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NLRP6 deficiency aggravates liver injury after allogeneic hematopoietic stem cell transplantation

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ABSTRACT

This study aims to observe the expression and role of NLRP6 in liver injury after allogeneic hematopoietic stem cell transplantation (Allo-HSCT). Allo-HSCT model was established through infusion of 5×10^6 bone marrow mononuclear cells into whole body irradiated mice. On days 7, 14, 21 and 28 after transplantation, the peripheral blood was collected to detect liver function. The liver of the mice was obtained to assess the pathological changes of liver tissues after allo-HSCT by H&E staining and Mason staining. Meanwhile, expression of NLRP6, phosphorylated p38-MAPK and IκBα, caspase-1 and NLRP3 in liver were detected by Western blot. ELISA was used for detection of the level of interleukin (IL)-1β, IL-18, tumor necrosis factor (TNF)-α, IL-6, myeloperoxidase (MPO) and tumor growth factor (TGF)-β1. Increased expression of NLRP6, phosphorylated Ikbα, phosphorylated p38-MAPK, pro-caspase-1, and p20, in liver tissue with injury and fibrosis in mice after allo-HSCT were observed. Meanwhile, the level of IL-1β, IL-18, IL-6 and TNF-α was also increased. However, NLRP6$^{-/-}$ mice showed more severe liver damage and liver fibrosis after transplantation together with higher level of phosphorylated Ikbα, phosphorylated p38-MAPK, Pro-caspase-1, p20 expression as well as IL-1β, IL-18, IL-6, and TNF-α secretion compared with wide-type. Interestingly, the expression of NLRP3 in the liver of NLRP6$^{-/-}$ mice was significantly higher than that of wild-type. In conclusion, the expression of NLRP6 in host's liver is associated with liver injury after allo-HSCT. NLRP6 deficiency in host's liver leads to more severe liver damage, indicating a protective role of NLRP6 in host's liver to liver damage after allo-HSCT.

1. Introduction

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is an effective approach to treat hematological diseases such as leukemia, multiple myeloma, and myelodysplastic syndrome and some hematopoietic disorders [1]. However, severe complications such as liver injury affect the long-term survival rate of patients after transplantation. It has been reported that the incidence of liver injury after allo-HSCT is about 60–80% [2,3], and the degree of liver injury is closely related to the prognosis of patients. If the total bilirubin is around 68–120 μmol/L (4–7 mg/dL), the patient's mortality rate reached 50% within 200 days after transplantation, and if the total bilirubin is higher than 170 μmol/L (10 mg/dL), the patient's mortality rate exceeded 70% [4]. Therefore, liver injury after transplantation has become an important issue.

There are many causes of liver damage after allo-HSCT, such as radiotherapy and chemotherapy preconditioning, hepatic vein occlusive disease and liver graft-versus-host disease, and these pathogenic factors often exist at the same time, eventually leading to liver damage. Our previous studies showed abnormal liver lobular structure, central vein and hepatic sinus stricture and obstruction, inflammatory cell infiltration in liver tissue, central vein and Fibrin deposition in the hepatic sinus, accompanied by increased expression of NLRP3, activation of caspase-1, increased secretion of inflammatory factors interleukin (IL)-1β and IL-18 in mice after allo-HSCT, suggesting that NLRP3

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inflammasome is involved in the development of liver injury after allo-HSCT [5,6]. The inflammasome complex is a multiprotein that has been shown to be involved in the development of a variety of liver-related diseases such as nonalcoholic steatohepatitis [7], liver fibrosis [8], and ischemic reperfusion injury [9], and liver infectious disease [10]. It consists of a receptor molecule NOD-like receptor, a linker protein AIM2, and an effector molecule pro-caspase-1 [11]. Both NLRP6 and NLRP3 belong to the NOD-like receptor family. When they recognize the pathogen-associated molecular patterns of pathogen-derived sources and the damage-related analysis patterns of host cell sources [12,13], pro-caspase-1 undergoes self-cleavage to generate active form p20 and p10, which then heterodimerizations to produce activated caspase-1 [14,15]. Activated caspase-1 cleaves the precursors of the inflammatory factors IL-1β and IL-18 to generate mature IL-1β, IL-18 [16–18]. However, recent studies have shown that NLRP6 not only participates in the inflammatory response by producing IL-1β and IL-18 [19], but also has been shown to inhibit NF-κB and mitogen-activated protein kinase signaling pathways [20], inhibit inflammation and prevent pathological damage [20–23].

