The Protection of Midazolam Against Immune Mediated Liver Injury Induced by Lipopolysaccharide and Galactosamine in Mice

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Objectives: Liver macrophages agitated by Lipopolysaccharide (LPS) can enhance immuno-inflammatory responses in the liver which mediate liver injury and result in dysfunction. Midazolam has been reported to have inhibitory effects on activated immunity and escalated inflammation, however, what the effects of midazolam on the liver injury caused by excessive immuno-inflammatory response in sepsis, and what influence it will exert on inflamed liver macrophages need to be elucidated.

Methods: In the present study, LPS and galactosamine-induced acute liver injury mice were used to observe the effect of midazolam in vivo. LPS-stimulated bone marrow cells were used to evaluate the influence of midazolam on monocytes in vitro.

Results: Midazolam prevented liver tissue injury and decreased serum alanine transaminase (ALT) level in LPS plus galactosamine treated mice. Mechanistically, midazolam suppressed tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) produced by LPS stimulated liver macrophages in vivo and bone marrow monocytes in vitro, and reduced the expression of major histocompatibility complex class II (MHC II), cluster of differentiation 40 and 86 (CD40 and CD86) on the cell surface. These results could be reversed by PK-11195, a peripheral benzodiazepine receptor (PBR) blocker.

Conclusion: Midazolam can prevent liver from LPS-induced immune mediated liver injury by inhibiting inflammation and immune activation in liver macrophages.

Keywords: immunity, inflammation, lipopolysaccharide, liver injury, macrophage, midazolam

Abbreviations: ALT, alanine transaminase; Arg-1, Arginase-1; BM, bone marrow; CCR2, CC chemokine receptor 2; CD40, cluster of differentiation 40; CD86, cluster of differentiation 86; DMSO, dimethyl sulfoxide; FACS, Fluorescence activated cell sorter; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL-1β, interleukin-1β; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MHC II, major histocompatibility complex class II; NF-κB, nuclear factor-κB; NS, normal saline; PBR, peripheral benzodiazepine receptor; PCR, polymerase chain reaction; Th1, T helper 1; TNF-α, tumor necrosis factor alpha.
INTRODUCTION

Liver dysfunction is a common complication of sepsis with an approximate incidence of 40% (van Gestel et al., 2004; Cheng et al., 2007; Kobashi et al., 2013). The mortality of septic patients with liver dysfunction remains at 54%−68%, which is higher than that of respiratory dysfunction in such patients (Cheng et al., 2007; Yan et al., 2014). In the process of sepsis, lipopolysaccharide (LPS) and the subsequently activated immunity and inflammation provokes liver macrophages in eliminating pathogens by phagocytosis, antigen presentation, high level cytokine and chemokine secretion, and oxygen and nitrogen radical production. However, these originally defensive reactions often result in liver tissue damage, which in turn can enhance the immuno-inflammatory responses in the injured liver. Thus, the reciprocal causation between the overreacted immuno-inflammatory responses and liver injury constructs a vicious circle and develops into liver dysfunction and even failure (Antoniades et al., 2008; Heymann and Tacke, 2016).

Midazolam, a benzodiazepine derivative, has been routinely used for sedation in critically ill patients in intensive care units. Recent studies have shown that midazolam depressed plasma levels of interleukin-1β, 6, 8 (IL-1β, 6, 8) and tumor necrosis factor-α (TNF-α) in critically ill patients (Helmy and Al-Attiyah, 2001). In murine models, midazolam reduced cluster differentiation 80 and 86 (CD80 and CD86), and major histocompatibility complex class II (MHC II) on dendritic cells and inhibited the proliferation of CD3+T cells and T helper 1 (Th1) cellular immune response (Ohta et al., 2011). Such evidence suggests that midazolam might be beneficial to septic patients who are suffering uncontrolled immuno-inflammatory responses. As for sepsis-induced liver injury, whether midazolam will aggravate liver dysfunction and what influence it might exert on liver macrophages during liver inflammation are still not clear. Therefore, we studied the effect of midazolam on acute liver injury induced by LPS and galactosamine in mice and investigated its effect on the immuno-inflammatory responses in liver macrophages in vivo and in bone marrow monocytes in vitro. Our results demonstrated that midazolam prevented acute liver injury and protected liver function mainly by inhibiting immuno-inflammatory responses in liver macrophages, especially in the liver infiltrating monocytes. A possible mechanism of midazolam could be, at least partially, due to blocking the nuclear factor-κB (NF-κB) signaling pathway after midazolam binding to the peripheral benzodiazepine receptor (PBR) in macrophages.

