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Original Article

CCR2 dependent neutrophil activation and mobilization rely on TLR4-p38 axis during liver ischemia-reperfusion injury

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Abstract: Liver ischemia-reperfusion injury (IRI) is a common clinical problem in which neutrophil recruitment is an essential event. Our previous study revealed the important role of C-C motif chemokine receptor 2 (CCR2) in neutrophils during liver IRI. The aim of the present study was to further investigate the underlying mechanisms mediating the changes in CCR2 expression in neutrophils during this pathophysiological process. Herein, we found that TLR4 ablation reduced neutrophil mobilization from the bone marrow and the subsequent infiltration into the liver during liver IRI; neutrophil-derived CCR2 expression was also repressed. In addition, neutrophil mobilization was dependent on CCR2 expression in neutrophils, which in turn relied on activation of the TLR4-p38 axis during liver IRI. In conclusion, neutrophil-derived CCR2 expression regulates neutrophil mobilization from the bone marrow and infiltration into the liver, which requires activation of the TLR4-p38 axis during liver IRI.

Keywords: CCR2, neutrophils, TLR4, ischemia-reperfusion

Introduction

Liver ischemia-reperfusion injury (IRI) is a common issue in clinical practice that arises from organ transplantation, liver resection, and hemorrhagic circulatory shock [1-3]. Liver IRI inevitably leads to abnormal liver function, acute liver failure, and multiple organ failure or even death [1]. Therefore, investigations into the detailed mechanisms underlying the pathophysiological processes that occur during liver IRI are of great clinical significance to optimize therapeutic measures.

Neutrophils form the first line of defense by the innate immune system, and neutrophil mobilization is one of the most important features of the innate immune response [4, 5]. Recruitment of neutrophils into inflamed tissues is a typical phenomenon observed in conditions of infectious or sterile inflammation [6]. Without exception, neutrophils mobilize from the bone marrow and infiltrate into the ischemic primed liver during liver IRI, which directly causes liver damage [7-9]. Therefore, studies investigating the mechanism of neutrophil migration are of great significance for understanding the pathophysiology of liver IRI.

In the physiological state, C-C motif chemokine receptor 2 (CCR2) is mainly expressed in monocytes and lymphocytes, but not in neutrophils [10, 11]. However, the expression of chemotactic receptors in neutrophils changes in response to acute inflammation [12]. Nevertheless, studies of the relationship between neutrophils and CCR2 have mainly been confined to cases of infectious inflammation [8]. In our recent study, we found that CCR2 regulates neutrophil mobilization during liver IRI [13]. Therefore, we performed the present study to further investigate the mechanism by which CCR2 is expressed in neutrophils.

Toll-like receptors (TLRs) are expressed in many cell types, particularly in the cells of the innate

immune system, where they recognize infections and dangerous potential invaders. Thirteen mammalian TLR analogues have been identified to date [14], including TLR4, which has been widely investigated in the context of various diseases [15, 16]. TLR4 plays a complex, cell type-specific role in liver IRI [17-20]. However, the dominant effect of TLR4 on liver IRI is to drive damage by promoting the inflammatory response to damage-associated molecular pattern molecules, such as high mobility group box 1 [21], and its role in mediating the expression of CCR2 and neutrophil infiltration remains unclear.

In this study, we established a liver IRI mouse model, and compared the extent of neutrophil mobilization from the bone marrow and infiltration in the liver between wild-type and TLR4-deficient ($TLR4^{-/-}$) mice. Moreover, we evaluated the gene and protein-level expression of CCR2 in the neutrophils of these mice, as well as the expression of other chemokines and their receptors that may be responsible for attracting neutrophils from the bone marrow into the ischemia injury-primed liver. These findings are expected to provide new insights into the relationship between TLR4 and CCR2 expression in neutrophils and the underlying mechanisms of liver IRI to highlight new therapeutic targets and establish clinical management strategies.

Materials and methods

Animals

Adult male C57BL/6 mice were obtained from the Center for Animal Experiment of Wuhan University. $TLR4^{-/-}$ mice in the C57BL/6 background were a gift from Dr. Billiar and were housed under specific-pathogen-free conditions at Huazhong University of Science and Technology (Wuhan, China). Eight to ten-week-old wild-type (WT) C57BL/6 and $TLR4^{-/-}$ (22-25 g) male mice were used. Previous studies have reported that less than 3% (3/84) of female mice successfully showed liver IRI in establishment of the model [22]. This female protective effect against renal, liver, and heart IRI has been attributed to estrogen and the activation of estrogen receptors [23-26]. Therefore, to avoid this estrogen effect, we deemed it reasonable to use only male mice in the present study. All animal experiments were approved by the Animal Care and Use Committee of Wuhan Union Hospital and were conducted in accordance with the National Institutes of Health Guidelines.

Liver IR model

Non-lethal segmental (70%) liver ischemia was induced using previously described methods [27]. Mice were anesthetized with pentobarbital (60 mg/kg, intraperitoneal injection). A midline laparotomy was performed, and the ligaments were carefully dissected. The portal vein, hepatic artery, and bile duct supplying the median and left lateral lobes of the liver were clamped with an artery microclamp (Fine Science Tools). The temperature was maintained at 32-33°C during ischemia using a warming incubator chamber. After 60 min of segmental liver ischemia, the clamp was removed to initiate liver reperfusion. The mice were sacrificed at 1 h or 6 h of reperfusion. The sham group received identical treatment but without microvascular clamp placement. Liver tissues were collected for subsequent analyses. The mice were injected with recombinant murine (rm) CCL2 (R&D Systems) immediately at the time of reperfusion via the tail vein, and the bone marrow or peripheral blood cells were examined by flow cytometry after 1 h of reperfusion. Mitogen-activated protein kinase (MAPK) inhibitors, including P38 inhibitor (SB-203580, Selleck, USA), c-Jun N-terminal kinase (JNK) inhibitor (SP600125, Selleck, USA), and extracellular-regulated kinase (ERK) inhibitor (PD98059, Selleck, USA), were intraperitoneally injected at a dose of 10 mg/kg 1 h before ischemia. A CCR2 inhibitor (RS504393, 2 mg/kg; Sigma-Aldrich) was administered 1 h before ischemia viaintraperitoneal injection according to previously described methods [28, 29].

