S100A6 Promotes Inflammation and Infiltration of Mononuclear/Macrophages via the *p*-P38 and *p*-JNK Pathways in Acute Liver Injury

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Research

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Abstract

Background

The phagocytic S100 protein, which mediates inflammatory responses and recruits inflammatory cells to sites of tissue damage, has long been known to be expressed in cells of myeloid origin. S100A6 belongs to the A group of the S100 protein family of Ca^{2+} -binding proteins. Currently, the mechanism by which S100A6 mediates the inflammatory response and recruits inflammatory cells to the tissue injury site is unknown.

Methods

A mouse model of carbon tetrachloride (CCl_4)-induced acute liver injury (ALI) was established, and the transcriptomes of postinjury 2d and 5d liver tissues were sequenced. Enzyme-linked immunosorbent assay was used to determine the expression of inflammatory factors (TNF- α , IL-1 β , IL-6, and IL-8) in the supernatant of the liver. Immunohistochemical analysis confirmed the expression of S100A6 in the liver cells. *In vitro* experiments proved the pro-inflammatory function of S100A6, and western blotting (WB) showed that the pathways were activated. The transwell experiment showed the infiltration of mononuclear/macrophages.

Results

We found that S100A6 is highly expressed in liver cells during the most severe period of ALI, suggesting that it acts as an endogenous danger signal and has a pro-inflammatory function. *In vitro*, the mouse S100A6 recombinant protein was used to stimulate liver Kupffer cells to promote the secretion of TNF- α , IL-1 β , IL-6, and IL-8. Further mechanistic experiments revealed that S100A6 acts as an endogenous danger signal to activate *p*-P38 and *p*-JNK downstream of the TLR4 and P65 pathways. Similarly, transcriptome data showed that S100A6 can activate the inflammatory response in Kuffer cells. WB revealed that S100A6 had no significant effect on cell apoptosis. To continue to explore the mechanism of monocyte/macrophage infiltration, we found that TNF- α stimulates liver cells as the main source of CCL2. TNF- α can initiate the *p*-P38 and *p*-JNK pathways of liver cells to produce CCL2, thereby recruiting the infiltration of mononuclear/macrophages.

Conclusions

Taken together, S100A6 is an endogenous danger signal that mediates inflammatory responses and recruits inflammatory cells to sites of tissue damage.

Background

Damage-associated molecular patterns (DAMPs) are endogenous danger signals derived from damaged or dead cells. In 2004, Seong and Matzinger first proposed the effect of DAMPs on the relationship between innate immunity and inflammation, and the inflammatory signaling pathway plays an important

role in diseases [1, 2]. DAMPs are recognized by specific members of the Toll-like receptor (TLR) family, as well as the multiligand receptor for advanced glycation end products (RAGE) [3, 4]. Alarmins such as high-mobility group protein B1 and heat shock proteins orchestrate key events in the inflammatory response not only in infectious diseases but also, more importantly, in sterile inflammation [5–7].

Excessive inflammation causes inflammatory damage and is closely related to the occurrence and development of various diseases [8, 9]. Mononuclear/macrophages, the primary inflammatory cells, are innate immune cells that play an important role in maintaining the immune balance of the body, and in liver injury and repair, and are considered a major feature of acute and chronic liver disease [10].

Mononuclear/macrophage infiltration is mainly regulated by the chemokine receptor CCL2 (also known as monocyte chemokine protein-1) and chemokine CC receptor (CCR2) [11]. Continuous chemotaxis of CCL2 induces a large number of mononuclear/macrophages to infiltrate the damaged liver site, attack hepatocytes, and lead to cell death or apoptosis [12, 13]. As a result, the site of liver injury cannot be repaired rapidly, and the degree of liver injury is further aggravated [14–16].

S100 proteins are a group of double-active Ca^{2+} -binding proteins with more than 20 members of the family that regulate cell proliferation, differentiation, migration, energy metabolism, calcium balance, inflammation, and death [17–20]. When S100 is released into the extracellular space, specific S100 proteins act as DAMPs to promote the migration and chemotaxis of immune cells through interaction with different receptors (such as TLR/RAGE). This promotes the release of a variety of inflammatory cytokines, regulating the body's inflammatory and immune responses [21, 22]. Some members of the S100 family, including S100A4, S100A6, S100A7, S100A8, S100A9, S100A12, S100B, and S100P, are secreted proteins that function as cytokines in peripheral blood. It has been reported that phagocyte-specific myeloid-related protein (MRP) MRP8 (S100A8) and MRP14 (S100A9) are DAMPs [23]. Studies involving mice deficient for MRP14 have uncovered a pivotal role for MRP8/MRP14 in the promotion of inflammation in many clinically relevant conditions [24]. S100A4 promotes inflammation but suppresses lipid accumulation via the STAT3 pathway in the chronic ethanol-induced fatty liver [25].