Our previous studies have shown that NLRP3 is closely related to liver damage after allo-HSCT, and liver damage is ameliorated when NLRP3 is inhibited [5], indicating that NLRP3 is closely related to liver damage after transplantation. NLRP6 is also a member of the NOD-like receptor family, and its role in liver damage after allo-HSCT remains unknown. This study aims to observe the expression of NLRP6 in liver tissue after allo-HSCT, and to explore its role in liver damage after transplantation.

2. Materials and methods

2.1. Animals

BALB/c mice (H-2Kd) and C57BL/6 mice (H-2Kb) were purchased from the Institute of Model Animals of Nanjing University, and NLRP6$^{-/-}$ (H-2Kb) mice were purchased from Saiye (Guangzhou) Biotechnology Co., Ltd. All mice were 8–10 weeks old and weighed 20–25 g. They were housed in SPF grade animal center in Xuzhou Medical University.

Both C57BL/6 mice (wild-type, WT) and NLRP6$^{-/-}$ mice were divided into two groups, control group (n = 3 for WT and n = 3 for NLRP6$^{-/-}$) and allo-HSCT group (n = 16 for WT and n = 16 for NLRP6$^{-/-}$). The normal control group did not receive any treatment, while the allo-HSCT group received a total body irradiation of 8.5 Gy followed by infusion of 5×10^6 bone marrow mononuclear cells of BALB/c mice through tail vein.

2.2. H&E staining

Liver tissues were collected from each group on the 7th, 14th, 21st and 28th days after transplantation and fixed by styrene, followed by dehydration and paraffin embedding with RM2126. The slicer was sectioned and sliced to a thickness of 4 μm, followed by H&E staining and analysis of pathological changes of liver tissue by light microscopy.

2.3. Masson staining

The 4 μm liver tissue sections were subjected to Masson staining using Masson kit (Zhuhai Besso, BA 4079A) according to manufacturer's instructions, and the degree of liver fibrosis was observed by an optical microscope.

2.4. Liver function analysis

Liver function (alanine aminotransferase ALT, aspartate aminotransferase AST and total bilirubin TBIL) were measured by biochemical analyzer.

2.5. Liver isolation after perfusion

To eliminate any influences of the donor blood cells, liver was isolated after perfusion as follows: mice in each group were anaesthetized with chloral hydrate on the 7, 14, 21,28th day after transplantation, and then the catheter was inserted into the portal vein with perfusate (0.9% sodium chloride). Perfusion was performed with 0.05% potassium chloride, HEPES 0.2%, 0.08 mg/L EGTA, pH 7.4. Once the perfusion was started, the hepatic vein was immediately cut off and the perfusate was perfused at a flow rate of 5 mL/min for about 15 to 20 min. The liver is then separated and collected for Western blot and ELISA.

2.6. Western blot

Total protein was isolated from liver tissues after perfusion using RIPA lysis buffer and quantified by BCA assay followed by separation on SDS-PAGE, and transferring to NC membrane. After blockage with the skim milk powder, the membrane was incubated with rabbit anti-mouse NLRP6, NLRP3, phosphorylated p38-MAPK, phosphorylated IκBα, caspase-1 and β-actin antibody, After washing the membrane with PBST, the corresponding HRP-conjugated secondary antibody was added. Protein was visualized through addition of ECL and the protein band was analyzed by Image J software.

2.7. ELISA

The perfused liver tissue was subjected to liquid nitrogen grinding, centrifuged, and the supernatant was collected for analysis of the level of IL-1β, IL-18, IL-6, tumor necrosis factor (TNF)-α, myeloperoxidase (MPO) and tumor growth factor (TGF)-β1 by ELISA kits according to manufacturer's instructions.

2.8. Statistical analysis

Data were presented as mean ± standard error (SE) and processed using GraphPad Prism software (version number 7.0). The comparison between the groups was performed by one-way ANOVA. The comparison of different time points of different groups was performed by two-way ANOVA. $P < 0.05$ indicates that the difference was statistically significant.

3. Results

3.1. Increased NLRP6 expression and liver injury after allo-HSCT

Our previous study found that NLRP3 is associated with liver damage after allo-HSCT [5]. Whether NLRP6 plays a role remains unclear. To explore the role of NLRP6 in liver injury after allo-HSCT, we examined the liver damage of WT mice and the expression of NLRP6 in liver tissue, and found that the protein expression of NLRP6 in liver tissue increased after transplantation, reaching a peak on the 14th day, followed by a gradual decrease (Fig. 1A). Meanwhile, liver function was impaired as shown by increased level of AST, ALT and TBIL after transplantation (Fig. 1B). Taken together, these data suggested that allo-HSCT can cause liver damage and increased expression of NLRP6 in liver tissue, indicating that NLRP6 may be involved in liver injury after transplantation.

