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Pre-activation of TLR3 enhances the therapeutic effect of BMMSCs through regulation the intestinal HIF-2α signaling pathway and balance of NKB cells in experimental alcoholic liver injury

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ABSTRACT

Increased intestinal permeability and immune disorder are important mechanisms of alcoholic liver disease (ALD). Recent evidences suggest bone marrow derived mesenchymal stem cells (BMMSCs) have protective effects on end-stage liver disease and intestinal barrier injury. Moreover, the activation of toll-like receptor 3 (TLR3) has been shown enhancing therapeutic effects of BMMSCs in inflammatory bowel disease (IBD). However, the mechanism remains unclear. In current study, chronic-binge alcohol abuse model was employed to investigate the therapeutic effects of BMMSCs and BMMSCs pre-activated with TLR3 (P-BMMSCs) on alcohol-induced liver and intestine damage. C57BL/6 mice were divided into four groups with normal control, alcohol-fed model, alcohol-fed model with BMMSCs treatment and alcohol-fed model with P-BMMSCs treatment. Alcohol-fed mice were fed Lieber-DeCali diet containing 5% alcohol for four weeks and given alcohol intragastrically on the 28th day, but control group were fed isocaloric diet. BMMSCs and P-BMMSCs were injected into the treatment group three times. Results showed alcohol diet causing significant damage to intestinal barrier and liver. These were reversed by the treatment of BMMSCs, especially P-BMMSCs. Moreover, alcohol increased the expression of intestinal HIF-2α, the proportion of NKB cells and the level of serum IL-18, while BMMSCs or P-BMMSCs reduced these factors. In conclusion, BMMSCs, especially TLR3 pre-activated BMMSCs could be used to protect alcohol-induced intestine and liver injury.

1. Introduction

Alcoholic liver disease (ALD) is caused by alcohol abuse, resulting in an increasing number of deaths. ALD is manifested as alcoholic steatosis, steatohepatitis, alcoholic cirrhosis and even hepatocellular carcinoma. Although a large number of studies have been devoted to understand ALD, the clinical treatment of ALD is still limited. Pathogenesis of ALD involves many aspects, such as increased intestinal permeability, bacterial translocation [1,2], immune disturbance [3,4], damage to hepatocytes by alcohol and its toxic metabolites. Among them, gut barrier dysfunction has been found playing an essential role in alcohol-induced liver injury.

One of the mechanisms of ALD is oxidative stress in the liver caused by oxygen consumption, which leads to local hypoxia and inflammation [5]. Hypoxia-inducible factor (HIF) is one of the nuclear transcription factors that maintains normal intestinal mucosal barrier function under hypoxic and inflammatory conditions. HIF is a heterodimer composed of an alpha and a beta subunit with HIF-1α and HIF-2α [6]. Recent studies have highlighted the role of HIF-2α in intestine and liver, which suggests that the activation of HIF-2α enhances pro-inflammatory responses, intestinal epithelial damage, liver steatosis, liver fibrosis, and tumor development [7]. These reveal that HIF-2α may play an important role in ALD, and its mechanism needs to be explored.

Immune disturbance is also an important pathological mechanism of ALD. Natural killer B (NKB) cells are a novel subpopulation of NK cells, mainly found in spleen and lymph nodes. NKB cells express both

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the NK cell marker - NK1.1 and the B cell markers - CD19 and IgM [8]. NKB cells involve in early microbial infection, autoimmunity and inflammation [9–11]. However, few research reported its association with ALD.

Mesenchymal stem cells (MSCs) have been described as a promising therapeutic approach to ALD based on limited ALD treatments and its pathological mechanisms [12]. MSCs are non-hematopoietic pluripotent stem cells having self-renewal and multi-directional differentiation potential. BMMSCs are one kind of MSCs used for the treatment of end-stage liver disease clinically, such as alcohol-induced cirrhosis [13]. But its specific effects are quite different and even unsatisfactory in various studies and clinical applications [14]. Polyinosinic-polycytidylic acid (poly(I:C)) is an RNA mimic ligand of TLR3. Treatment of BMMSCs with poly(I:C) exhibits lower immunogenicity, stronger immunosuppressive [15] and paracrine functions [16]. Moreover, researches demonstrated poly(I:C) treated BMMSCs showing a better efficacy in sepsis [17] and inflammatory bowel disease [18].

Therefore, in this study, we examined the effects of poly(I:C) treated BMMSCs, NKB cells, and HIF-2α in an experimental alcoholic liver disease model.

2. Material and methods

2.1. Culture and preparation of BMMSCs

BMMSCs (P6) were purchased from Cyagen Biosciences Inc. (Cyagen, Guangzhou, China). It was isolated from the bone marrow of C57BL/6 mice and cultured in the complete medium (Cyagen). BMMSCs were plated on 6-well plates at 1×10^5 cells per well. When the confluence was reached to 80–90%, BMMSCs were treated with 4μg/ml, 20μg/ml and 200μg/ml poly(I:C) (sigma, USA) respectively for 24 h. After that, the cells were trypsinized, centrifuged and collected for expression of TLR3 in vitro. In addition, all media were collected and stored at -80 °C. The cells with the highest expression of TLR3 (abbreviated as P-BMMSCs) were selected for in vivo experiments.