Here, we showed that S100A6 is secreted by liver cells and exerts inflammation-promoting functions in injured livers. Furthermore, S100A6 accumulates during the progression of liver injury and induces upregulation of TNF- α and other inflammatory factors, which in turn promotes the activation of CCL2 and the infiltration of mononuclear/macrophages. Further mechanistic studies found that S100A6 activates liver inflammation mainly by activating the *p*-P38, *p*-JNK, and P65 pathways and upregulating inflammatory factors. The inflammatory factor TNF- α can activate liver cells to produce CCL2 through the *p*-P38 and *p*-JNK pathways and recruit macrophages to infiltrate. Thus, S100A6 is an endogenous danger signal that mediates inflammatory responses and recruits inflammatory cells to sites of tissue damage.

Materials And Methods

Animal

Specific-pathogen-free male C57BL/6 mice, aged 6–8 weeks, were purchased from Beijing Vital River Laboratory Animal Technology Co. (Beijing, China). All mice were kept in a room with constant temperature (20–22 °C), humidity (60%±10%), light/dark cycle for 12 h, and had free access to water and food. To establish an acute liver injury (ALI) model of carbon tetrachloride *in vivo*, male C57BL/6 mice were intraperitoneally injected with 1 mL/kg carbon tetrachloride. Mice in the control group were intraperitoneally injected with the same volume of olive oil. At the designated time point after injection, the mice were anesthetized with isoflurane, and blood was collected from the ocular vein. The mice were euthanized, and the livers were removed. All animal experimental protocols used in this study were conducted under the standards of the Ethics Committee of Inner Mongolia Medical University (SCXK2016-0001).

Determination of the serum liver enzyme and inflammatory factor activities

ALT and AST serum levels were assessed using commercially available diagnostic kits to reflect the activities of ALT and AST (Nanjing Jiancheng, China). The liver levels of inflammatory factors TNF- α , IL-1 β , IL-6, IL-8, and CCL2 were detected using an enzyme-linked immunosorbent assay (ELISA) kit (Jiangsu Meibiao China and R&D Systems, USA). The steps of the ELISA kit protocol were strictly followed, and the optical density was measured at 450 nm. Liver TNF- α , IL-1 β , IL-6, IL-8, and CCL2 levels were calculated according to the standard curve.

HE staining and Masson's trichrome staining

Liver tissue sections from the same part of the liver lobe were cut, fixed in 4% buffered formalin at 25°C for 24 h, embedded in paraffin, and cut into 4 μ m thick sections. HE and Masson's trichrome staining protocols were used to stain the sections. Histopathological changes were observed under a microscope.

Immunohistochemical analysis

Immunohistochemical analysis was performed using deparaffinized liver sections. The sections were blocked with 3% goat serum at room temperature for 30 min. The primary antibody (F4/80, Invitrogen, China) was then added, and the sections were incubated overnight at 4°C. After washing with PBS, the sections were incubated with a secondary antibody for 50 min at room temperature. They were then immersed in PBS for 5 min and stained with DAB and hematoxylin. The sections were immersed in 1% hydrochloric acid and ethanol for 3 s and rinsed in tap water; when the rinsing was stopped, the ammonia water returned to blue for 5–10 s. Further, the sections were rinsed with tap water, photographed, and preserved.

Flow cytometric detection of macrophages in the liver

Animals (five mice per group) were sacrificed at 2 d, 5 d, 7 d, and 14 d after ALI, cells from the liver were prepared, and surface staining with anti-APC-CD11b, -PE-F4/80 antibody at 1:100 dilution was performed for 30 min at 4°C. Then, 1×10^6 events were acquired using FACS, and data were analyzed using the De Novo software program.

RNA-seq

The same liver lobes of mice in the control and model groups (the second and fifth days after ALI) were chosen to create an RNA library of each phenotype and perform RNA sequencing. The differentially expressed genes that displayed a >1.5-fold change in expression and a *p*-value < 0.01 based on at least two pairwise comparisons with the same trend, were selected for further examination in the test phase.