3.2. More severe liver damage after allo-HSCT in NLRP6$^{-/-}$ mice

To investigate the role of NLRP6 in liver injury after transplantation, we observed the liver function of NLRP6$^{-/-}$ mice after transplantation, and found that the liver function was significantly impaired as demonstrated by higher level of AST, ALT and TBIL after transplantation (Fig. 2A). Analysis of liver histopathology showed that NLRP6$^{-/-}$ mice

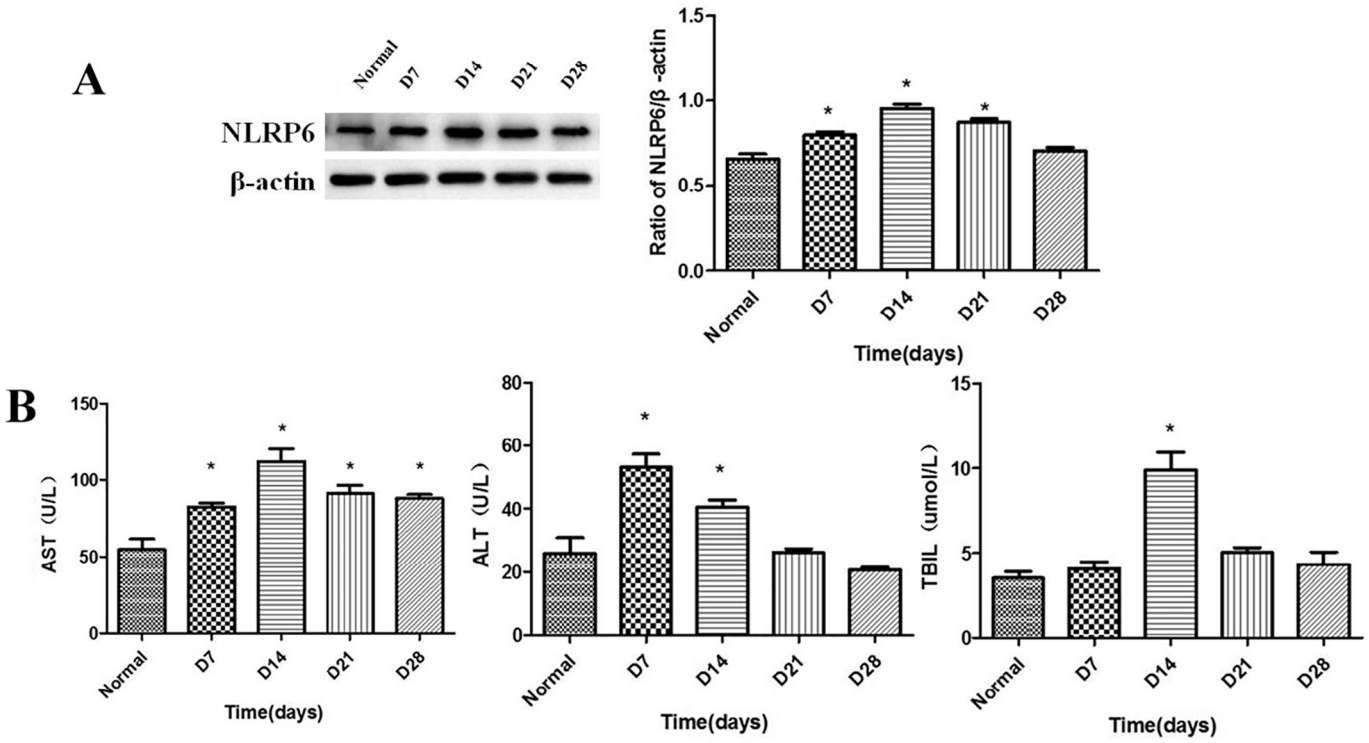

Fig. 1. Expression of NLRP6 in liver tissue and liver function after allo-HSCT. The protein in the liver tissues was extracted from mice on the 7th, 14th, 21st and 28th day after allo-HSCT followed by analysis of the expression of NLRP6 by Western blot (A). Meanwhile, peripheral blood was collected for measuring the level of AST, ALT and TBIL (B). Compared to normal mice (N), $^{*}P < 0.05$. n = 3 for normal group and n = 4 for each time point after allo-HSCT.

developed more severe hepatocyte edema after transplantation together with inflammatory cell infiltration and thrombosis (Fig. 2B), and more fibrin deposition in liver tissue (Fig. 2C). In addition, we also assessed the inflammatory cell marker MPO and liver fibrosis-associated cytokine TGF-β1 level in liver on the 14th day after transplantation, in which NLRP6 was highly expressed and found that MPO and TGF-β1 were all higher in the liver of NLRP6$^{-/-}$ mice compared with WT (Fig. 2D). These data indicated that the expression of NLRP6 can alleviate liver damage after allo-HSCT, reduce inflammatory cell infiltration and liver fibrosis.

