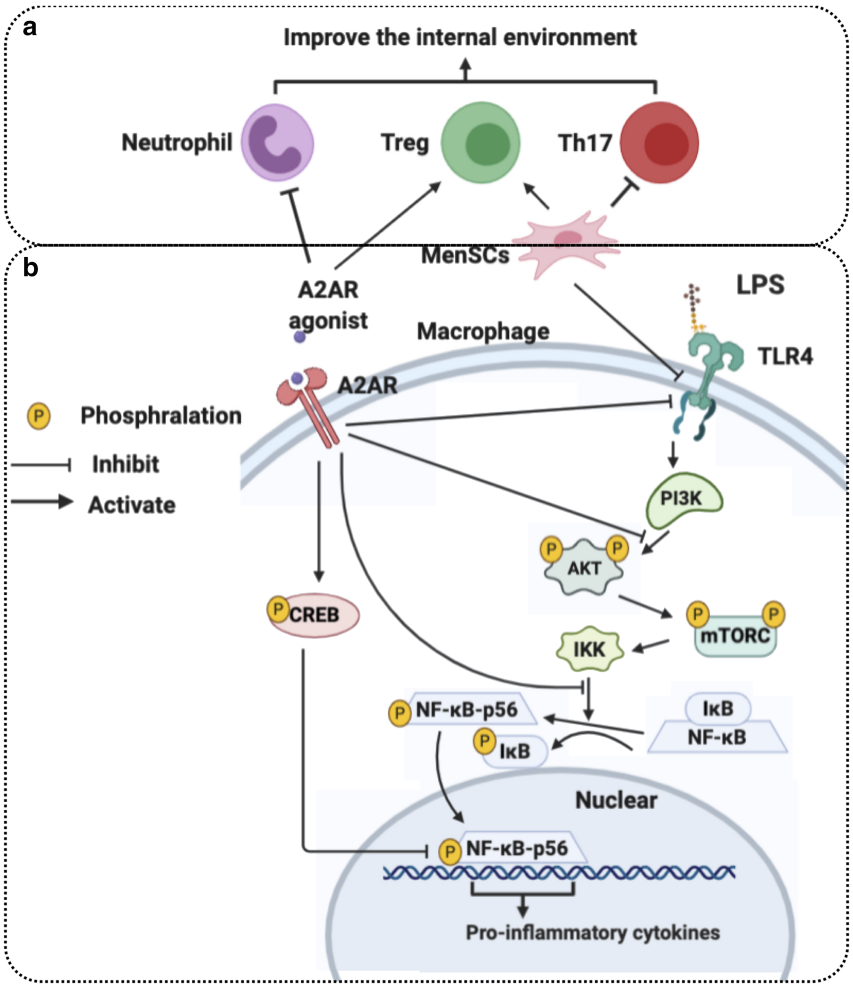

Figure 6 The speculated schematic of the synergic therapeutic effect of adenosine A2AR and menstrual blood-derived mesenchymal stem cells (MenSCs) transplantation through the (b) regulation of other immune cells, such as Treg and Th17, and (a) inhibition of TLR4/NF-κB pathway in macrophages. LPS, lipopolysaccharide.