Quantitative PCR

Total RNA was isolated from the livers to verify the mRNA expression level. The relative mRNA levels were determined using the comparative Ct method with β -actin as the reference gene and the formula $2^{-\Delta\Delta C_t}$. All primers used are listed in Table 1.

Table 1
Primer sequences used for quantitative real-time PCR analysis.

Gene Name	Forward Primer	Reverse Primer
S100A6	GCCCGCTAAACTCCCTCATT	GCAAGGCAAACGAACATCCC
S100A3	CCAGCCTCTGTCTCTACTGC	GGTGGGGTTAGCACATCAAGA
S100A4	TCCTCTCTCTTGGTCTGGTCT	GTCACCCTCTTTGCCTGAGT
S100A10	TGAAGCAGAAGGGGAAGAAATAGGC	CAAGAAGCAGTGGGGCAGATTC
S100A11	GCGGGAAGGATGGAAACAACA	TCATCATGCGGTCAAGGACAC
TNF- α	CAGGCGGTGCCTATGTCTC	CGATCACCCCGAAGTTCAGTAG
IL-1 β	GAAATGCCACCTTTTGACAGTG	TGGATGCTCTCATCAGGACAG
IL-6	CTGCAAGAGACTTCCATCCAG	AGTGGTATAGACAGGTCTGTTGG
IL-8	TCGAGACCATTCTACTGCAACAG	CATTGCCGGTGGAAATTCCTT
CCL2	AAGCAGAAGTGGGTTCAGGATT	GGGTTGTGGAGTGAGTGTTT
β -actin	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT

Cell culture and conditions

Mouse Kupffer cells, hepatocyte cell line FL83B, and macrophage RAW264.7 were cultured in the Dulbecco's Modified Eagle's medium (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The cells were cultured at 37°C and 5% CO₂. Mouse Kupffer cells were stimulated with a 6 mg/ml dose of S100A6 for 2 h. To block the TLR4 pathway, anti-TLR4 (Abcam, USA) was used at a concentration of 0.2 μ g/ml. Mouse Kupffer cells were treated for 12 h for mRNA expression analysis.

SDS-PAGE and immunoblot analysis

Total protein extracted from liver tissues with RIPA buffer was separated by SDS-polyacrylamide gel electrophoresis and transferred onto PVDF membranes (Millipore, MA, USA). After blocking with non-fat milk, membranes were incubated overnight with the following primary antibodies: anti-P38, anti-ERK, anti-JNK, anti-STAT3, anti-*p*-P38, anti-*p*-ERK, anti-*p*-JNK, anti-*p*-STAT3, anti-P65, and anti- β -actin (Abcam, USA). Membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies. The analysis was conducted using an enhanced chemiluminescence system (Bio-Rad, Hercules, CA, USA).

Macrophages transwell migration

Cells were harvested at the exponential growth phase, prepared into a suspension of 1×10^6 cells/mL, and 100 μ L of this suspension was added to each well of transwell plates. The upper layer was macrophages RAW264.7, the lower layer was FL83B cells. FL83B cells in the experimental group were stimulated with 40 ng/mL TNF- α for 2 h.

Statistical analysis

All data were expressed as the mean $\pm$ SEM and analyzed using the GraphPad Prism software. Significant differences between the mean values were obtained from three independent experiments. Differences between the two groups were compared using a two-tailed unpaired Student's t-test. One-way ANOVA with Bonferroni correction was used for multiple comparisons. Significance of differences was classified as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, or ns (not significant).

Results

Severe inflammation during acute liver injury

To evaluate whether the ALI model was successfully constructed, the levels of ALT and AST in the serum of mice were detected on days 2, 5, 7, and 14. ALT and AST levels increased significantly on day 2 (Figure 1A). The levels of ALT and AST on day 5 were very similar to the control group. HE staining revealed that the structures of liver lobules of the control group mice were intact. The liver parenchymal cells were radial, the outlines were clear, the nucleus and cytoplasm were evenly distributed, and the hepatic cords were neatly arranged, while the tissues in the injury group showed large areas of diffuse degeneration along the central hepatic vein on day 2. Necrosis, the arrangement of liver cells, is disordered, and most of them are in a state of edema. Observed by hepatoscopy, the damaged area was up to 2/3, and some inflammatory cell infiltration was observed. After 5 days post the injury, the area of damage and necrosis was reduced, and there was liver cell proliferation along the edge of the injury area. On the fourteenth day post-injury, the liver tissue had almost completely repaired the necrotic area (Figure 1B). Similarly, MASSON staining revealed that compared with the control group, the injury group had a large number of blue signals in the diffuse necrosis area on the second day, indicating that there was collagen deposition, and a large number of vascular-derived immune cells infiltrated around the central vein on the fifth day. Part of the immune infiltration still exists near the Tianmen tube area, but most of the necrotic area has been repaired, and the tissue repair is not completed until the fourteenth day. The above results indicated