3.3. NLRP6 negatively regulates activation of p38-MAPK and NF-κB after allo-HSCT

NLRP6 has been shown to negatively regulate the activation of MAPK and NF-κB to reduce inflammatory damage. Therefore, we measured the level of phosphorylated p38-MAPK and NF-κB as well as the downstream inflammatory cytokines IL-6 and TNF-α in liver tissues of WT mice and NLRP6$^{-/-}$ mice on day 14 post-transplantation. As seen in Fig. 3, the expression of phosphorylated p38 and IκBα (Fig. 3A), IL-6 and TNF-α (Fig. 3B) was increased in both WT and NLRP6$^{-/-}$ mice after transplantation with significantly higher levels being observed in NLRP6$^{-/-}$ mice than those in WT mice, indicating that NLRP6 inhibits the activation of NF-κB signaling pathway and p38-MAPK signaling pathway.

3.4. Increased expression of NLRP3, pro-caspase-1, and p20 in NLRP6$^{-/-}$ mice after allo-HSCT

To confirm whether NLRP6 involves in liver damage following allo-HSCT via the inflammatory complex pathway, we examined the inflammatory complex effector molecule pro-caspase-1 and its activated form p20 and its downstream inflammatory factors in liver. The expression of pro-caspase-1, p20, IL-1β and IL-18 in the liver of WT mice was increased on the 14th day after transplantation (Fig. 4A). Similarly, we also examined the expression of the corresponding indicators in the NLRP6$^{-/-}$ group of mice, and found that the expression of pro-caspase-1, p20, IL-1β, IL-18 was significantly increased compared with the WT group (Fig. 4A). As NLRP3 is also involved in liver injury after allo-HSCT, we examined the expression of NLRP3 and found that the expression of NLRP3 was increased in the liver tissues of WT mice and NLRP6$^{-/-}$ mice, with much higher level in liver tissues of NLRP6$^{-/-}$ mice than that in WT mice (Fig. 4C), suggesting that NLRP6 deficiency up-regulates the expression of NLRP3 in liver tissue after allo-HSCT, which then activates caspase-1 to promote the secretion of inflammatory cytokines IL-1β and IL-18.

4. Discussion

Allo-HSCT is an effective treatment for several malignant or non-malignant hematological diseases such as leukemia, myeloma, solid tumor and autoimmune diseases [23–25]. However, liver damage is one of the complications after allo-HSCT and has a serious impact on the patient's prognosis. Our previous study found that the expression of NLRP3 in liver tissue was increased after transplantation and inhibition of NLRP3 ameliorated liver injury after transplantation [5]. NLRP6 and NLRP3 belong to the NOD-like receptor family, but whether NLRP6 is also involved in liver damage after allo-HSCT remains unclear. This study investigated the role of NLRP6 in liver tissue and liver damage after allo-HSCT and found increased levels of AST, ALT, TBIL, hepatocyte edema, increased fibrin deposition in liver tissue in mice after allo-HSCT, indicating that allo-HSCT can cause liver damage and liver fibrosis, which is consistent with our previous studies [5,26]. In addition, we also found that the expression of NLRP6 in liver of mice after allo-HSCT was increased, suggesting that NLRP6 might be involved in the liver damage after allo-HSCT. In order to investigate this, we selected NLRP6$^{-/-}$ mice as recipients and found that NLRP6$^{-/-}$ mice had more severe liver damage, impaired liver function, and hepatocyte edema