References

1 Trey C, Davidson CS. The management of fulminant hepatic failure. *Prog. Liver Dis.* 1970; **3**: 282–98.
2 Lefkowitch JH. The pathology of acute liver failure. *Adv Anat Pathol* 2016; **23**: 144–58.
3 Romero M, Palmer SL, Kahn JA *et al*. Imaging appearance in acute liver failure: correlation with clinical and pathology findings. *Dig. Dis. Sci.* 2014; **59**: 1987–95.
4 Fontana RJ, Ellerbe C, Durkalski VE *et al*. Two-year outcomes in initial survivors with acute liver failure: results from a prospective, multicentre study. *Liver Int.* 2015; **35**: 370–80.
5 Bernal W, Hyyrylainen A, Gera A *et al*. Lessons from look-back in acute liver failure? A single centre experience of 3300 patients. *J. Hepatol.* 2013; **59**: 74–80.
6 Donnelly MC, Hayes PC, Simpson KJ. Role of inflammation and infection in the pathogenesis of human acute liver failure: clinical implications for monitoring and therapy. *World J. Gastroenterol.* 2016; **22**: 5958–70.
7 Lee WM. Recent developments in acute liver failure. *Best Pract. Res. Clin. Gastroenterol.* 2012; **26**: 3–16.
8 Wasmuth HE, Kunz D, Yagmur E *et al*. Patients with acute on chronic liver failure display "sepsis-like" immune paralysis. *J. Hepatol.* 2005; **42**: 195–201.
9 Woolbright BL, Jaeschke H. The impact of sterile inflammation in acute liver injury. *J. Clin. Transl. Res.* 2017; **3**: 170–88.
10 Kim SJ, Park JS, Lee DW, Lee SM. Trichostatin A protects liver against septic injury through inhibiting Toll-like receptor signaling. *Biomol. Ther.* 2016; **24**: 387–94.
11 van der Mark VA, Ghiboub M, Marsman C *et al*. Erratum to: Phospholipid flippases attenuate LPS-induced TLR4 signaling by mediating endocytic retrieval of Toll-like receptor 4. *Cell. Mol. Life Sci.* 2017; **74**: 1365.
12 Hu C, Zhao L, Wu Z, Li L. Transplantation of mesenchymal stem cells and their derivatives effectively promotes liver regeneration to attenuate acetaminophen-induced liver injury. *Stem Cell Res Ther.* 2020; **11**: 88.
13 Zhang ZH, Zhu W, Ren HZ *et al*. Mesenchymal stem cells increase expression of heme oxygenase-1 leading to anti-inflammatory activity in treatment of acute liver failure. *Stem Cell Res Ther.* 2017; **8**: 70.
14 Shao M, Xu Q, Wu Z *et al*. Exosomes derived from human umbilical cord mesenchymal stem cells ameliorate IL-6-induced acute liver injury through miR-455-3p. *Stem Cell Res Ther.* 2020; **11**: 37.
15 Cui CH, Uyama T, Miyado K *et al*. Menstrual blood-derived cells confer human dystrophin expression in the murine model of Duchenne muscular dystrophy via cell fusion and myogenic transdifferentiation. *Mol. Biol. Cell* 2007; **18**: 1586–94.
16 Meng X, Ichim TE, Zhong J *et al*. Endometrial regenerative cells: a novel stem cell population. *J. Transl. Med.* 2007; **5**: 57.
17 Alcayaga-Miranda F, Cuenca J, Luz-Crawford P *et al*. Characterization of menstrual stem cells: angiogenic effect, migration and hematopoietic