that the liver tissue was most severely damaged on the second day, accompanied by partial immune infiltration on the fifth day, and the repair was completed on the fourteenth day (Figure 1B). To further confirm the inflammation and infiltration of the liver, we performed F4/80 staining of liver tissue. Interestingly, a large number of macrophages infiltrated the hepatic veins on the fifth and seventh days after injury (Figure 1B). Similarly, flow cytometry data showed that there were more macrophages in the liver on the fifth and seventh days of injury (Figure 1C). These results indicate that severe inflammation is accompanied by ALI. Additionally, we confirmed our findings at the protein level for five secreted cytokines (TNF- α , IL-1 β , IL-6, IL-8, and CCL2) by ELISA of liver tissue suspension (Figure 2A). Transcriptome data also showed that many inflammatory responses were activated during ALI (Figure 2B). The other ALI models were based on C57BL/6 mice treated with paracetamol (APAP) (Figure S1). The results obtained suggest that the accumulation of inflammation in the liver is a common occurrence during ALI, independent of the mouse model used.

S100A6 protein increased significantly on the second day of acute liver injury

To analyze how the inflammatory response is activated during ALI, we performed transcriptome sequencing of liver tissues on the second and fifth days. On the second day, 3577 genes were upregulated and 3377 genes were downregulated. On the fifth day, 3202 genes were upregulated and 2826 genes were downregulated (Figure 3A). We screened gene families with the potential to activate inflammation from the upregulated genes, such as the HMGB family histones, heat-shock protein (Hsp) family, and S100 protein family (Figure 3B), and arranged them according to the results of transcriptome sequencing (Figure 3C). We will focus on the S100A protein family. Further transcriptome sequencing results showed that A6, A11, A8, A10, A9, A3, and A4 in the S100A protein family increased sharply on the second day after injury (Figure 3D and E). Then qPCR verification was performed (Figure 3F). Among the S100 family, the expression of S100A6 in normal liver tissues was not very high, but the increase in ALI was most significant on the second day. The prime focus was on studying the S100A6 protein. S100A6 was significantly overexpressed on the second day after APAP ALI (Figure S2). It was expressed in the liver cells after immunohistochemical localization of the injury (Figure 4). Thus, S100A6 may be used as an endogenous danger signal, which is expressed significantly when the injury is the most serious.

S100A6 stimulates Kuffer cells to produce TNF- α through p-P38, p-JNK, and P65 pathways

To further analyze the role of S100A6, secreted by liver cells. We used S100A6 recombinant protein from mice to stimulate mouse liver Kupffer cells *in vitro* (Figure S3A). qPCR was used to detect a significant increase in the gene expression levels of TNF- α , IL-1 β , IL-6, and IL-8 produced by Kuffer cells (Figure 5A). Additionally, we confirmed that the four secreted cytokines (TNF- α , IL-1 β , IL-6, and IL-8) were also significantly increased at the protein level through ELISA of cell culture supernatant (Figure 5A). To address whether the S100A6 induced effects on cell survival identified *in silico* were indeed functionally relevant, we confirmed the change in apoptotic/antiapoptotic genes by qRT-PCR (Figure 5B). S100A6 can inhibit the expression of apoptotic genes (Caspase3, Caspase7, BAX) and promote the expression of anti-