MATERIALS AND METHODS

Mice
Male C57BL/6 wild type mice, which were 8-week-old and specific-pathogen-free, were purchased from Beijing Vital River Laboratory (Beijing, China). The mice were housed under pathogen-free conditions with a 12-h light-dark cycle and free access to food and water in the animal facilities at the Beijing Friendship Hospital. All experimental procedures were conducted in accordance with the protocol approved by the Institutional Animal Care and Use Committee, Beijing Friendship Hospital, Capital Medical University.

Reagents and Antibodies
Midazolam injection (batch No. H19990027) was purchased from Jiangsu Nhwa Pharmaceutical Co., Ltd., (Jiangsu, China). Escherichia coli LPS (O111:B4) and galactosamine were purchased from Sigma-Aldrich (St Louis, MO, United States). PK11195 (ab109497) was purchased from Abcam (Cambridge, United Kingdom). Fluorochrome-conjugated antibodies against mouse CD45, CD11b, Ly6G, Ly6C, F4/80, TNF-a, CD86, MHC II, CD40, CC chemokine receptor 2 (CCR2), Biotin anti-IL-1β, streptavidin-APC, TER-119, GR1, and B220 were listed in the Supplementary Table S1.

LPS and Galactosamine Induced Acute Liver Injury
Mouse acute liver injury was induced by intraperitoneal injection of LPS (5 ug/kg body weight) plus galactosamine (200 mg/kg body weight) (LG group, n = 5). To assess the effects of midazolam, mice were administered with midazolam intraperitoneally (8 mg/kg body weight) 30 min before and subcutaneously (4 mg/kg body weight) 30 min after the injection of LPS and galactosamine (MLG group, n = 5). Mice received only midazolam without LPS and galactosamine were regarded as the Mida group (n = 5). All reagents above were dissolved in normal saline (NS) at the indicated concentrations. NS was used as the blank vehicle in the mice of the Control group (n = 5). The mice were killed 12 h after the LPS and galactosamine injection. Blood and liver samples were harvested at the time of execution.

Serum ALT Level
Serum ALT levels were measured using the Alanine Aminotransferase Assay Kit (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) according to the manufacturer’s instructions.

Histological Analysis
Liver tissue was fixed in 4% paraformaldehyde overnight, embedded in paraffin and cut into 4-μm sections. The sections were stained with hematoxylin and eosin.

Analysis of Immunocytes From Mouse Liver in vivo
The liver was perfused with NS by inserting a syringe into the left ventricle. Livers were excised, minced using a gentle MACS Dissociator (Miltenyi Biotec, Italy), and digested by collagenase IV (Sigma-Aldrich, United States)/DNase I (Roche, Germany), and then intrahepatic immunocytes were isolated and purified by percoll density gradient centrifugation according to standard procedures (Zhang et al., 2005). All cells
were stained with the following fluochrome-conjugated antibodies: phycoerythrin-Cyanine7 anti-CD45, fluorescein isothiocyanate anti-CD11b, allophycocyanin-Cy7 anti-Ly6G, allophycocyanin anti-F4/80. The experimental conditions of the antibodies are listed in **Supplementary Table S1**. Liver infiltrating monocytes (CD45^+Ly6G^-CD11b^high/F4/80^low^), Kupffer cells (CD45^+Ly6G^-CD11b^low/F4/80^high^), and liver neutrophils (CD45^+CD11b^-Ly6G^+) subsets were examined on an Aria II flow cytometer with a fluorescence-activated cell sorter (FACS) (BD Biosciences, San Jose, CA, United States) from liver immunocytes. The gating strategy used for flow cytometry on liver immunocytes is shown in **Supplementary Figure S1**. The data were analyzed using FlowJo software (Treestar, Ashland, OR, United States).