Isolation of non-parenchymal cells (NPCs)

Hepatic NPCs were obtained from the liver using a previously described collagenase digestion method [13, 17]. The purity of the NPCs exceeded 95% and the viability was typically greater than 90%, as determined by flow cytometry and trypan blue exclusion, respectively. In brief, each liver was perfused *in situ* with phosphate-buffered saline (PBS) containing collagenase IV (1 mg/mL; Sigma-Aldrich) via the portal vein. The liver was removed, placed in PBS, and incubated at 37°C for 15 min. The liver was then torn using cell scrapers. The cell suspension was shaken on a shaking table at 37°C for 20 min and then filtered through a 70-μm nylon

mesh. The cell suspension was centrifuged (300 *g* for 11 min) and the supernatant was removed. NPCs were obtained after density-gradient centrifugation (400 *g* for 15 min) using OptiPrep™ (OptiPrep: PBS = 1:2; Axis-Shield) and then washed by high-speed centrifugation (1500 rpm for 5 min).

Real-time reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from the ischemic lobes with TRIZOL reagent (Invitrogen) and was reverse-transcribed using an RT reagent Kit (Thermo Fisher Scientific). Real-time PCR was conducted using primers targeting specific genes with SYBR Green Master Mix (Bio-Rad). Each sample was assessed in triplicate, and mean values were used for quantification. Relative gene expression profiles were analyzed by normalizing the levels of the target gene to those of the β-actin gene using the $2^{-(\Delta\Delta Ct)}$ method. The gene-specific primers were: *Ccl2*, F: 5'-GGTGTCCCAAAGAAGCTGTAGTT-3', R: 5'-GAGGTGGTTGTGGAAAAGGTAGT-3'; *Ccl7*, F: 5'-TGTCCCTGGGAAGCTGTTAT-3', R: 5'-GGAGTTGGGGTTTTCATGTCTA-3'; *Cxcl1*: F: 5'-CCAAACCGAAGTCATAGCCA-3', R: 5'-TGGGGACACCTTTTAGCATCT-3'; *Cxcl2*: F: 5'-GCCCAGACAGAAGTCATAGCC-3', R: 5'-TCTTTGGTTCTTCCGTTGAGG-3'; *Cxcr2*: F: 5'-GGGTCGTACTGCGTATCCTG-3', R: 5'-AGACAAGGACGACAGCGAAG-3'; *Ccr2*: F: 5'-ATCCACGGCATACTATCAACATC-3', R: 5'-CAAGGCTCACCATCATCGTAG-3'; and β-actin: F: 5'-AGCCATGTACGTAGCCATCC-3', R: 5'-CTCTCAGCTGTGGTGGTGAA-3'.

Western blot analysis

Total proteins were extracted from the cells using RIPA lysis buffer (Beyotime) supplemented with 1 mmol/L phenylmethylsulfonyl fluoride, followed by electrophoretic separation on 10% polyacrylamide gels and transfer onto nitrocellulose membranes (Life Science). The membranes were then blocked in Tris-buffered saline with Tween 20 (TBS-T) containing 5% milk for 2 h at room temperature and incubated with primary antibodies against p38, ERK, and JNK (Cell Signaling Technology) overnight at 4°C. The following day, the blots were washed with TBS-T and incubated with a peroxidase-conjugated secondary antibody (HuaAn Biotechnology) for 1 h at room temperature. Immunoreactive bands were assessed using a chemiluminescence imaging system (ChemiQ 4800 mini) after incubation with horseradish peroxidase.

Flow cytometry

Mouse femurs were flushed with PBS containing 0.5% bovine serum albumin to separate the bone marrow cells. Red blood cells were lysed using Hybri-Max red blood cell lysis buffer (Sigma-Aldrich). NPCs (isolated as described above) and cells from the bone marrow or blood were incubated with fluorochrome-conjugated anti-mouse antibodies for 30 min at 4°C in PBS containing 1% bovine serum albumin. Flow cytometry was performed using the following antibodies: Pacific Blue-conjugated anti-CD45, PE/Cy7-conjugated anti-CD11b, PE-conjugated anti-mouse Ly-6G (BD Biosciences), or anti-CCR2-APC (R&D Systems). Cells were washed with PBS and resuspended in PBS containing 1% paraformaldehyde at a density of 5×10^6 cells/mL. Flow cytometry was performed using a CyAn ADP analyzer (Beckman Coulter) for acquisition and compensation. Data analysis was performed off-line using FlowJo version 7.6 software.

Statistical analysis

All values are reported as means ± standard errors of the means. Significance was determined using Student's t-test and one-way analysis of variance, as appropriate. All analyses were performed using SPSS 15.0 software. Statistical significance was defined as $P < 0.05$.