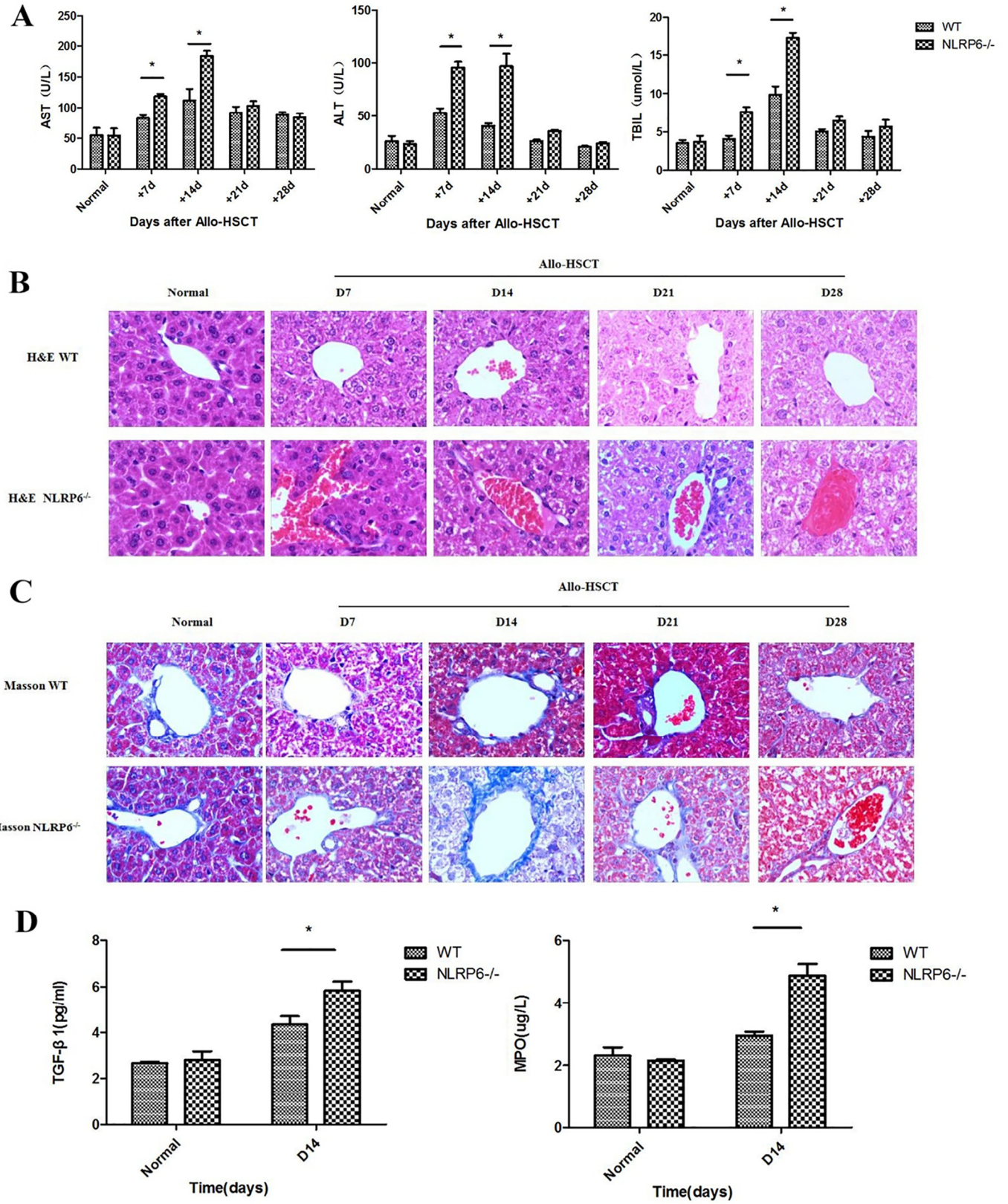

Fig. 2. Liver function and pathological changes of liver after allo-HSCT. Peripheral blood was collected on the 7th, 14th, 21st and 28th day after transplantation and plasma was obtained for measuring the level of AST, ALT and TBIL were detected by biochemical analyzer (A). Meanwhile, liver tissues were extracted, fixed by formaldehyde, dehydrated, embedded in paraffin, and then cut into 4 μm slices for H&E staining (B) and Mason staining (C). Protein was isolated from WT and NLRP6$^{-/-}$ normal mice and liver tissue of mice on the 14th day after transplantation and the supernatant was collected after homogenization for detection of inflammatory cell marker MPO and liver fibrosis-associated cytokine TGF-β by ELISA (D). *$P < 0.05$ compared to WT mice. n = 3 for each in normal group and n = 4 for each time point after allo-HSCT.