2.2. Animal experiment

Thirty male C57BL/6 wild type (WT) mice (4–6 weeks of age) were purchased from the Shanghai Laboratory Animal Center (Shanghai, China). As described previously [19], all mice were first given Lieber-DeCali liquid control diet (Trophic Animal Feed High-tech Co., Jiangsu, China) for 5 days. Mice in other groups, except in control group, were given different proportion of Lieber-DeCali ethanol diet (5% ethanol). The ratio of Lieber-Decarli control diet to ethanol diet was from 2:1 1:1 to 1:2 at days 2, 4 and 6 respectively for one week, and then mice were randomly divided into different groups. There are four experimental groups: (1) control group (C, n = 6): Lieber-DeCali control diet for 4 weeks. (2) Alcoholic liver injury model group (M, n = 6): Lieber-DeCali alcohol diet for 4 weeks. (3) BMMSCs treated group (B, n = 6): Lieber-DeCali alcohol diet for 4 weeks plus BMMSCs ($> 1 \times 10^5$ cells/mouse) injected into the caudal vein 3 times on the 14th, 21st and 28th days respectively. (4) P-BMMSCs treated group (PB, n = 6): Lieber-DeCali alcohol diet for 4 weeks plus P-BMMSCs ($> 1 \times 10^5$ cells/mouse) injected into the caudal vein 3 times on the 14th, 21st and 28th days respectively. Moreover, except the control group, mice in other three groups were given gavage of alcohol (5 g/kg body weight) on the 28th day. After 10 h of alcohol gavage, all mice were euthanized to collect blood, liver, spleen and intestinal tissue. Mice body weights and liver weights were also measured.

2.3. Liver and small intestine histopathology

Once the liver and small intestine were collected, they were fixed in 4% paraformaldehyde and then embedded in paraffin. Paraffin tissues were cut into a 4um tissue section and stained with hematoxylin/eosin (HE). Sections of the liver and small intestine were quickly frozen after they were collected, and then cut into 4um sections and stained with Oil Red O. Both two were analyzed by light microscopy.

Table 1
Primer sequences used in RT-PCR.

Name	Sequences of primers	Tm	Length of product
TLR 3:	Sense: TCCTTCTCCTATCTCCCAA Antisense: GTAAATGCTCGCTTCAAACT	60 °C	125BP
HIF-2α:	Sense: GTCTTCCTGCTTTCCAACTGC Antisense: CTTGGCACCCTACATCCTAT	60 °C	207BP
ZO-1:	Sense: GAGTGGACTATCAAGTGAGCCTAA Antisense: ATCCAAGTTGCTCGTCAATCTAA	60 °C	137BP
Occludin:	Sense: CATCTTTCTTCGGGTTTTCAC Antisense: TCTGGATCTATGTACGGCTCA	60 °C	204BP
Claudin-1:	Sense: AGCGTAAAACCAGTAAGAATCCCA Antisense: CAAACAAGACAGCTCCAGACCAAA	60 °C	132BP
GAPDH:	Sense: AAGAAGGTGGTGAAGCAGG Antisense: GAAGGTGGAAGAGTGGGAGT	60 °C	111 BP

TLR3: Toll-like receptor 3; HIF-2α: hypoxia-inducible factor-2α; GAPDH: glyceraldehydes-3-phosphate dehydrogenase.

2.4. Real-time reverse transcriptase polymerase-chain reaction (RT-PCR) assay

Total RNA in tissues or cells was separately extracted according to the instructions of the RNA extraction kit (Aidlab Biotechnologies Co, Beijing, China) and reverse-transcribed using the PrimeScriptTM RT reagent Kit (Perfect Real Time), (Aidlab Biotechnologies Co, Beijing, China). The relevant cDNA was then amplified using the SYBR Premix Ex Taq II (TOYOBO, Osaka, Japan) with specific primers as listed in Table 1. RT-PCR was performed by a 7500 real-time PCR system (Applied Biosystems, Life Technologies, USA). Each mRNA expression was calculated based on glyceraldehyde-3-phosphate dehydrogenation (GAPDH) as a reference gene. The final data was analyzed by the $2^{-\Delta\Delta Ct}$ method.

2.5. Western blot analysis

Total protein from either cells or tissues were extracted in a mixture of ice-cold RIPA (Beyotime Biotechnology, China) and protease inhibitor. Proteins were separated by 10% SDS-PAGE and electro-transferred to PVDF membrane (Millipore, USA). The membranes were blocked for 2 h at room temperature with 5% BSA in TBST solution and then incubated with primary antibody anti-TLR3 (1:1000, CST), anti-HIF-2α (1;1000, Abcam), anti-ZO-1 (1:1000, Sigma), anti-Claudin-1 (1:1000, Abcam), anti-Occludin (1:1000, Abcam) and anti-GAPDH (1:1000, Goodhere, China) respectively at 4 °C overnight. After washing with TBST, the membranes were incubated with the secondary antibody (1:5000, Biosharp) for 1 h. Washing with TBST 3 times. Then the bands were detected and analyzed by WesternBright ECL (Advansta, USA) and Image Lab 4.1 software (Bio-Rad).