Results

CCR2 mediates the effects of TLR4 on neutrophil mobilization from the bone marrow and infiltration into the ischemic-primed liver

The infiltration of neutrophils into the liver is an important event during liver IRI. We used fluorescence-activated cell sorting to investigate cell groups gated on the CD45+ NPCs to explore this process. Although both groups of mice showed significant neutrophil mobilization from the bone marrow after 6 h of reperfusion, neutrophil mobilization in the $TLR4^{-/-}$ mice was significantly decreased compared with that in the WT mice (**Figure 1A**). Similarly, neutrophil infiltration was significantly inhibited in the livers of the $TLR4^{-/-}$ mice after 6 h of reperfusion compared to those of WT mice (**Figure 1B**). The mRNA expression levels of all chemokines and

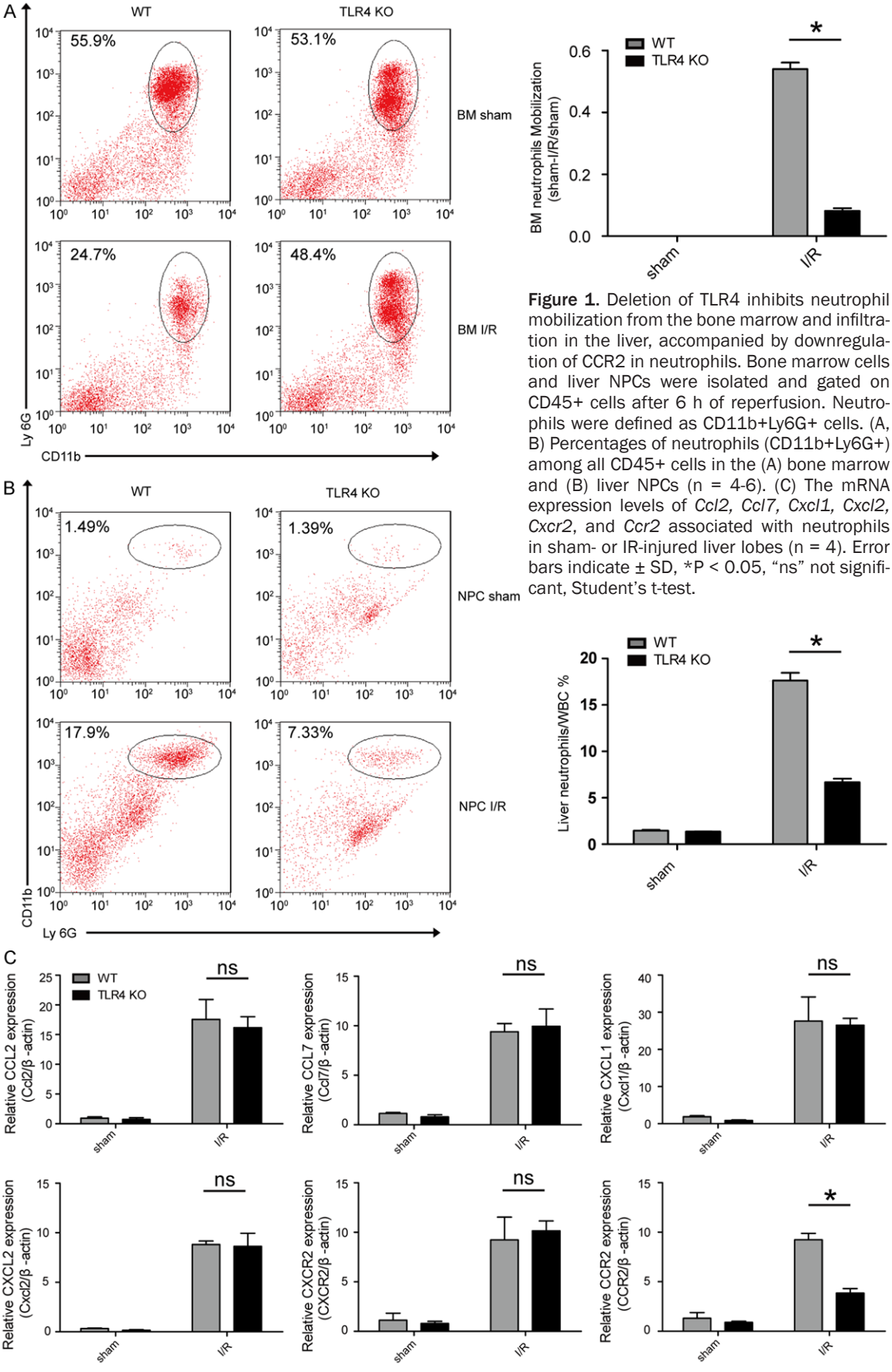

Figure 1. Deletion of TLR4 inhibits neutrophil mobilization from the bone marrow and infiltration in the liver, accompanied by downregulation of CCR2 in neutrophils. Bone marrow cells and liver NPCs were isolated and gated on CD45+ cells after 6 h of reperfusion. Neutrophils were defined as CD11b+Ly6G+ cells. (A, B) Percentages of neutrophils (CD11b+Ly6G+) among all CD45+ cells in the (A) bone marrow and (B) liver NPCs (n = 4-6). (C) The mRNA expression levels of *Ccl2, Ccl7, Cxcl1, Cxcl2, Cxcr2*, and *Ccr2* associated with neutrophils in sham- or IR-injured liver lobes (n = 4). Error bars indicate ± SD, *P < 0.05, "ns" not significant, Student's t-test.