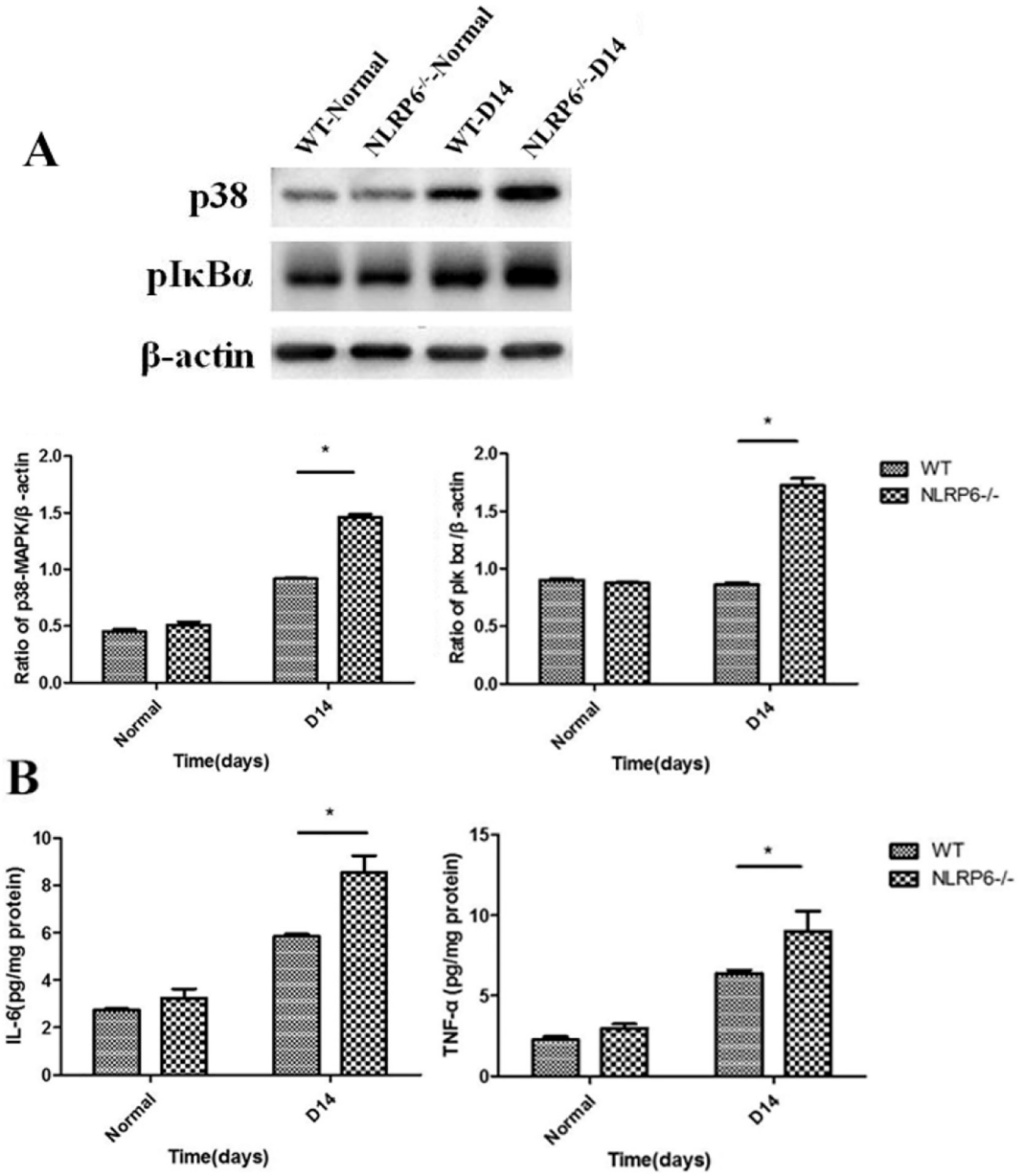

Fig. 3. NLRP6 affects the activation of NF-κB and p38-MAPK signaling pathway. Proteins were extracted from liver tissue of mice on day 14 after transplantation, followed by analysis of the expression of phosphorylated p38 and phosphorylated IκBα by Western blot (A), the expression of inflammatory cytokines IL-6 and TNF-αby ELISA (B). *$P < 0.05$ compared with WT mice. n = 3 for each in normal group and n = 4 for each in D14 group after allo-HSCT.

and liver fibrosis compared with WT mice after transplantation. In addition, there were more inflammatory cell infiltrations in liver tissue, suggesting that the protective role of NLRP6 in liver damage after allogeneic hematopoietic stem cell transplantation.

The immune system has a series of germline-encoded pattern recognition receptors that recognize infection, tissue damage, metabolic stress, and other factors [27], and NOD-like receptors act as one of the pattern recognition receptors. The inflammatory response can be initiated when it recognizes the above stimulating factors [27]. NOD-like receptors include NLRP1, NLRP2, NLRP3, and NLRP6. Once activated, it forms a multiprotein complex called inflammasome. Studies have shown that inflammasomes are involved in infection, inflammation, and autoimmunity via caspase-1, which mediates the production of the inflammatory cytokines IL-1β and IL-18 [28]. However, unlike other NOD-like receptors, NLRP6 not only mediates the production of the inflammatory cytokines IL-1β and IL-18 through the formation of inflammatory complexes [19,29,30], but is also shown to inhibit NF-κB and MAPK signaling pathways, thus inhibiting inflammatory responses [22,31] and reducing pathological damage [10]. In order to explore how NLRP6 affects liver injury after transplantation, we selected the 14th day with the highest expression of NLRP6, and detected the expression of pro-caspase-1, p20 and NF-κB signaling pathway (phosphorylated IκBα) and phosphorylated p38-MAPK, which is closely associated with inflammatory response [32]. We found that the level of IL-6, TNF-a, TGF-β1 (associated with liver fibrosis) [33,34], phosphorylated IκBα and p38-MAPK in NLRP6$^{-/-}$ mice were significantly higher than that in WT mice, indicating that NLRP6 inhibits NF-κB and p38MAPK signaling pathway.