2.6. Measurement of cytokines, lipopolysaccharide (LPS), ALT and AST levels

The collected blood was centrifuged at 1500 rpm at room temperature for 10 min to obtain serum. Then serum and the collected culture medium were preserved at -80 °C until use. The levels of serum AST and ALT were measured by using an automated biochemical analyzer (Abbott Laboratories, USA) in the clinical biochemical laboratory of the First Affiliated Hospital of Wenzhou Medical University. LPS level in serum was measured using ELISA kit (Xiamen Reagent Laboratories, Xiamen, China) according to instructions. And ELISA kits from

MultiSciences (Hangzhou, China) were used to measure the concentration of cytokines in serum and the collected culture medium.

2.7. Flow cytometry

Mice spleens from different groups were removed and washed with PBS. After using lymphocyte separation kit (TBDscience, Tianjin, China) isolated lymphocytes from spleens, 500 μl of cell suspension was used to incubate with fluorescent labeled monoclonal antibodies CD3-FITC (eBioscience, CA, USA), CD19-APC (eBioscience) and NK1.1-PE (eBioscience) respectively at room temperature for 30 min. Mouse IgG2a Kappa labeled with PE (eBioscience) was used as isotype control to avoid non-specific binding. NKB cells are defined as CD3-CD19 + NK1.1 + NK cells. The ratio of NKB cells to total CD3- NK cells was analyzed using the BD FACSCalibur platform (BD Bioscience, CA, USA) and analytical software.

2.8. Statistics and data analysis

Comparisons between multiple sets of data were performed using one-way analysis of variance (ANOVA), LSD and Dunnett T3 tests in SPSS20.0 software. Data are expressed as mean ± SEM. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Preparation of BMMSCs

BMMSCs have been documented to play a role in liver diseases however whether BMMSCs release alcohol-induced liver injury and whether activation of TLR3 are critical in BMMSCs' treatment of ALD remain to be investigated. As shown in Fig. 1, the TLR3 protein and mRNA levels were detected by Western blotting and RT-PCR after BMMSCs were cultured with different concentration of poly(I:C) (0, 4, 20 and 200 μg/ml) for 24 h. The highest expression of TLR3 was observed at the concentration of 20 μg/ml. So BMMSCs treated with 20 μg/ml were used as P-BMMSCs for the rest of the experiments (Fig. 1A, B).

3.2. Effect of poly(I:C) on the secretion of IL-10 and PGE2 in BMMSCs

The contents of PGE2 and IL10 in cell culture medium were measured by ELISA to speculate the possible influence of poly(I:C) on BMMSCs' paracrine ability in vitro. The results showed IL-10 and PGE2 levels in the cell supernatant of the poly(I:C)-stimulated group were higher than the unstimulated group (Fig. 1C, D).

3.3. BMMSCs and P-BMMSCs attenuated alcohol-induced liver damage

Alcohol-induced liver injury was examined and shown in Fig. 2. The body weight and liver weight of mice were documented in Fig. 2A. Alcohol caused a significant decrease in body weight and a significant increase in liver weight. Treatment of either BMMSCs or P-BMMSCs elevated a little bit of body weight but not reach statistical significance. But treatment of BMMSCs and P-BMMSCs respectively significantly reduced the increased liver weight caused by alcohol intake. Liver aminotransferases – ALT and AST were also significantly increased in alcohol-fed mice (Fig. 2B). Treatment of either BMMSCs or P-BMMSCs significantly reduced alcohol-induced elevation of these enzymes, but with more reduction in mice treated with P-BMMSCs especially to ALT level. Liver histologies also showed the liver damage of alcohol, the improvement of liver injury and lipid deposition after treatment of BMMSCs or P-BMMSCs (Fig. 2C). More improvement was observed in the liver treated with P-BMMSCs.

3.4. BMMSCs and P-BMMSCs attenuated alcohol-induced intestinal barrier dysfunction

One of the mechanisms of ALD is intestinal barrier dysfunction. Therefore, intestinal histology and intestinal tight junction (TJ) proteins were examined and documented in Fig. 3. As shown in Fig. 3A, alcohol intake caused the intestinal villi thinner, shorter and irregular. Treatment with both BMMSCs and P-BMMSCs alleviated alcohol-induced intestinal villi destruction. The intestinal barrier function was evaluated by examining the expression of the three TJ proteins (claudin-1, occludin and ZO-1) and bacterial translocation. As shown in Fig. 3B, alcohol intake resulted in a significant decrease in protein and mRNA levels of these TJ proteins. BMMSCs treatment could only significantly reverse claudin-1 protein although BMMSCs could reverse all three mRNA levels. However, P-BMMSCs treatment could significantly reverse the protein and mRNA levels of these three genes. Furthermore, HIF-2α protein and gene expression in the intestine were shown in Fig. 3C. Alcohol increased the HIF-2α protein in intestine, that suggests a role of HIF-2α in alcohol-induced intestinal injury. BMMSCs or P-BMMSCs could reduce alcohol-induced overexpression of HIF-2α protein in intestine.

Bacteria translocation was assessed by detection of E. coli protein in the liver and the level of LPS in serum. As shown in Fig. 4A, alcohol caused a significant elevation of bacteria protein compared to normal. Only P-BMMSCs treatment reduced bacteria protein significantly but not BMMSCs. LPS could be detected in the serum of mice and alcohol intake resulted in a significant increase in LPS while treatment of either BMMSCs or P-BMMSCs reduced LPS level (Fig. 4B).