apoptotic genes (BCL-2, BCL-XL, BCL-W). However, the protein level did not change significantly (Caspase3, BCL-2, BCL-XL) (Figure 5D). Recent studies have found that TLRs can bind to endogenous molecules (i.e., endogenous ligands) produced by the body itself. We use TLR4 antibody to incubate Kuffer cells overnight, and S100A6 was used to stimulate Kuffer cells and detect the gene expression levels and protein expression levels of several inflammatory factors. After the TLR4 receptor on the surface of Kuffer cells was blocked, the gene expression and protein expression levels of TNF- α , IL-1 β , IL-6, and IL-8 were significantly reduced (Figure 5C). To further confirm the role of TLR4 in S100A6-mediated stimulation of Kuffer cells, we analyzed the pathway members downstream of TLR4 signaling. Several members of the mitogen-activated protein kinase (MAPK) family, such as P38 and JNK, are activated by phosphorylation during TLR4 signaling, which was observed in the S100A6-stimulated Kuffer cells (Figure 5D). Alongside the induction of activation of P65 and STAT3, *p*-STAAT3 (Figure 5D). Similarly, the transcriptome data of S100A6 stimulated Kuffer cells also showed that it can activate the inflammatory response. At the same time, it promoted cell metabolism (Figure 5E). The results demonstrated that inflammatory cytokines such as TNF- α , IL-1 β , IL-6, and IL-8, which are known to be induced via TLR4-dependent pathways, are upregulated after S100A6 treatment. Overall, this demonstrates that both known TLR4 signaling pathways are activated by S100A6.

TNF- α recruits the infiltration of mononuclear macrophages through the bypass activation effect

In the transcriptional tissue data, we found that the chemokine pathway in the liver was also activated on the fifth day of injury, which may be the cause of the infiltration of monocytes/macrophages. To determine the source of the chemokine CCL2, we used S100A6 to stimulate the supernatant of Kuffer cells to stimulate normal liver cells FL83B, which can highly express CCL2 (Figure S3B). Through extensive screening, we found that stimulating normal liver cells with mouse recombinant TNF- α protein promoted the expression of CCL2 (Figure 7A). Liver cells stimulated by TNF- α can cause the infiltration of monocyte/macrophage RAW64.7 (Figure 7B). We next tested the signaling pathways that TNF- α may participate in during the accumulation of monocytes/macrophages recruited by the liver. As shown in Figure 7C, the P38- and JNK-phosphorylated proteins in liver cells stimulated by TNF- α increased significantly. Therefore, these results indicate that TNF- α recruits mononuclear/macrophage via the *p*-P38 and *p*-JNK pathways. The results showed that the infiltration of mononuclear/macrophages on the fifth day of injury may be due to S100A6 stimulating Kuffer cells to produce TNF- α and acting on liver cells to produce CCL2, thereby recruiting mononuclear/macrophage to infiltrate.

Discussion

ALI is a major global health concern. ALI can be caused by exogenous compounds, such as drugs, such as acetaminophen (APAP)-induced liver injury and sepsis-induced liver disease [26]. Numerous studies have shown that, in addition to direct liver cell damage caused by injury factors, the immune-inflammatory response activated in the liver plays an important role in the subsequent liver injury process

[27, 28]. In CCl₄-induced liver injury, inflammation is another important pathological mechanism. CCl₄-mediated oxidative stress can activate inflammatory cells, causing them to release excessive pro-inflammatory factors, such as TNF- α and IL-6 [29, 30]. In the current study, we explored the unique immunological mechanism of CCl₄-induced acute hepatic injury in mice. Namely, CD11b⁺ Kupffer cells produce TNF, as well as FasL, and induce hepatic injury, in which IFN- γ , NK cells, and NKT cells are not involved [31]. Our findings reveal that IL-1 β production and hepatic inflammation in HCV infection are linked and driven by virus-induced TLR7 and NLRP3 inflammasome signaling in liver macrophages [32]. IL-8 levels are elevated in APAP overdose patients [33, 34], and MIP-2 and KC levels are increased in mice after APAP overdose [35]. In this study, inflammatory infiltration was recorded on the second, fifth, and seventh days after ALI with carbon tetrachloride. Further detection is accompanied by severe inflammation during acute liver damage, such as infiltration of macrophages and excessive secretion of inflammatory factors. KEGG and GO analyses of the transcriptome data of upregulated genes on the fifth day after the injury, and the pathways were enriched in immune response and P65 pathways. This phenomenon is not limited to the carbon tetrachloride ALI model, inflammatory infiltrates during APAP-induced ALI caused by APAP were also observed.