**Bone Marrow (BM) Cell Culture and Treatment**

BM cells were extracted from femurs and tibias of healthy C57BL/6 mice; red blood cells were removed by RBC lysis buffer (Qiagen, Valencia, CA, United States). Then, GR1^+^, TER119^+^, and B220^+^ cells were depleted using magnetically conjugated antibodies. After that, BM cells were suspended in complete medium (RPMI 1640 supplemented with 10% fetal bovine serum, 1% L-glutamine, 100U/ml penicillin, and 100 ug/ml streptomycin), and were seeded into 24-well-plates with the number of 3 \times 10^5/well. Cells were incubated in 95% air–5% CO_2 humidified atmosphere at 37°C. In evaluation of the cell responses to midazolam and LPS, BM cells were cultured in complete medium with or without midazolam (100 uM) for total 16 h, and were challenged by LPS (100 ng/ml) in the last 4 of 16 h. BM cells incubated only in complete medium were regarded as the control. BM cells incubated in complete medium with midazolam only were regarded as the Mida group. In addition, to examine if cells could response to midazolam via the PBR, PK-11195 (100 uM), a selective antagonist of PBR, was applied 30 min before the incubation of midazolam followed by LPS stimulation. PK-11195 was first resolved in dimethyl sulfoxide (DMSO), and then diluted 1000-fold in complete medium to ensure the final concentration of DMSO would not exceed 0.2% (v/v) (Anderson et al., 2011).

**Real-Time Polymerase Chain Reaction (Real-Time PCR)**

Total RNA was extracted from liver tissues and cultured cells using the RNeasy Mini-Kit (Qiagen, Valencia, CA, United States) and reverse transcribed to cDNA using the PrimeScript™ RT Reagent Kit (TaKaRa Bio, Shiga, Japan). TNF-α, IL-1β, CCR2, inducible nitric oxide synthase (iNOS), and Arginase-1 (Arg-1), CD86, CD40, NF-κB (p105) and p65 (Rel A) mRNA were quantified by real-time PCR using the ABI 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, United States). The expression of each gene was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and quantified using the 2^−ΔΔCt method. The primer sequences are shown in **Supplementary Table S2**.
The ALT levels and mRNA expressions had no apparent differences between the Control group and the Mida group (Figure 1A,C).

**Midazolam Inhibited Liver Inflammatory Responses Mainly by Its Effects on Liver Macrophages**

In LPS plus galactosamine induced liver injury, the number of liver immunocytes, the proportions of liver infiltrating monocytes (Liver-Mono, CD45+Ly6G−CD11b<sup>high</sup>F4/80<sup>low</sup>) and neutrophils (Liver-Neu, CD45<sup>+</sup>CD11b<sup>+</sup>Ly6G<sup>+</sup>) were increased markedly; and midazolam led to a significant reduction in the number of liver immunocytes and the proportion of infiltrating monocytes, but did not affect intrahepatic neutrophils. Also, the proportion of Kupffer cells (CD45<sup>+</sup>Ly6G<sup>−</sup>CD11b<sup>low</sup>F4/80<sup>high</sup>) was not affected by LPS and galactosamine; and midazolam expanded this portion but without significance (Figure 2A–D). Based on the evidence above, we speculated that midazolam might restrict liver innate inflammatory responses mainly by its effect on liver macrophages.