3.5. BMMSCs and P-BMMSCs reverse the alcohol-induced disorder of spleen NKB cells

Alcohol not only alters intestinal barrier function but also affects immune and inflammatory function in mice. To assess these changes in mice, flow cytometry was used to detect the subpopulation of NKB cells in the spleen. As shown in Fig. 5A and B, alcohol exposure significantly increased the proportion of NKB cells in the spleen as well as the concentration of IL-18 in serum (Fig. 5C). Treatment of either BMMSCs or P-BMMSCs reduced alcohol—induced elevation of NKB cells with a more significant reduction in P-BMMSCs.

4. Discussion

Liver is the most important detoxification organ in body, where different toxins can cause liver damage. Since the enterohepatic axis was proposed in 1998, the intestine has been found related to liver diseases. Abnormal changes in the intestine can be found in different kinds of liver diseases such as ALD [1], nonalcoholic fatty liver disease (NAFLD) [20] and autoimmune liver disease (AIH) [21] etc. Especially in ALD, alcohol abuse can cause intestinal ecological disorders and intestinal barrier dysfunction [2], which could lead to bacterial translocation, exacerbate immune disorders and liver damage.

Normal intestinal barrier function is essential to maintain the stability of internal environment. Intestinal barrier function is mainly maintained by intestinal mucosal barrier, which is the mechanical barrier of intestine. Moreover, tight junction proteins (including ZO-1, Occludin and Claudin-1, etc.) are important structures to maintain the mechanical barrier and permeability of mucosal epithelium [22,23]. The intestinal barrier dysfunction caused by alcohol abuse is usually manifested as decrease in TJ protein, increase in plasma endotoxin, translocation of intestinal microbe and histological changes of intestinal mucosa [23,24]. In our study, we found alcohol causes intestinal villi being thinner and disordered. Also, alcohol abuse decreased the expression of TJ proteins, impaired intestinal barrier function and increased bacterial translocation. In addition, HIF-2α is necessary to maintain the epithelial barrier [25]. Activation of HIF-2α in the