Damage-associated molecular patterns (DAMPs) are endogenous danger molecules that are released from damaged or dying cells and activate the innate immune system by interacting with pattern recognition receptors. Recent studies have suggested that various DAMPs, such as high-mobility group box 1 (HMGB1), S100 proteins, and HSPs, are increased and are considered to play a pathogenic role in inflammatory diseases [36]. Numerous studies have revealed that damaged hepatocytes can release HMGB1 upon APAP dosing [37–39]. Additionally, the secreted HMGB1 acts as an activator of macrophages and induces local inflammation in the liver, thereby leading to hepatocellular necrosis. HMGB1 can induce autophagy to maintain cell survival and the functional stability of CHB-Tregs by activating the pathways of RAGE-ERK/MAPK (JNK, ERK, and p38) and inactivating the mTOR pathway (G β L, raptor, rictor, mTOR), thereby suppressing specific antiviral immunity and causing persistent inflammation [40]. It has been reported that the levels of HSP70 were elevated in the synovial fluid of rheumatoid arthritis (RA) patients [41], and HSP gp96 was increased in the synovial fluid of RA patients; this is considered to promote inflammation by activating macrophages through TLR2 signaling [42]. Soluble HSP60 was increased in patients with early carotid atherosclerosis [43], and HSP60 promoted atherosclerosis by inducing VSMC migration via TLR4 and ERK MAPK activation [44]. S100A8 and S100A9 exist in plaques, and they increase atherogenesis by activating neutrophils and monocytes in arterial lesions [45, 46]. We analyzed the Hmgb family proteins, HSPs, and S100 in the transcriptome and found that these proteins were all significantly upregulated on the second day of ALI. Here, we are concerned with the S100 family of proteins. In particular, S100A6 has a significant increase in the proportion of ALI on the second day, so it is speculated that it plays a role as an endogenous danger molecule. Furthermore, S100A6 protein expression was localized in the liver. In the liver tissues of mice in the control group, the expression of S100A6 was less distributed; however, we observed that a large amount of S100A6 was expressed and secreted in hepatocytes on the second day after the injury. On the fifth and seventh days after injury, there were cells along the hepatic vein expressing S100A6, but not all

hepatocytes. Here, we focused only on the expression of hepatocytes, in which reduced expression of S100A6 was observed. After the fourteenth day, there was no difference from the control group. To further illustrate that S100A6 can be used as an endogenous danger molecule, mouse S100A6 recombinant protein was used to stimulate Kuffer cells. These inflammatory factors, such as TNF- α , IL-1 β , IL-6, and IL-8, are either genetic level and the protein level increases significantly. Further mechanistic experiments revealed that S100A6 can activate the downstream *p*-P38 and *p*-JNK pathways of TLR4/MYD88, however, it had no significant effect on the expression of TLR4/MYD88. At the same time, WB showed that the apoptotic and anti-apoptotic proteins of Kuffer cells had no significant effect. Transcriptome data also showed that S100A6 can not only activate the innate immune response but also promote the metabolic response of cells. Interestingly, we found that there was no significant change in the CCL2 of Kuffer cells after S100A6 stimulation. However, it was found from the tissue section that after the fifth day of liver injury, a large number of macrophages infiltrated the hepatic vein. To explore this, we used S100A6 to stimulate the supernatant of Kuffer cells to induce normal liver cells and found that the expression of CCL2 was increased. Further screening revealed that TNF- α recombinant protein can significantly stimulate liver cells to secrete CCL2 and recruit the infiltration of mononuclear macrophage RAW264.7. Moreover, experimental results show that TNF- α can activate the *p*-P38 and *p*-JNK pathways in liver cells.

Conclusion

Here, we observed significantly high expression of S100A6 in ALI models (CCl₄ and APAP), which, as endogenous danger molecules, can stimulate Kuffer cells to produce inflammatory factors through the *p*-P38, *p*-JNK, and NF- κ B pathways. Among them, TNF- α can stimulate liver cells to produce chemokine CCL2 through the *p*-P38 and *p*-JNK pathways and recruit macrophages to infiltrate. In summary, our findings prove that S100A6 promotes inflammation and recruits mononuclear/macrophage infiltration.

Abbreviations

CCL2

Chemokine (C-C motif) ligand2

ELISA

Enzyme-linked immunosorbent assay

ERK

Extracellular regulated protein kinases

JNK

C-Jun N-terminal kinase

IL-1 β

Interleukin-1 β

IL-6

Interleukin-6

STAT

Signal Transducer and Activator of Transcription

TNF- α

Tumoral Necrosis Factor - α

TLR4

Toll-like receptors

Declarations

Ethical Approval and Consent to participate

The study was conducted according to the guidelines of the Declaration of Helsinki. All animal-related experimental protocols applied in this study were conducted under the standards of the Ethics Committee of Inner Mongolia Medical University (YKD202001030).