We examined pro-inflammatory molecules and cell markers in Kupffer cells and liver infiltrating monocytes by flow cytometry. Compared with the Control group, LPS plus galactosamine obviously upregulated the expression of CCR2, IL-1β, IL-6, CD40, CD86, and CCR2, whereas midazolam downregulated those gene expressions with significance except CCR2 (Figure 3B). Moreover, midazolam upregulated the Arg-1 mRNA in LPS stimulated BM cells, while LPS alone had no such effect. Contrary to Arg-1, midazolam markedly inhibited iNOS transcription in LPS stimulated BM cells (Figure 3B). We noticed that these changes of Arg-1 and iNOS acquired in vitro were similar to those obtained in vivo, verifying that midazolam could restrain the pro-inflammatory function of macrophages in response to LPS.

In addition, gene transcripts of NF-κB subunits p105 and p65, which were upregulated after LPS stimulation, were remarkably downregulated in the presence of midazolam (Figure 3B). The mRNA alteration of NF-κB subunits was parallel to that of TNF-α and IL-1β described in this study, suggesting that...
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FIGURE 2 | Midazolam reduced liver infiltrating monocytes and inhibited the production of the inflammatory molecules by liver macrophages especially by liver infiltrating monocytes. (A) The number of liver immunocytes in each group. Statistical analysis of the proportion of (B) liver neutrophils, and (C) liver infiltrating monocytes (Liver-Mono) and Kupffer cells (Liver-KC), measured by flow cytometry. (D) Representative flow cytometry images of liver infiltrating monocytes and Kupffer cells (lower) in the liver tissues of each group. (E) Statistical analysis of inflammatory molecules produced by liver infiltrating monocytes (Mono) and Kupffer cells (KC), respectively, which were measured by flow cytometry. The data are presented as the mean ± SD, n = 4 in each group. The difference among groups was examined by one-way ANOVA analysis and comparison between two groups was analyzed by t-test. ∗p < 0.05, ∗∗p < 0.01, NS no significance. LG = LPS+galactosamine. MLG = midazolam+LPS+galactosamine. Mida = midazolam.

the inhibitory effect of midazolam on inflammatory responses of macrophage might partially be due to the attenuation of NF-κB activity by the reduction of p105 and p65 mRNA transcription.

The Anti-inflammatory Effect of Midazolam on BM Monocytes Was Exerted via the PBR

It is well known that the binding sites of midazolam exist inside the central nerve system and also in peripheral tissues (Kaynar et al., 2013). Our flow cytometry results exhibited the existence of the PBR in BM monocytes (Figure 4A). Therefore, we studied the cultured BM monocytes in the presence and the absence of PK-11195. PK-11195, a specific PBR blocker, almost fully switched TNF-α, CD40, and MHC II to the previous levels induced by LPS, and promoted IL-1β and CD86 to a level obviously higher than that in the absence of PK-11195 (Figure 4B). These results demonstrated that midazolam could inhibit the inflammatory response by binding to the PBR in the macrophages.

DISCUSSION

In the inflamed liver, the activated immunity and the excessive inflammation are always accompanied by liver tissue injury and liver dysfunction (Possamai et al., 2014; Heymann and Tacke, 2016). In this study, LPS plus galactosamine treated mice had an apparent elevation of serum ALT and classical histopathological manifestations of liver injury. Consistent with the acute liver injury, there was an accumulation of inflammatory cells and increased gene expression of pro-inflammatory cytokines. Compared to the mice treated by LPS plus galactosamine, the serum ALT was decreased, and liver histological features had no apparent damage in the mice pre-treated with midazolam followed by LPS and galactosamine. It suggested that midazolam might have preventive effect on LPS and galactosamine induced
FIGURE 3 | Effect of midazolam on BM cells cultured in vitro. (A) Statistical analysis of inflammatory molecules produced by the cultured BM cells, which were measured by flow cytometry. (B) Relative mRNA expression levels in the cultured BM cells, which were detected by real-time PCR. The data are presented as the mean ± SD, n = 3 in each group. The difference among groups was examined by one-way ANOVA analysis and comparison between two groups was analyzed by t-test. ∗p < 0.05, ∗∗p < 0.01, NS no significance. LPS = lipopolysaccharide. MLS = midazolam+LPS. Mida = midazolam.