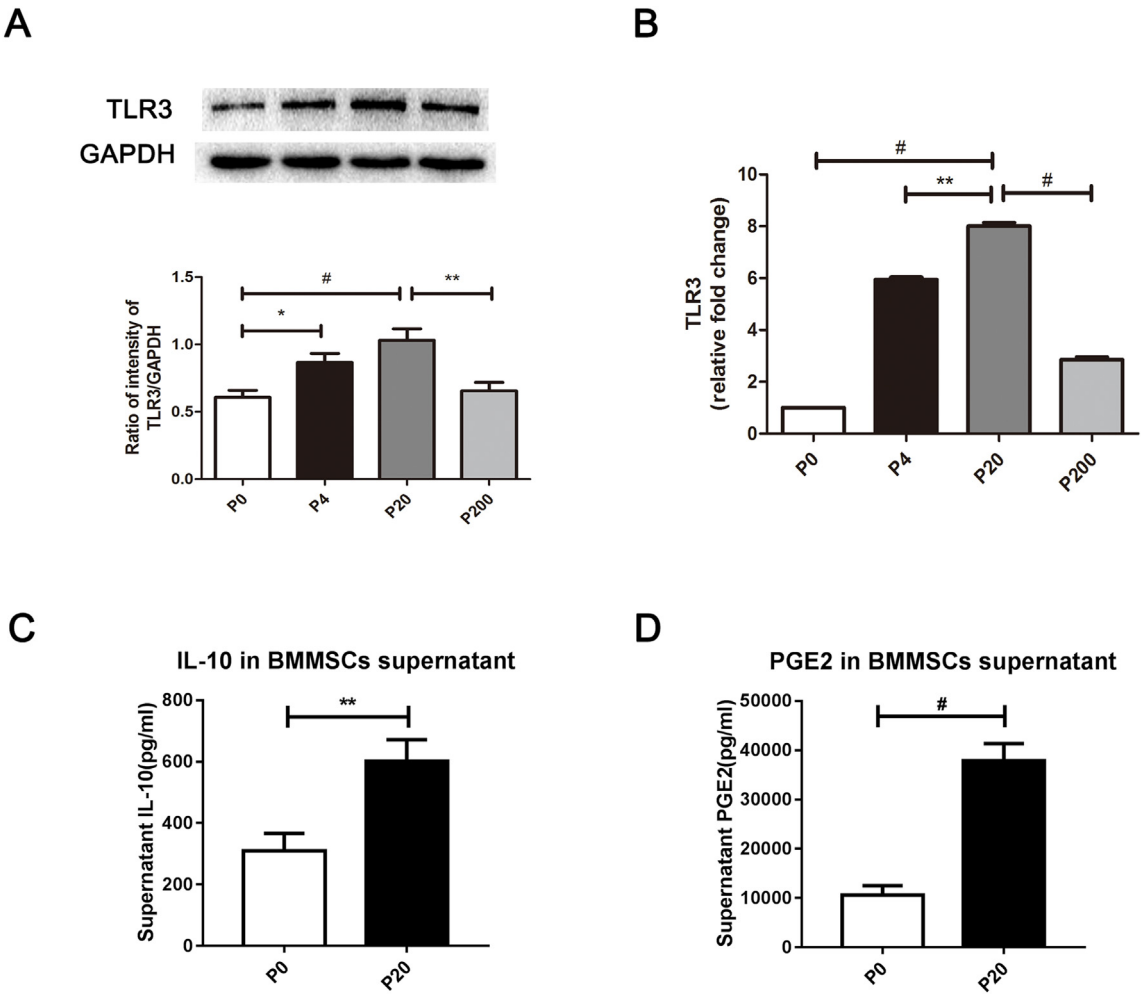

Fig. 1. Effect of poly I:C on TLR3 protein expression and IL10, PGE2 levels in BMMSCs supernatant. Panel A shows the level of TLR3 protein evaluated by western blotting and panel B displays mRNA level of TLR3 quantitated by RT-PCR. Both protein and mRNA were normalized to the level of GAPDH. Panel C and D show IL-10 and PGE2 levels in BMMSCs supernatant quantitated by ELISA. Results were mean ± SEM from six samples. *P < 0.05, **P < 0.01 and #P < 0.001.

intestine can lead to pro-inflammatory reaction, epithelial barrier dysfunction, and even lead to the occurrence and development of cancer [7]. The abnormal expression of HIF-2α in the liver can cause lipid accumulation, inflammation, and fibrosis [26]. Increased expression of HIF-2α has been reported in many diseases, including viral hepatitis [27], fatty liver disease [26,28] and IBD [29]. Consistent with other studies, our experimental results also showed the increased expression of intestine HIF-2α in ALD mice, which may promote ALD. However, after BMMSCs and P-BMMSCs injection, intestinal HIF-2α decreased, intestinal TJ protein increased, bacterial translocation decreased, and intestinal epithelial barrier function recovered. All these findings indicated that BMMSCs and P-BMMSCs could alleviate intestinal and liver damage caused by alcohol, and P-BMMSCs are better than BMMSCs in the therapeutic efficacy.

In the past 30 years, mesenchymal stem cells (MSCs) have been proposed as a treatment of different liver diseases [30]. BMMSCs have been demonstrated to alleviate liver injury in NAFLD and AIH, and help patients with alcoholic cirrhosis to recover liver structure and function [13]. BMMSCs can also alleviate IBD [18]. Moreover, researches have conducted to explore the possible mechanism of MSCs in the treatment of liver and intestinal diseases. MSCs can migrate to the injured site selectively, differentiate into corresponding cells [31,32] and secrete many kinds of growth factors. All of these can create an environment for healing and regeneration [33,34]. Furthermore, MSCs can play an immunomodulatory role through direct contact or paracrine action between cells to reduce inflammation [35–38]. MSCs can modulate not only innate immunity but also adaptive immunity by secreting different kinds of cytokines, such as IL10, IL6. Our study confirms that BMMSCs can attenuate chronic-binge alcohol induced liver injury and support the notion that MSCs act through regulation of immunity.

Although our study shows that BMMSCs has a therapeutic effect on ALD, its effect is limited. A number of meta-analyses evaluated the role of MSCs in liver repair, and the specific efficacy showed a wide gap [39–41]. Since the immunomodulatory properties of MSCs are malleable [42], ligands bound to TLRs have been used to regulate differentiation and maintain MSCs' pluripotency [43,44]. When TLR3 is activated, MSCs will pole into immunosuppressive MSC2 phenotype, and produce cytokines which inhibit inflammatory response [45]. Poly(I:C) is the activator of TLR3. Pretreatment with poly(I:C) enhanced the protective effect of MSCs on colitis and damaged hamster heart [18]. Activation of TLR3 makes MSCs produce multiple cytokines (such as IL-10, PGE2, etc.), which could inhibit liver inflammation, fibrosis and alleviate alcohol-induced liver injury [46,47]. In our study, BMMSCs were treated with three different concentrations of poly(I:C), and BMMSCs with the highest activation of TLR3 were obtained to compare with untreated BMMSCs. We found that the highest activation level of TLR3 was at the concentration of 20 mg/μl. And BMMSCs with pre-activated TLR3 had better protective effects on alcohol-induced liver injury than these untreated. Moreover, we also found that BMMSCs with pre-activated TLR3 can produce more PGE2 and IL-10 in vitro. It