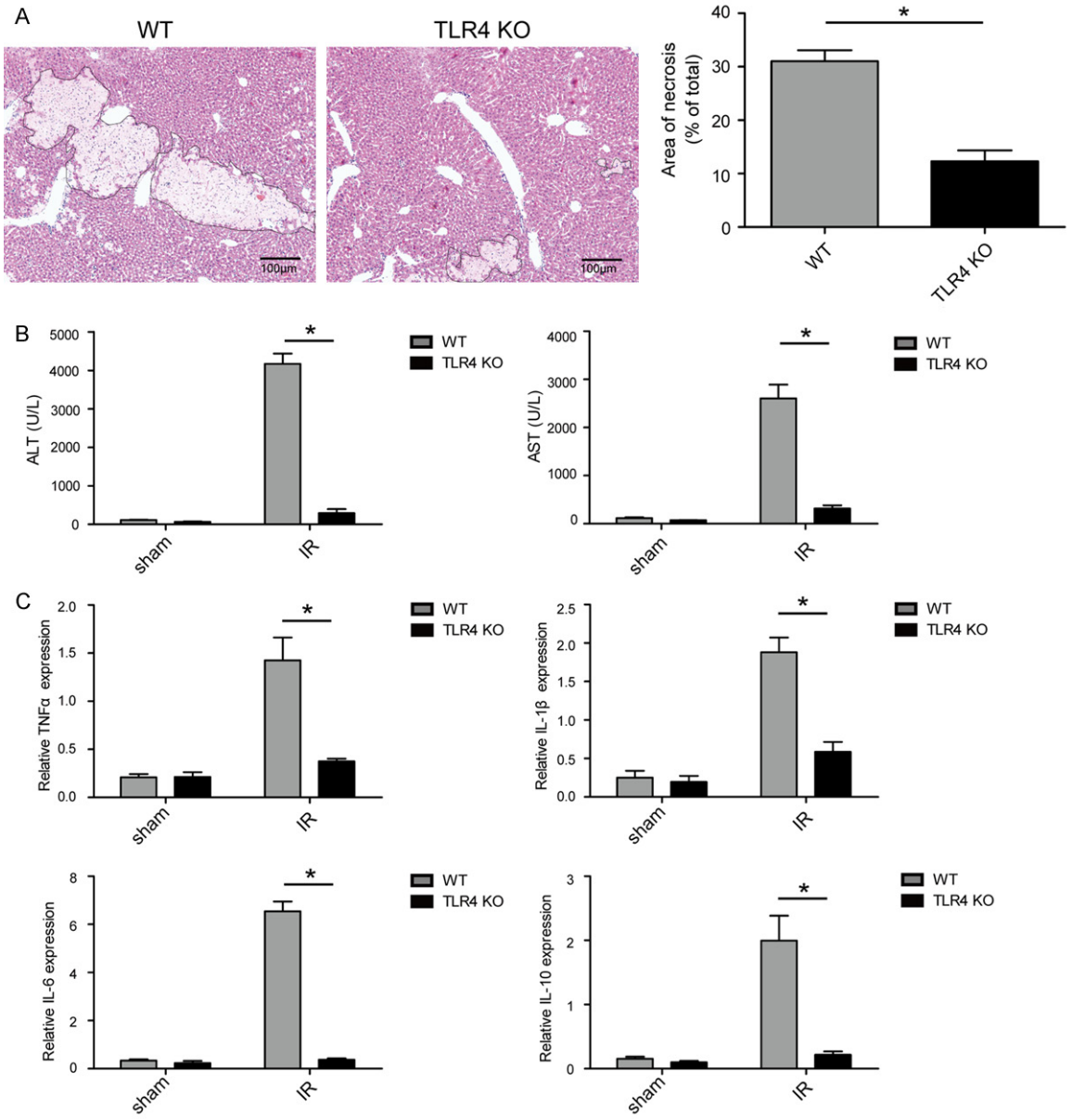

Figure 2. Deletion of *Tlr4* reduces liver injury in IR. Wild-type and TLR4 knockout (KO) mice were subjected to warm liver IR or a sham procedure, and then blood and liver tissues were collected. Mouse blood was obtained via cardiac puncture. Serum alanine aminotransferase (ALT) and aspartate transaminase (AST) levels were measured at the Clinical Laboratories of Wuhan Union Hospital (Wuhan, China). A. H&E staining after liver IR treatment in the two groups of mice. The necrotic area/whole area ratio was normalized in > 10 random fields. Nonviable tissue is marked by black lines. Scale bar, 100 μm. N = 3; error bars indicate ± SD, *P < 0.05, Student's t-test. B. ALT and AST enzyme levels in peripheral blood samples. N = 4-6; error bars indicate ± SD, *P < 0.05, Student's t-test. C. Real-time PCR analysis of the mRNA expression of cytokines in the IR ischemic liver lobes or sham liver of the two groups of mice. N = 4; error bars indicate ± SD, *P < 0.05, Student's t-test.

chemokine receptors were increased in the ischemic livers at 6 h of reperfusion compared with those in the sham group. However, only *Ccr2* expression was significantly different between the WT and TLR4$^{-/-}$ mice, with lower expression in TLR4$^{-/-}$ mice after liver IRI (**Figure 1C**).

Moreover, the histological necrosis and levels of liver enzymes were significantly reduced in the TLR4-deficient mice compared with the WT mice after IRI (**Figure 2A**, **2B**), and the mRNA expression levels of inflammatory cytokines were much lower in the ischemic liver lobes from the TLR4-deficient mice than those of WT