FIGURE 4 | Midazolam might exert its anti-inflammatory function via PBR in the BM monocytes. (A) Representative flow cytometry images and statistical analysis of PBR in the cultured BM monocytes. (B) Statistical analysis of inflammatory molecules produced by BM monocytes pretreated with or without PK-11195, which were measured by flow cytometry. The data are presented as the mean ± SD, n = 3 in each group. The difference among groups was examined by one-way ANOVA analysis and comparison between two groups was analyzed by t-test. ∗p < 0.05, ∗∗p < 0.01, NS no significance. LPS = lipopolysaccharide. MLS = midazolam+LPS. PML = PK11195+midazolam+LPS.
liver injure. We also found that midazolam decreased TNF-α and IL-1β mRNA transcripts and reduced the fraction of liver infiltrating monocytes. These findings suggested that midazolam might protect the liver by suppressing the immuno-inflammatory responses in the liver. However, midazolam did not display an inhibition on liver neutrophils. This finding led us to speculate that midazolam might protect liver mainly by its effects on liver macrophages.

According to the difference of origin, liver macrophages are composed of resident Kupffer cells and infiltrating monocytes from circulation (Karlmark et al., 2009; Tacke and Zimmermann, 2014; Ju and Tacke, 2016). In the normal liver, Kupffer cells prevail in the macrophage pool and preserve immunological tolerance (Karlmark et al., 2009). In the injured liver, bone marrow-derived monocytes infiltrate into the liver and differentiate into macrophages, then dominate the subsequent inflammation together with the Kupffer cells (Dal-Secco et al., 2015). In the current study, liver macrophages were activated in the LPS plus galactosamine treated mice with an accumulation of TNF-α and IL-1β; and midazolam hindered macrophages from escalating inflammatory responses by suppressing the production of these two cytokines in liver infiltrating monocytes. The findings suggested that midazolam had the ability to downregulate the innate immunity and pro-inflammatory action of the macrophages in response to inflammatory insults.

MHC II (Antoniades et al., 2008; Varin and Gordon, 2009), together with costimulatory molecules CD86 (Yang et al., 2013; Ruiz-Rosado Jde et al., 2016) and CD40 (Seino and Taniguchi, 2005; Shibata et al., 2016) on the macrophages are essential in antigen presentation, which can activate T cells and elicit subsequent cellular and humoral immune responses during the liver injury (Kimura et al., 2006; Antoniades et al., 2008; Elgueta et al., 2009). In our study, the expressions of MHC II, CD40, and CD86 on the liver macrophages were elevated in LPS and galactosamine injured liver, and depressed in the treatment of midazolam; and the change of these molecules was mainly found in the liver infiltrating monocytes. These results suggested that midazolam impeded the inherent ability of antigen presentation in liver macrophages.

Numerous murine liver injury models indicate that the migration and accumulation of macrophages in the injured liver are dependent on CCR2 (Zigmond et al., 2014; Mossanen et al., 2016; Wang et al., 2016). Our study showed that CCR2mRNA was increased in the injured liver, specifically, CCR2 was highly expressed on both Kupffer cells and liver infiltrating monocytes in the LPS plus galactosamine treated mice. With the treatment of midazolam, CCR2 was reduced both in the injured liver and on liver macrophages, which might impede chemotaxis and migration of macrophages.

Ly6C<sup>high</sup> monocytes are often massively recruited into the injured area of liver, and are considered to be pro-inflammatory M1 phenotype (Karlmark et al., 2009; Zigmond et al., 2012). With the treatment of midazolam, the fraction of Ly6C<sup>high</sup> monocytes in the mouse liver was reduced.

Our data from the in vivo experiments revealed that midazolam could prevent liver injury probably via regulating liver macrophage functions, such as pro-inflammatory cytokines production, antigen presentation, and macrophage migration. Further, we found that midazolam had a greater impact on liver infiltrating monocytes than on Kupffer cells.

To verify the results acquired in vivo, we further isolated BM cells and cultured in the presence or absence of midazolam followed by LPS stimulation. For the BM monocytes, midazolam had similar effects in vitro as to those in vivo, except for CCR2.