In order to clarify whether NLRP6 also affects liver damage after allogeneic hematopoietic stem cell transplantation through inflammasome pathway, we examined pro-caspase-1, p20 and its downstream inflammatory cytokines IL-1β, IL-18 in liver tissue. In response to inflammatory stimulus, inflammasome undergoes oligomerization, leading to conversion of pro-caspase-1 to active caspase-1 (p20), which

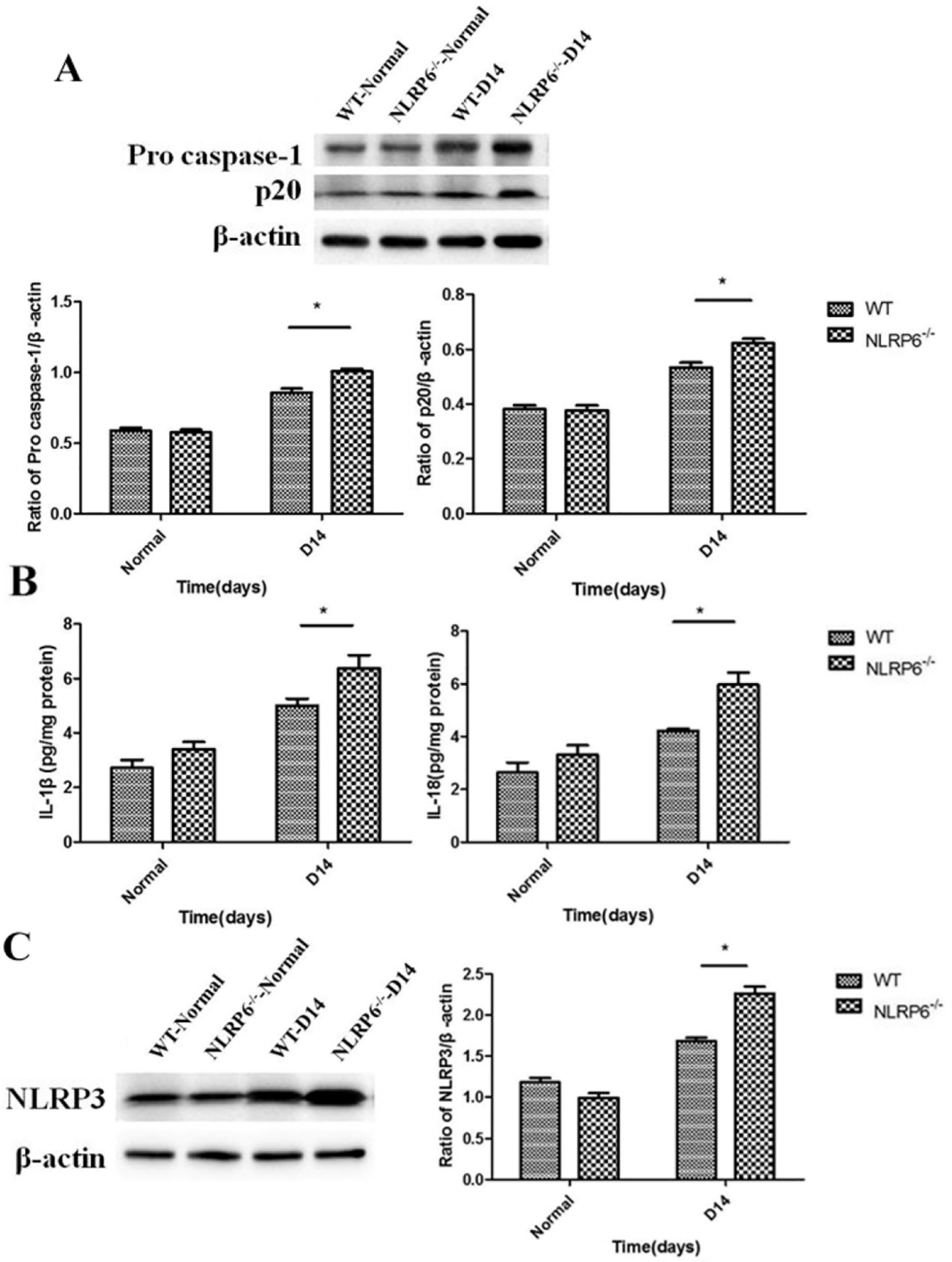

Fig. 4. NLRP6 affects the expression of Pro caspase-1, p20, IL-1β, IL-18 and NLRP3. Total protein was extracted from liver tissue of mice on the 14th day after transplantation followed by measuring the expression of Pro caspase-1, p20 (A) and NLRP3 (C) by Western blot, the expression of inflammatory cytokines IL-1β and IL-18 (B) by ELISA. *$P < 0.05$ compared with WT mice. n = 3 for each in normal group and n = 4 for each in D14 group after allo-HSCT.