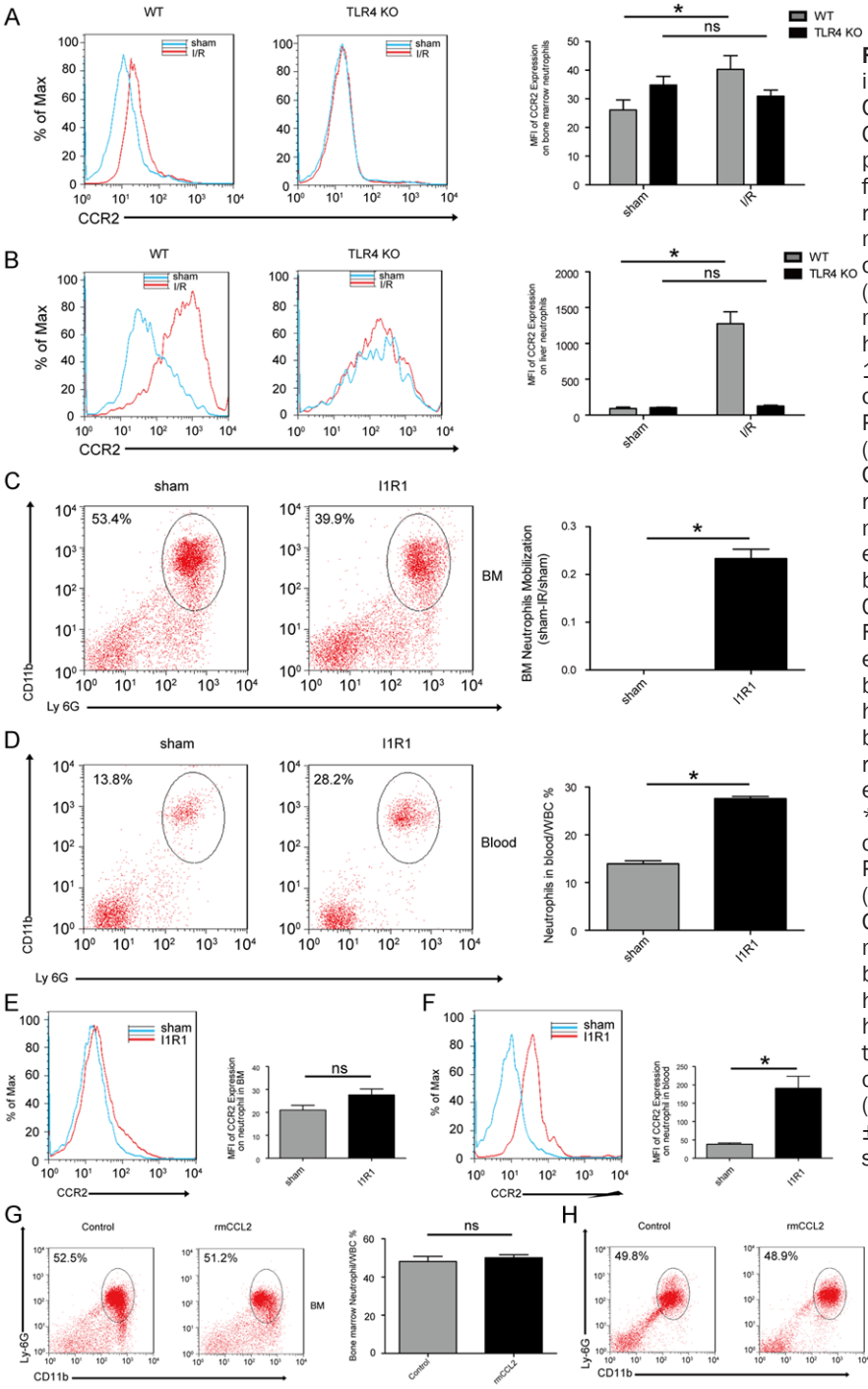

Figure 3. Knockout of TLR4 inhibits the expression of CCR2 in neutrophils. (A, B) Quantification of CCR2 expression in neutrophils by flow cytometry after 6 h of reperfusion in the (A) bone marrow and (B) liver NPCs of WT and TLR4$^{-/-}$ mice (n = 4-6). Wild type (WT) mice were subjected to 1 h of ischemia followed by 1 h of reperfusion (I1R1) or a sham procedure. (C, D) Percentage of neutrophils (CD11b+Ly6G+) among all CD45+ cells after 1 h of reperfusion in the (C) bone marrow and (D) peripheral blood (n = 4-6); error bars indicate ± SD, *P < 0.05, Student's t-test. (E, F) Quantification of CCR2 expression in neutrophils by flow cytometry after 1 h of reperfusion in the (E) bone marrow and (F) peripheral blood (n = 4-6); error bars indicate ± SD, *P < 0.05, "ns", not significant, Student's t-test. (G, H) Percentage of neutrophils (CD11b+Ly6G+) among all CD45+ cells in the (G) bone marrow and (H) peripheral blood of TLR4$^{-/-}$ mice after 1 h of ischemia followed by 1 h of reperfusion with injection of 500 ng of rmCCL2 or control IgG via the tail vein (n = 4-6). Error bars indicate ± SD, *P < 0.05, "ns", not significant, Student's t-test.

mice (**Figure 2C**). This result is consistent with previous studies [17, 20].

TLR4 ablation inhibits CCR2 expression in neutrophils during liver IRI

Based on the results above, we next investigated the potential role of TLR4 in mediating CCR2 expression in neutrophils during liver IRI. CCR2 expression was significantly increased in neutrophils derived from the bone marrow and liver NPCs after the IR process compared with that in the sham group only in the WT mice and not in the TLR4$^{-/-}$ mice (**Figure 3A**, **3B**). This finding indicated that neutrophil-derived CCR2 expression requires functional TLR4. To further confirm this result, we injected rmCCL2 to promote the mobilization of CCR2-expressing neutro-