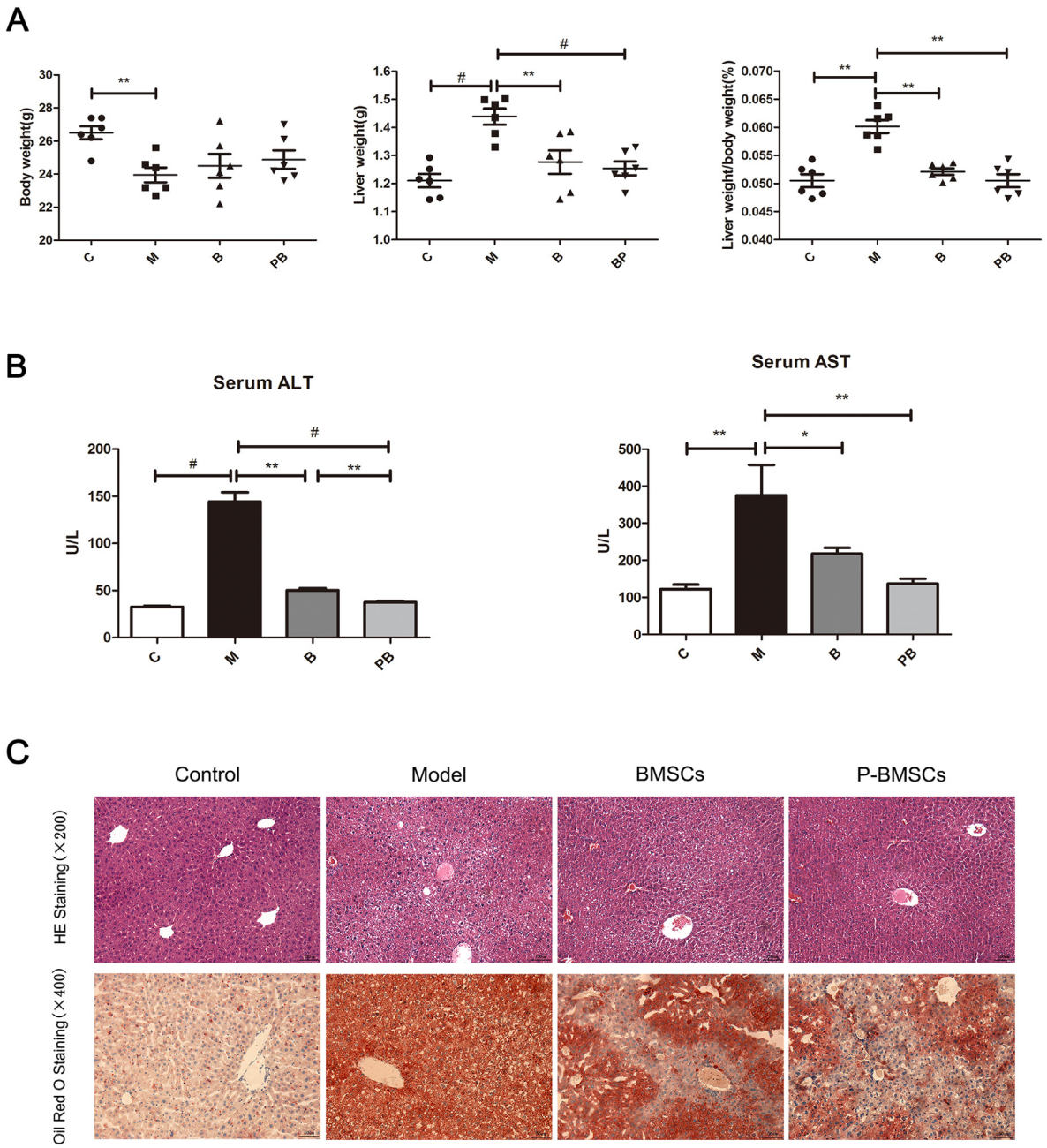

Fig. 2. Effect of BMMSCs and P-BMMSCs treatment on serum transaminase, body weight, liver weight and liver histology. Panel A shows body weight, liver weight, and the ratio of liver to body weight. Panel B displays serum ALT and AST levels from left to right. Data were presented as mean ± SEM from six mice. *P < 0.05, **P < 0.01 and #P < 0.001. Panel C shows the liver histology stained with HE and Oil Red O (magnification: HE ×200, scale bar = 100 μm; Oil red O × 400, scale bar = 50 μm).

proves that activation of TLR3 can promote BMMSCs secrete IL-10 and PGE2, which may enhance the therapeutic effect of BMMSCs on ALD. But the specific mechanism of this remains to be further studied.

MSCs can not only inhibit adaptive immunity (such as T lymphocyte proliferation), but also reduce NK cell proliferation and toxicity [47,48]. Moreover, TLR3 activated MSCs showed better immunosuppressive ability [15] and had more paracrine function than inactivated MSCs [16]. For example, Poly(I:C)-treated MSCs impaired Th1/17 cell expansion and suppressed Treg cell through producing PGE2 [49]. Compared with normal mice, our study showed the number of NKB cells in spleen and the level of IL-18 in serum were significantly increased in alcohol-fed mice. These changes were reversed after BMMSCs and P-BMMSCs treatment. Others also showed that co-culture with NKB cells could increase NK and ILC1 cells' proliferation by producing IL-18 and IL-12. They also demonstrated NKB cells play a crucial role in early innate immunity by secreting IL-18 in IL-18 deficient mice [8]. These innate lymphoid cells were involved in intracellular parasites [50], autoimmune disorders, allergic disease [51,52], and protective immunity against bacteria [53,54]. They can also be driven to shift from innate immunity to adaptive immunity [10]. Therefore, mass production of NKB cells and IL-18 in ALD may lead to the subsequent activation of a large number of NK cells and ILC1 cells, which may further aggravate chronic liver inflammation and lipid deposition in ALD. This could be the reason of why MSCs alleviate liver injury caused by alcohol. Because after BMMSCs' treatment, there were decreases in the content of spleen NKB cells and serum IL-18 level. So, pre-activation of TLR3 in BMMSCs can enhance the immunosuppression in the early innate immune cells - NKB cells.