stem cell support in comparison with bone marrow mesenchymal stem cells. *Stem Cell Res Ther.* 2015; **6**: 32.
18 Chen L, Xiang B, Wang X, Xiang C. Exosomes derived from human menstrual blood-derived stem cells alleviate fulminant hepatic failure. *Stem Cell Res Ther.* 2017; **8**: 9.
19 Fozard JR, Ellis KM, Villela Dantas MF, Tigani B, Mazzoni L. Effects of CGS 21680, a selective adenosine A2A receptor agonist, on allergic airways inflammation in the rat. *Eur. J. Pharmacol.* 2002; **438**: 183–8.
20 Odashima M, Bamias G, Rivera-Nieves J *et al*. Activation of A2A adenosine receptor attenuates intestinal inflammation in animal models of inflammatory bowel disease. *Gastroenterology* 2005; **129**: 26–33.
21 Xiao X, Wang Y, Chen Z *et al*. Effect of adenosine A2A receptor agonist combined with bone marrow mesenchymal stem cells transplantation on the negative immune regulation in mice with acute liver failure. *Chin. J. Infect. Dis.* 2017; **1**: 15–21.
22 Chen L, Qu J, Xiang C. The multi-functional roles of menstrual blood-derived stem cells in regenerative medicine. *Stem Cell Res. Ther.* 2019; **10**: 1.
23 Tan L, Qiu T, Xiang R *et al*. Down-regulation of Tet2 is associated with Foxp3 TSDR hypermethylation in regulatory T cell of allergic rhinitis. *Life Sci.* 2019; **241**: 117101.
24 Lin WC, Deng JS, Huang SS *et al*. Anti-inflammatory activity of sanghuangporus sanghuang mycelium. *Int. J. Mol. Sci.* 2017; **18**: 347.
25 Li D, Wang C, Chi C *et al*. Bone marrow mesenchymal stem cells inhibit lipopolysaccharide-induced inflammatory reactions in macrophages and endothelial cells. *Mediators Inflamm.* 2016; **2016**: 2631439.
26 Wang YH, Wu DB, Chen B, Chen EQ, Tang H. Progress in mesenchymal stem cell-based therapy for acute liver failure. *Stem Cell Res. Ther.* 2018; **9**: 227.
27 Marra F, Aleffi S, Galastri S, Provenzano A. Mononuclear cells in liver fibrosis. *Semin. Immunopathol.* 2009; **31**: 345–58.
28 Hammerich L, Heymann F, Tacke F. Role of IL-17 and Th17 cells in liver diseases. *Clin. Dev. Immunol.* 2011; **2011**: 345803.
29 Bettelli E, Korn T, Oukka M, Kuchroo VK. Induction and effector functions of T(H)17 cells. *Nature* 2008; **453**: 1051–7.
30 Awasthi A, Kuchroo VK. Th17 cells: from precursors to players in inflammation and infection. *Int. Immunol.* 2009; **21**: 489–98.
31 Zhai S, Zhang L, Dang S *et al*. The ratio of Th-17 to Treg cells is associated with survival of patients with acute-on-chronic hepatitis B liver failure. *Viral Immunol.* 2011; **24**: 303–10.
32 Niu YH, Yin DL, Liu HL *et al*. Restoring the Treg cell to Th17 cell ratio may alleviate HBV-related acute-on-chronic liver failure. *World J. Gastroenterol.* 2013; **19**: 4146–54.
33 Odashima M, Otaka M, Jin M *et al*. A selective adenosine A2A receptor agonist, ATL-146e, prevents concanavalin A-induced acute liver injury in mice. *Biochem. Biophys. Res. Commun.* 2006; **347**: 949–54.
34 Ohta A, Madasu M, Subramanian M *et al*. Hypoxia-induced and A2A adenosine receptor-independent T-cell suppression is short lived and easily reversible. *Int. Immunol.* 2014; **26**: 83–91.
35 Fredholm BB, Chern Y, Franco R, Sitkovsky M. Aspects of the general biology of adenosine A2A signaling. *Prog. Neurobiol.* 2007; **83**: 263–76.
36 Tang LM, Zhu JF, Wang F *et al*. Activation of adenosine A2A receptor attenuates inflammatory response in a rat model of small-for-size liver transplantation. *Transplant. Proc.* 2010; **42**: 1915–20.
37 Ke RH, Xiong J, Liu Y, Ye ZR. Adenosine A2a receptor induced gliosis via Akt/NF-kappaB pathway in vitro. *Neurosci. Res.* 2009; **65**: 280–5.
38 Ahmad SF, Ansari MA, Nadeem A, Bakheet SA, Al-Ayadhi LY, Attia SM. Toll-like receptors, NF-kappaB, and IL-27 mediate adenosine A2A receptor signaling in BTBR T(+) Itpr3(tf)/J mice. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 2017; **79**: 184–91.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Expression of IL-6, IL-1β and TNF-α in RAW264.7 cells after treatment of LPS. A and D represent histogram of IL-6 protein and mRNA respectively. B and E display the amount of IL-1B protein and mRNA respectively. C and F indicate the levels of TNF-a protein and mRNA respectively. Data are expressed as mean ± SD. * Indicates $P < 0.05$ between the control and other treatments; # indicates $P < 0.05$ between LPS model and other treatment groups.

Figure S2. The KM plot of the overall survival rate among those 6 groups, including control group, ALF model group, MenSCs transplantation group, TLR4 inhibitor group, MenSCs transplantation + A2AR agonist (CGS21680) group, and A2AR agonist (CGS21680) group.

Figure S3. Spleen profile of the CD4 (+) CD25 (+) FoxP3 (+) Treg cells and CD4 (+) IL17 (+) Th17 cells in mice model. A shows flow cytometry pictures of Treg cells and panel C displays scatter profile of Treg cells in different groups. B represents flow cytometry pictures of Th17 cells and panel D indicates scatter profile of Th17 cells. Data are expressed as mean ± SD. * Indicates $P < 0.05$ between the control group and the ALF model group; # indicates $P < 0.05$ between the ALF model group and the other treatment groups; △ indicates $P < 0.05$ between the MenSCs + A2AR agonist group and the other groups.

Table S1. RT-qPCR primers sequence. [Correction added on 15 April 2021, after first online publication: Table S1 caption has been amended]