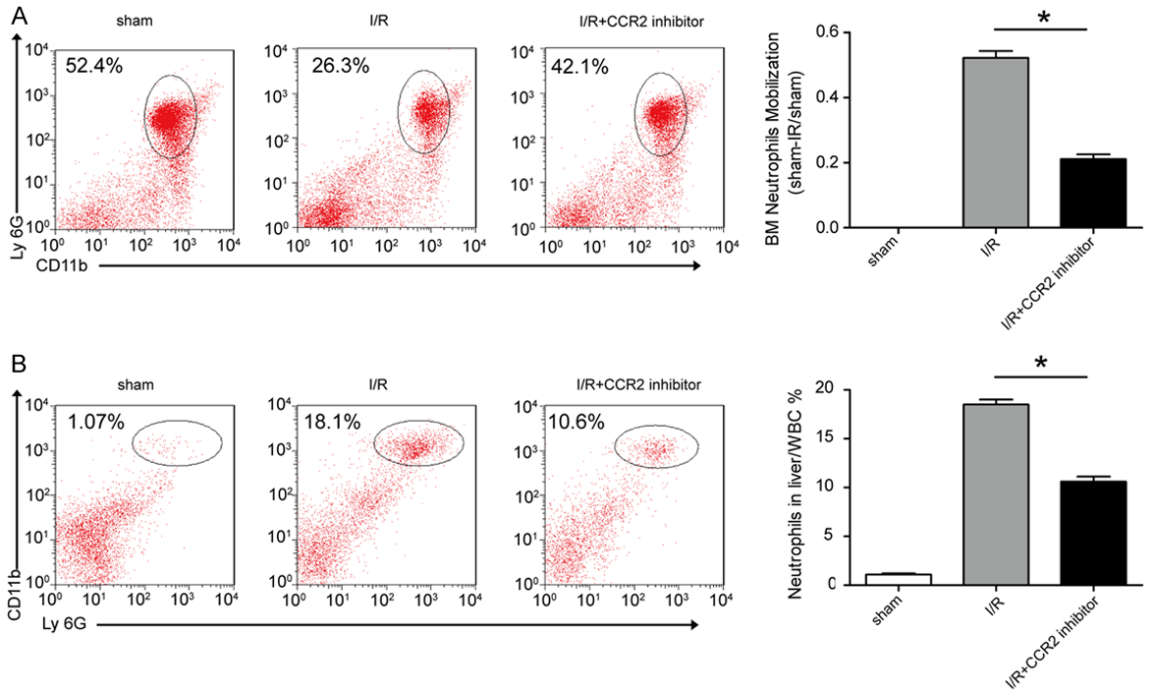

Figure 4. CCR2-expressing neutrophils play an important role in neutrophil infiltration of the liver during IR. The percentages of neutrophils (CD11b+Ly6G+) among all CD45+ cells in the (A) bone marrow and (B) liver NPCs in the sham, IR, and IR + CCR2 inhibitor groups (n = 4); error bars indicate ± SD, *P < 0.05, one-way ANOVA.

phils from the bone marrow to the peripheral blood. After 1 h of reperfusion, there was significant neutrophil mobilization from the bone marrow into the blood, along with increased CCR2 expression in peripheral neutrophils in the WT mice (**Figure 3C-F**). In addition, rmCCL2 injection promotes the mobilization of CCR2-expressing neutrophils from the bone marrow to the peripheral blood in WT mice [13]. By contrast, the administration of rmCCL2 did not promote the mobilization of TLR4$^{-/-}$ neutrophils from the bone marrow to the peripheral blood, indicating that CCR2 expression in neutrophils requires intact TLR4 function (**Figure 3G**, **3H**).

Inhibition of CCR2 function in neutrophils blocked their mobilization from the bone marrow and infiltration into the liver during IRI

We used a CCR2 inhibitor to further confirm the role of CCR2 in neutrophils during liver IRI. As expected, neutrophil mobilization from the bone marrow was significantly decreased in mice injected with the CCR2 inhibitor compared with that in control mice after 6 h of reperfusion (**Figure 4A**). Similarly, we observed a remarkable reduction in neutrophil infiltration in the livers of mice injected with the CCR2 inhibitor (**Figure 4B**). Thus, CCR2 expression in neutrophils is required for the mobilization of neutrophils from the bone marrow and infiltration into the liver.

CCR2 expression in neutrophils requires activation of the TLR4-p38 axis

We used specific inhibitors targeting the MAPK pathway, operating downstream of TLR4, to determine the signaling pathway that regulates CCR2 expression. First, we observed obvious up-regulation of the MAPK pathway in the ischemic liver lobes of the WT mice after IR compared with that in the sham mice, including p38, ERK, and JNK (**Figure 5A**). We then used inhibitors targeting the MAPK pathways to identify the specific signaling pathways associated with CCR2 expression in neutrophils. As shown in **Figure 5**, inhibition of the p38 pathway induced an obvious blockade of neutrophil mobilization from the bone marrow and neutrophil infiltration in the liver compared with the vehicle-injected mice (**Figure 5B**, **5C**). Similarly, CCR2 expression was inhibited in neutrophils derived from the bone marrow and liver NPCs of mice treated with the p38 inhibitor (**Figure**