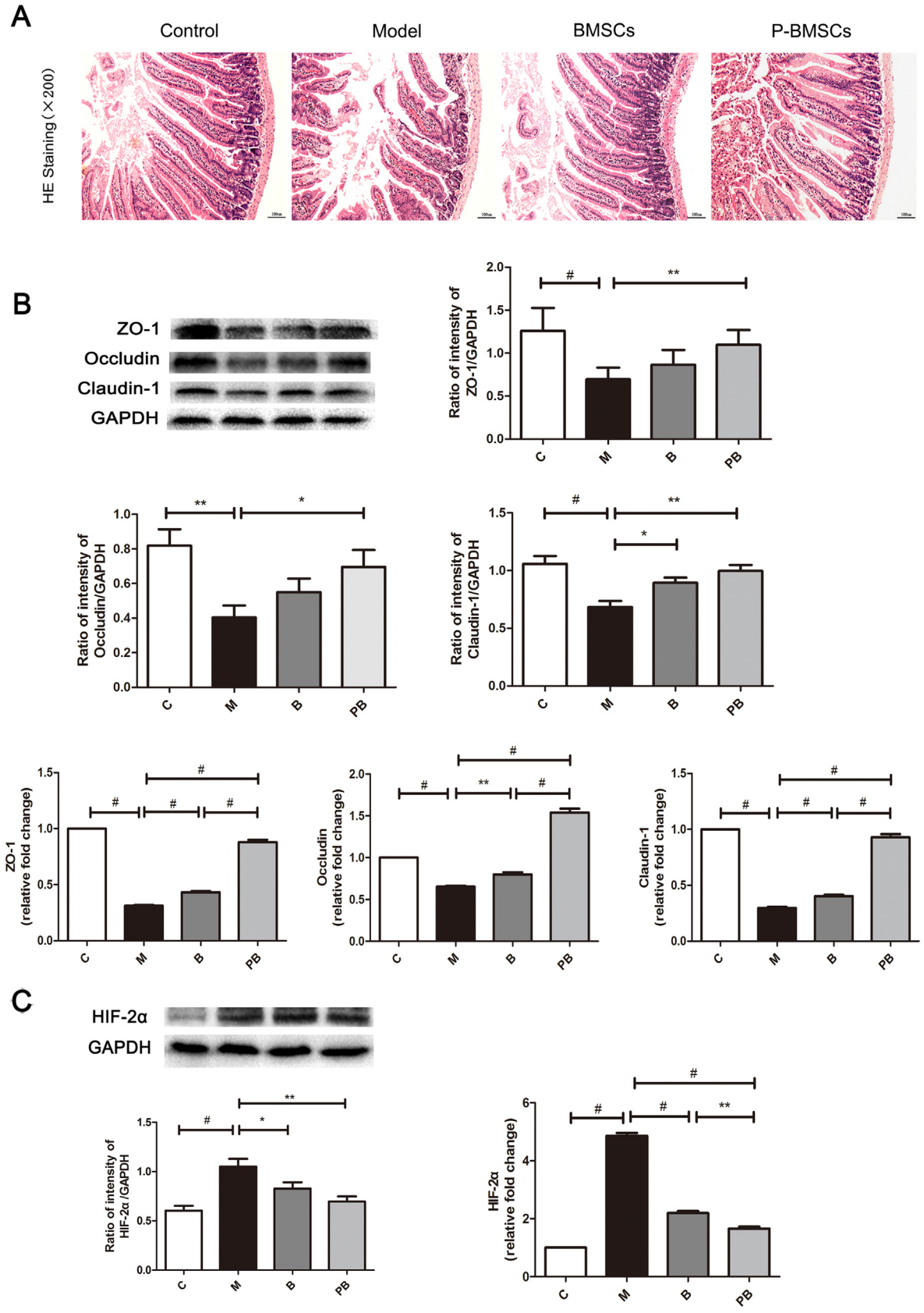

Fig. 3. Effect of BMMSCs and P-BMMSCs treatment on intestinal barrier. Panel A shows the histology of small intestine stained with HE (magnification: ×200, scale bar = 100 μm). Panel B displays the protein levels of intestinal ZO-1, Occludin and Claudin-1 evaluated by WB and RT-PCR. Panel C shows the protein and mRNA levels of intestinal HIF-2α evaluated by WB and RT-PCR, which were normalized to the levels of GAPDH. Results were presented as mean ± SEM from six mice. *P < 0.05, **P < 0.01 and #P < 0.001.