in turn cleaves the precursors of IL-1β and IL-18 to produce biologically active IL-1β and IL-18. IL-1β and IL-18 have been shown to mediate a variety of pathophysiological processes such as infection, inflammation, and autoimmunity [28]. Our previous study found IL-1β and IL-18 was significantly elevated in liver tissue after allogeneic hematopoietic stem cell transplantation and caused severe liver inflammatory damage [5]. In the present study, we found that the expression of pro-caspase-1, p20 and its downstream inflammatory cytokines IL-1β and IL-18 in NLRP6$^{-/-}$ mice after allo-HSCT were higher than those in WT mice. Consistently, a previous study found that NLRP6$^{-/-}$ mice have more severe brain damage and higher expression of IL-1β in brain tissue around the hematoma when intracranial hematoma occurs [35]. In order to explore its possible mechanism, we examined the expression of NLRP3 in liver tissue, and found that NLRP3 expression in liver tissue of NLRP6$^{-/-}$ mice was significantly higher than that in WT mice, suggesting that increased expression of pro-caspase-1, p20, IL-1β and IL-18 in NLRP6$^{-/-}$ mice may be due to increased expression of NLRP3, which is supported by a previous study demonstrating that the expression of pro-caspase1, NLRP3, and pro-IL-1β in human periodontal ligament cells is significantly increased after inhibition of NLRP6

expression by NLRP6 siRNA [36]. Recent studies have found that NF-κB signaling pathway can up-regulate NLRP3, pro-caspase-1, and pro-IL-1β [37,38]. Therefore, the increase of NLRP3, pro-caspase-1, p20, IL-1β and IL-18 expression in NLRP6$^{-/-}$ mouse liver tissue may be due to activation of NF-κB signaling in liver tissue of NLRP6$^{-/-}$ mice.

The data from our present study demonstrate that the expression of NLRP6 can attenuate liver damage and inflammation after allo-HSCT, supporting a protective role of NLRP6 which was consistent with several previously studies [35,36,39]. For example, Wang et al. reported that NLRP6 can inhibit NF-κB signaling pathway and attenuates brain damage after ICH [35]. Elke Ydens et al. found that NLRP6 promotes the recovery of damaged peripheral nerves by inhibiting the activation of ERK-MAPK [39], whereas WL Lu et al. also found that NLRP6 inhibits NF-κB and ERK-MAPK signaling pathway to alleviate the inflammatory response of human periodontal ligament cells [36]. These results indicate that NLRP6 plays a protective role in the defense against injury. However, liver cells have complicated cellular components, such as hepatocytes, sinusoidal endothelial cells, intrahepatic macrophages, large granular lymphocytes, and bile duct epithelial cells, although Maciej Lech et al. have confirmed that NLRP6 is present in mouse liver tissue [40], and our study also shows the expression of NLRP6 in liver tissue, which is increased after allo-HSCT. However, it is still unknown which cells express NLRP6, which requires further research. In contrast to the protective role of NLRP6, a previous study demonstrated that NLRP6-deficient mice had lower gastrointestinal intestinal graft-versus-host disease (GVHD) and longer survival time compared with WT mice [41], indicating that NLRP6 plays a role in aggravating damage in the gastrointestinal GVHD model. These data indicate that NLRP6 may play different roles in different disease models. The exact mechanism by how NLRP6 protects liver injury after allogeneic hematopoietic stem cell transplantation remains to be further elucidated.

In conclusion, our study shows that NLRP6 expression in host's liver negatively regulates liver damage after allogeneic hematopoietic stem cell transplantation possibly through inhibiting p38-MAPK and NF-κB signaling pathway, reducing the production of IL-6, TNF-α, IL-1β, IL-18 and TGF-β1 to reduce Inflammatory cell infiltration and liver fibrosis, indicating that NLRP6 may be a new target for the treatment of liver damage after allogeneic hematopoietic stem cell transplantation.

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Declaration of Competing Interest

All authors have no conflict of interest to declare.

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