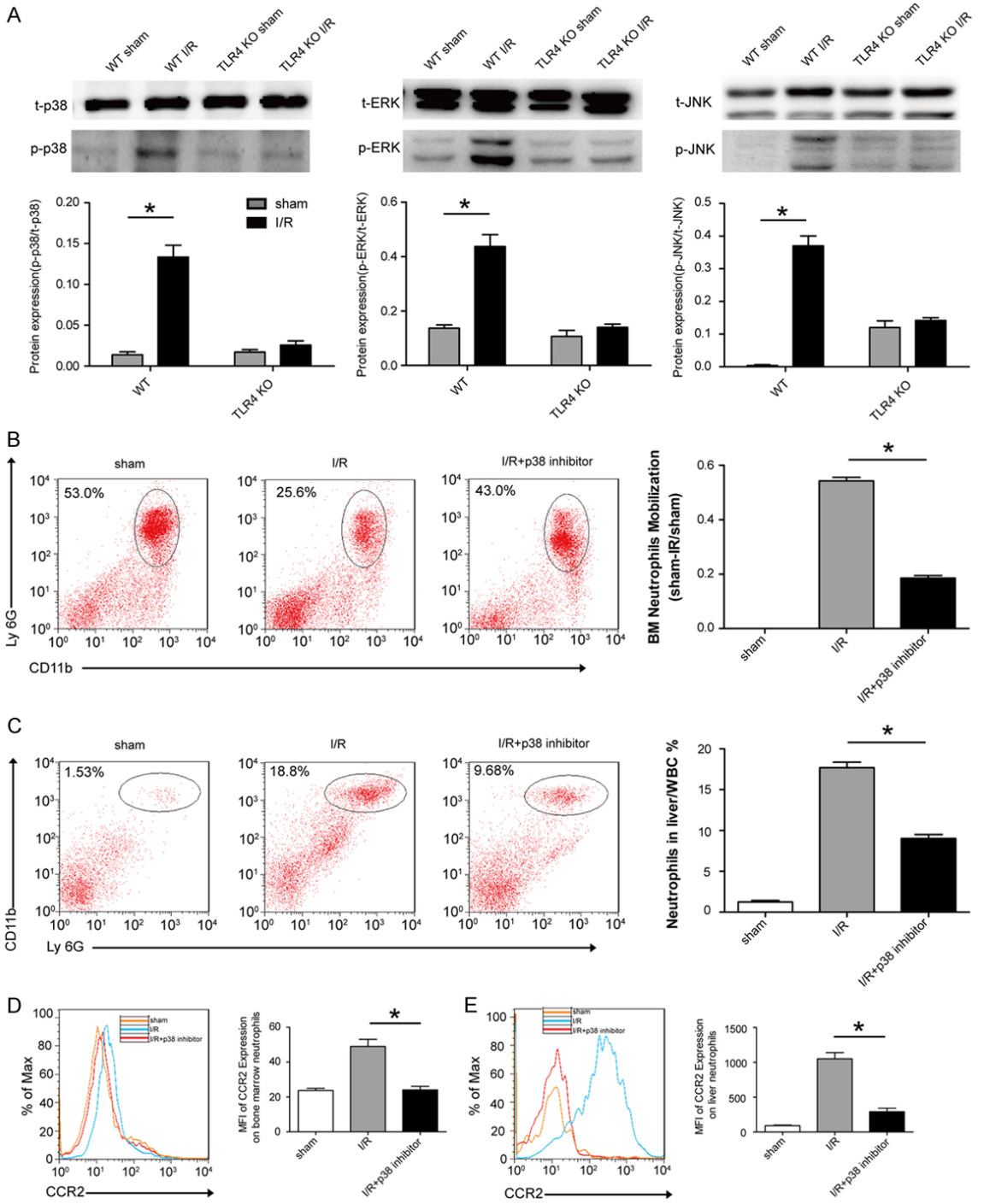

Figure 5. CCR2 in neutrophils is triggered by the TLR4-p38 axis during liver IR. (A) Intracellular MAPKs (p38, ERK, JNK) activation in sham- or IR-injured liver lobes of WT and TLR4$^{-/-}$ mice after 6 h of reperfusion determined by western blot. Protein expression was normalized by phosphorylated protein/total protein (e.g., p-p38/t-t38); n = 3, error bars indicate ± SD, *P < 0.05, Student's t test. (B, C) Percentages and (D, E) CCR2 expression quantification of neutrophils (CD11b+Ly6G+) among all CD45+ cells in the bone marrow (B, D) and liver NPCs (C, E) in the sham, IR, and IR + P38 inhibitor groups (n = 4); error bars indicate ± SD, *P < 0.05, one-way ANOVA.

5D, **5E**). In addition, we did not observe a change in CCR2 expression in neutrophils following injections of the ERK or JNK inhibitors (**Figure 6**). Thus, CCR2 expression in neutrophils during liver IRI requires the p38 signaling pathway.