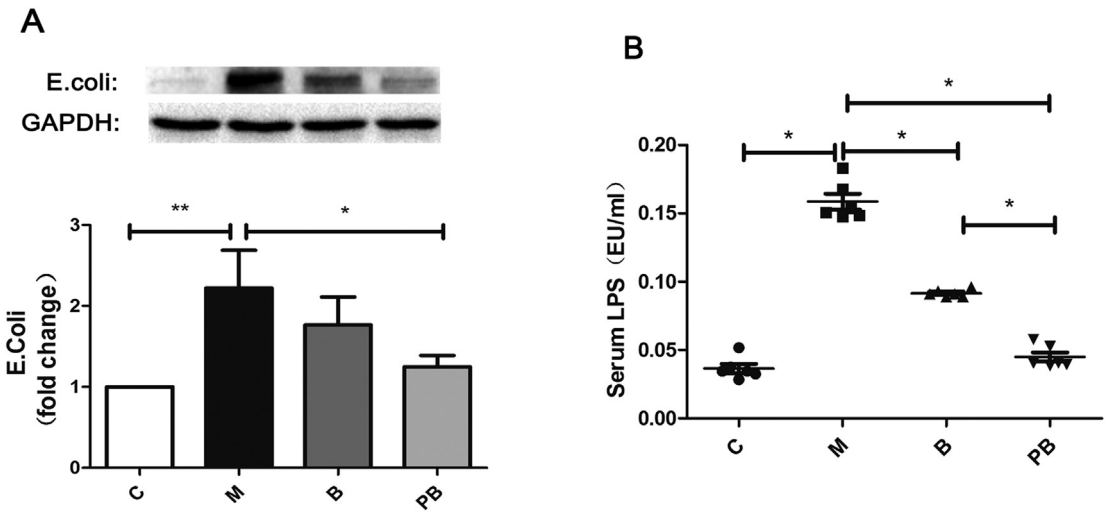

Fig. 4. Effect of BMMSCs and P-BMMSCs treatment on bacteria translocation. Panel A shows the level of *E. coli* protein in the liver evaluated by western blotting and normalized to GAPDH. Data were presented as fold changes relative to control group. Panel B displays serum LPS level quantitated by ELISA. Results were mean ± SEM from six mice. *P < 0.05, **P < 0.01.

In conclusion, our findings confirm that BMMSCs can have protective effects on intestinal barrier dysfunction and liver injury caused by alcohol. The therapeutic activity of BMMSCs may be related to its regulation of intestinal HIF-2α gene expression and innate lymphoid cells-NKB cells. Therefore, BMMSCs especially TLR3 activated BMMSCs could be a potential therapeutic strategy for patients with ALD.

Declarations of interest

None.

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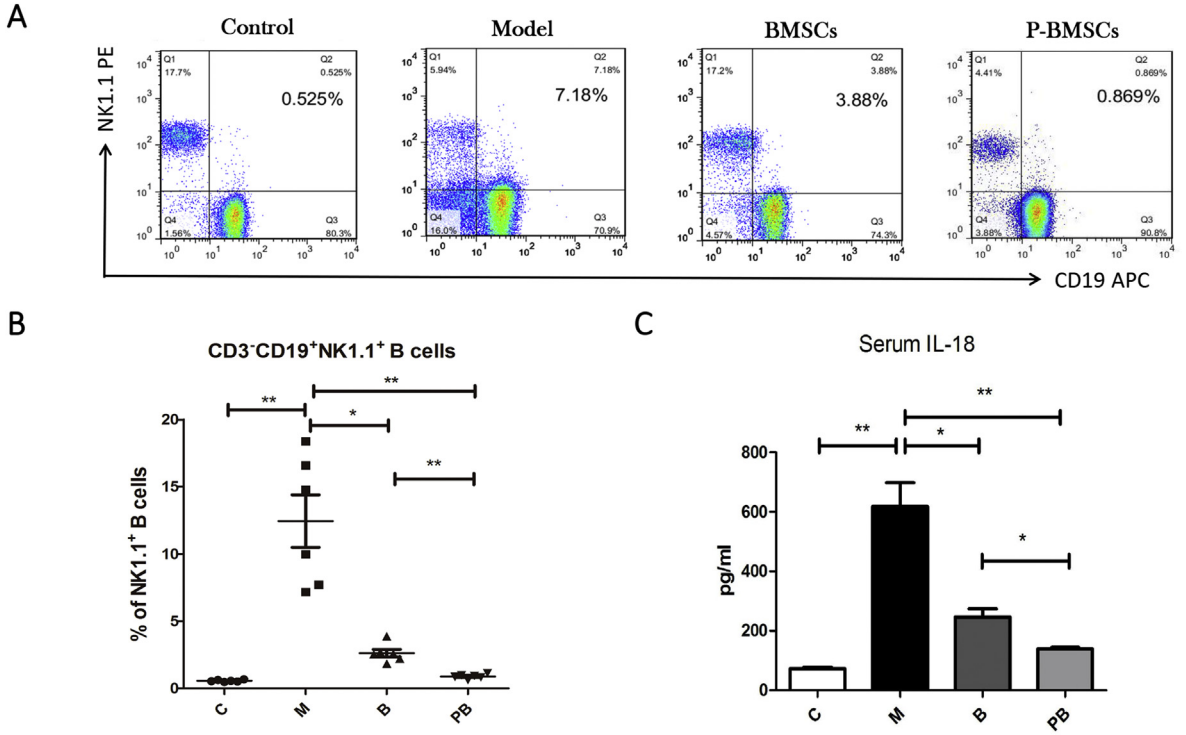

Fig. 5. Effect of BMMSCs and P-BMMSCs on NKB cells and Serum IL-18. Panel A shows a typical flow cytometric diagrams of CD3-CD19 + NK1.1 + NKB cells relative to the corresponding percentage of CD3- NK cells. Panel B displays the ratio of CD3-CD19 + NK1.1 + NKB to CD3- NK cells in scatter plot. The plot was obtained from > 20,000 cells. Results were presented as mean ± SEM from six independent experiments. *P < 0.05, **P < 0.01 and #P < 0.001. Panel C shows serum IL-18 level quantitated by ELISA. Results were expressed as mean ± SEM from six mice. *P < 0.05 and **P < 0.01.

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