Consent for publication

Not applicable.

Availability of supporting data

All data generated or analysed during this study are included in this published article [and its Additional files].

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

H.T. and L.W. contributed to the conception and design of the work, acquired the majority of the data and drafted the manuscript. K.Z. contributed to the conception and design of the work, and acquired some of the data. J.S. and Y.S. contributed to the design of the work and to interpretation of data. C.W. and Y.B. contributed to the design of the work, to interpretation of data and substantively revised the manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1

Increased macrophages in the liver during acute liver injury.

(A) Detect the content of ALT/AST in mouse serum at different time points (2d–5d–7d–14d) (n = 5). (B) Liver tissues were harvested and stained using H&E, MASSON or anti-F4/80 antibodies. Scale bar, 50 μ m, (2d–5d–7d–14d) (n = 5). (C) FACS (fluorescence-activated cell sorting) analysis of the percentage and

number of macrophages in the liver (2d-5d-7d-14d) (n = 5). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ or ns (not significant).

Figure 2

Accompanied by severe inflammation during acute liver injury.

(A) TNF- α , IL-1 β , IL-6, IL-8 and CCL2 levels, determined by ELISA, in the supernatant of the liver were analyzed after acute liver injury (ALI) (2d~5d~7d~14d) (n = 5). (B) KEGG and GO were used to analyze the up-regulation of differential genes on the fifth day after ALI (2d~5d) (n = 5). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ or ns (not significant).

Figure 3

S100A of transcriptional regulation in liver tissue.

(A) Differential gene changes in the second and fifth days after injury and hierarchical clustering of the differentially expressed genes (2d and 5d) (n = 5). (B) Hierarchical clustering of Hmgb, Hsp, and S100 genes (n = 5). (C) Significant order of difference between Hmgb, Hsp, and S100 genes (2d,5d). (D) Hierarchical clustering of the S100 genes (2d and 5d) (n = 5). (D) and (E) Expression levels of S100 genes in the transcriptome data. (F) qRT-PCR analysis of the expression of S100A6, S100A4, S100A10, and S100A11 (2d–5d–7d–14d) (n = 5). Results are presented relative to baseline expression in control mice, and β -actin was used as a housekeeping control gene. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ or ns (not significant).

S100A6

Figure 4

CCl₄ exposure promotes S100A6 expression in the liver cells.

Representative analysis of immunohistochemical staining of S100A6 in liver tissues (2d–5d–7d–14d) (n = 5). Scale bar, 50 µm.

Figure 5

S100A6 promotes the secretion of inflammatory factors by upregulating *p*-P38, *p*-JNK, and P65.

(A) qRT-PCR analysis of the expression of TNF- α , IL-1 β , IL-6, IL-8, and secretion of TNF- α , IL-1 β , IL-6, and IL-8 in the supernatants was analyzed by ELISA. (B) qRT-PCR analysis of the expression of Caspase3, Caspase7, BAX, BCL-2, BCL-XL, and BCL-W. (C) TNF- α , IL-1 β , IL-6, IL-8, gene, and protein expression levels

after blocking TLR4. (D) WB shows the change in P38, *p*-P38, ERK, *p*-ERK, JNK, *p*-JNK, P65, BCL-2, BCL-XL, Caspase3, and β -actin protein in Kuffer cells after S100A6 stimulation. (E) KEGG and GO analyses of differential genes stimulated by S100A6 in Kuffer cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ or ns (not significant).

Figure 6

TNF- α stimulates hepatocyte FL83B to release CCL2 and recruits mononuclear/macrophage infiltration through the *p*-P38 and *p*-JNK pathways.

(A) Protein expression and gene expression of CCL2 in liver cells FL83B with TNF- α . (B) Transwell experiment showed that TNF- α stimulated liver cells FL83B to recruit mononuclear macrophages RAW264.7 infiltration. (C) Western blot showing changes in P38, *p*-P38, ERK, *p*-ERK, JNK, *p*-JNK, STAT3, *p*-STAT3, P65, and β -actin protein in FL83B cells after TNF- α stimulation. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ or ns (not significant).

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