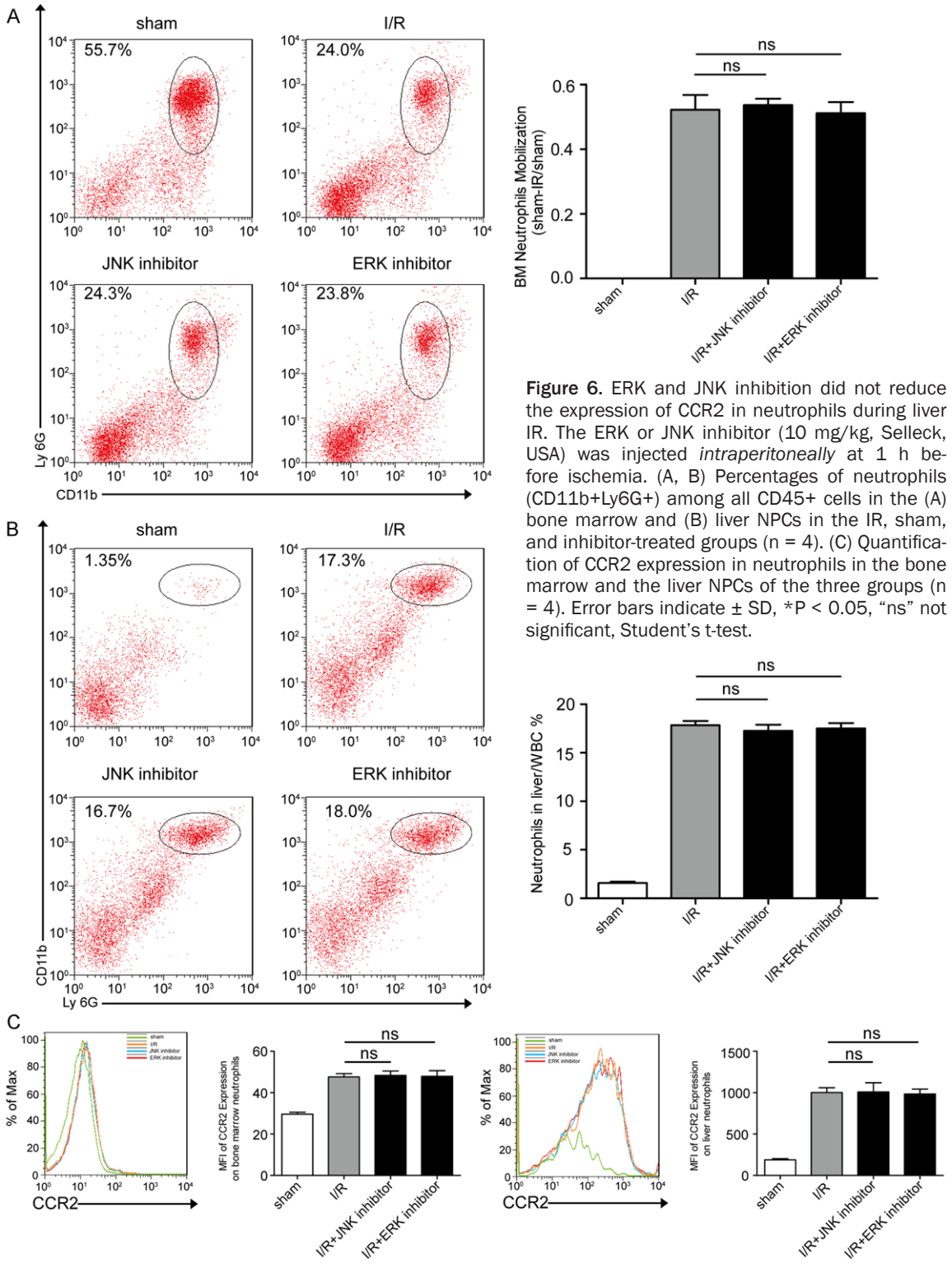

Figure 6. ERK and JNK inhibition did not reduce the expression of CCR2 in neutrophils during liver IR. The ERK or JNK inhibitor (10 mg/kg, Selleck, USA) was injected *intraperitoneally* at 1 h before ischemia. (A, B) Percentages of neutrophils (CD11b+Ly6G+) among all CD45+ cells in the (A) bone marrow and (B) liver NPCs in the IR, sham, and inhibitor-treated groups (n = 4). (C) Quantification of CCR2 expression in neutrophils in the bone marrow and the liver NPCs of the three groups (n = 4). Error bars indicate ± SD, *P < 0.05, "ns" not significant, Student's t-test.

Discussion

Liver IRI is a common issue, and has been associated with diverse and complex mechanisms [30]. A murine model of warm partial liver IRI is commonly used to investigate the underlying mechanisms, which shows good stability and repeatability [17, 27]. However, the clinical

translation of these experimental results has thus far been difficult because of anatomical and physiological differences between mice and humans, and the inevitable simplification of experimental work [31]. Based on the literature, we used 70% liver lobe ischemia for 1 h and reperfusion for 6 h as a suitable model to elucidate the underlying mechanism [21, 22, 32-34]. In addition, environmental temperature is an important factor that impacts the degree of liver injury observed during liver ischemia [35, 36]. Thus, we placed the mice into a baby incubator to maintain their temperature at 32°C during the ischemia procedure based on the results from our previous study [13].

Neutrophil mobilization from the bone marrow and infiltration into the liver is an important phenomenon that occurs during liver IRI and directly induces hepatocyte damage. CCR2 is highly expressed in neutrophils and plays a pivotal role in all major steps during the entry of neutrophils into peripheral tissues, including egress from the bone marrow into peripheral blood, movement from the blood into peripheral tissues, and recruitment to the inflammatory sites [37, 38]. By contrast, CXCR2 mainly mediates the recruitment of neutrophils into the infection focus [39, 40]. Our previous study revealed the relationship of CCR2 expression in neutrophils with neutrophil mobilization and infiltration in a liver IRI model [13]. Here, we investigated the underlying mechanisms responsible for these observations.

A previous study demonstrated that TLR4 expressed in neutrophils regulates neutrophil activation and lifespan, and that TLR4 mediates early neutrophil survival in a manner dependent on NF-κB and MAPK signaling cascades [41]. This suggested that TLR4 might play an important role in neutrophils function. Indeed, Parker et al. [42] demonstrated that activation of TLR4 results in down-regulation of CCR2 expression in human monocytes, and Souto et al. [8] found that TLR4 activation induced CCR2 expression in neutrophils during sepsis. In support of this role, our present study demonstrated that the activation of CCR2 in neutrophils is TLR4-dependent under sterile conditions as well as in liver IRI.

We further showed that TLR4 ablation in neutrophils inhibited both neutrophil mobilization from the bone marrow and CCR2 expression in neutrophils. Since CCL2 is the most important ligand for CCR2, we also injected the mice with rmCCL2 to promote the mobilization of CCR2-expressing neutrophils from the bone marrow to the peripheral blood. This method was proven to be effective in our previous study, in which rmCCL2 injection obviously promoted the mobilization of CCR2-expressing neutrophils from the bone marrow into the peripheral blood after 1 h of reperfusion in WT mice [13]. These results were confirmed in the present study in the WT mice, whereas rmCCL2 failed to accomplish its mission in the $TLR4^{-/-}$ mice. Thus, CCR2 expression clearly depends on TLR4 signaling.

TLR2 and TLR4 signaling were found to mediate the up-regulation of CCR2 expression in neutrophils in acecal ligation and puncture model through the MyD88/NF-kB pathway [8]. However, to our knowledge, this is the first report to show the relationship between CCR2 expression in neutrophils and TLR4 in a septic inflammation model. To clarify the pathway downstream of TLR4 that was responsible for the observed effects, we used specific chemical inhibitors targeting the MAPK pathway, and found that the p38 inhibitor reduced neutrophil mobilization and blocked CCR2 expression in neutrophils. These results suggest that p38 is important for this crucial event, and that CCR2 expression in neutrophils requires activation of the TLR4-p38 pathway during liver IRI.

In conclusion, our study provides the following mechanistic insights into CCR2-expressing neutrophils in a liver IRI model: (1) increased CCR2 expression in neutrophils plays an important role in neutrophil mobilization from the bone marrow and infiltration into the liver during liver IRI, and (2) CCR2 expression in neutrophils requires activation of the TLR4-p38 axis. Based on these findings, our study helps to gain a better understanding of neutrophil recruitment during liver IRI as well as the role of CCR2 in neutrophils under aseptic conditions. Although the detailed mechanism requires further study, these insights can suggest new targets for therapeutic intervention.

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Disclosure of conflict of interest

None.

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