Increasing evidence demonstrates that liver macrophages have pro-inflammatory actions (M1) in pathogen elimination and tissue destruction, and also possess anti-inflammatory properties (M2) in resolving inflammation and promoting tissue repair (Ramachandran et al., 2012; Sica et al., 2014). Since the M1 macrophages advocate the Th1 immune response in association with iNOS (Karlmark et al., 2009; Raber et al., 2012) and M2 can reduce inflammation by upregulating Arg-1 (Cassetta et al., 2011), we detected the mRNA of iNOS and Arg-1 in the mice livers and cultured BM cells. In the LPS plus galactosamine injured livers, the mRNA level of iNOS was increased, whereas the Arg-1 remained steady; when the liver injured mice were treated with midazolam, the mRNA of iNOS was decreased, but Arg-1 was upregulated. Similar changes of iNOS and Arg-1 were also seen in the cultured BM cells. These results suggested that midazolam could facilitate monocytes to switch the phenotype into M2 with an imaginable reduction of inflammation in the liver.

NF-κB is a major intracellular signaling pathway involved in the progress of inflammation. The activated NF-κB pathway may result in the products of pro-inflammatory cytokines and mediators (O’Neill and Bowie, 2007; Qin et al., 2016). In our study, we found a massive production of pro-inflammatory cytokines (such as TNF-α, IL-1β, and iNOS) and increased antigen presenting molecules (such as MHC II, CD86, and CD40) both in the liver macrophages stimulated by LPS and galactosamine in vivo and in the BM monocytes agitated by LPS in vitro. Consistent with the changes above, the mRNA levels of NF-κB components p105 and p65 were also increased in the LPS stimulated BM cells, indicating the activation of NF-κB. These findings are consistent with the results in different experiments (Krappmann et al., 2004; Hansen et al., 2015; Qin et al., 2016; Park et al., 2017). With the treatment of midazolam, the escalated inflammation was suppressed both in vivo and in vitro, and the hindered pro-inflammatory responses of monocytes in vitro were accompanied by a decrease in mRNA transcripts of NF-κB. Kim et al. found that midazolam inhibited NF-κB activation in LPS-stimulated RAW264.7 cells by blocking IκBα degradation and inhibiting translocation of NF-κB p65 subunit into the nucleus (Kim et al., 2006). Therefore, we speculated that the anti-inflammatory effects of midazolam on macrophages were exerted, at least in part, by blocking the NF-κB pathway.

Midazolam has the equal ability in binding to the PBR and to the receptors in central nerve system. Therefore, we first detected the existence of PBR in the BM monocytes by flow cytometry. With a positive outcome, we subsequently applied PK-11195, an antagonist of PBR, to the cultured monocytes. Then we found that TNF-α, IL-1β, MHC II, CD86, CD40 and the fraction of Ly6C<sup>high</sup> monocytes, which were downregulated by midazolam...
in the LPS-stimulated monocytes, were upregulated by PK-11195. These reversal manifestations of PK-11195 indicated that binding to the PBR was responsible for the anti-inflammatory effect of midazolam on LPS-activated macrophages.

In summary, midazolam can prevent LPS induced immune mediated liver injury by suppressing the immune response of liver macrophages, and polarizing monocytes/macrophages from pro-inflammatory M1 to anti-inflammatory M2. These results can be, at least partially, attributed to the reduced activity in the NF-kB pathway after midazolam binding to the PBR in the macrophages.

AUTHOR CONTRIBUTIONS

All listed authors participated meaningfully in this study and that they have seen and approved the submission of this manuscript. JL, HT, and XiaZ participated in performing the research, analyzing the data, and initiating the original draft of the article. CZ, HJ, YT, XinZ, and XL participated in performing the research and collecting the data. DZ, MD, and XS established the hypotheses, supervised the studies, analyzed the data, and co-wrote the manuscript.

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SUPPLEMENTARY MATERIAL

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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