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Short-term use of MyD88 inhibitor TJ-M2010-5 prevents D-galactosamine/lipopolysaccharide-induced acute liver injury in mice

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ABSTRACT

Excessive activation of the TLR/MyD88 signaling pathway contributes to several inflammation-related diseases. Previously, our laboratory synthesized a novel thiazaol-aminoramification MyD88 inhibitor named TJ-M2010-5. In this study, we interrogated the role of MyD88, as well as the protective effect of TJ-M2010-5, in a D-gal/LPS-induced acute liver injury mouse model. In order to induce acute liver injury, BALB/c mice received intraperitoneal injection of D-gal and LPS at a dose of 800 mg/kg and 80 μg/kg body weight, respectively. All mice died within 48 h of injection without intervention. However, pre-treatment with TJ-M2010-5 as well as knockout (KO) of the MyD88 gene significantly improved mouse survival rate to 73.3% and 80% at 48 h, respectively, and both treatments protected liver function. These pathological results demonstrated that TJ-M2010-5 and MyD88 KO reduced the infiltration of inflammatory cells and protected hepatocytes against apoptosis. Furthermore, TJ-M2010-5 remarkably inhibited NF-κB and MAPK signaling in vivo. LPS-induced activation of macrophages as well as pro-inflammatory factors were also shown to be decreased after TJ-M2010-5 treatment in vivo and in vitro. Taken together, these results suggested that blockage of the TLR/MyD88 signaling pathway by TJ-M2010-5 has an important role in the prevention of inflammation-related acute liver injury.

1. Introduction

Acute hepatitis injury is a liver disease commonly seen in clinical practice. If left untreated the condition may develop into acute liver failure (ALF) which is life-threatening. Causation for this condition can be multi-factorial [1,2]. In intensive care units, septicemia is the main cause of acute liver injury, which continues to have high morbidity and mortality rates despite the availability of advanced clinical management [3,4].

Evidence has shown that macrophages play a key role in the development of sepsis-induced liver injury [5]. Kupffer cells (KCs) are macrophages resident in the liver; they help maintain immunological balance in the liver as well as the whole body. These cells act as a double-edged sword, mediating the immune response and clearing pathogens, but also causing inflammation in response to sepsis [6]. Normally, these cells capture and destroy bacteria from the intestines through the hepatic portal to maintain systematic homeostasis. Under pathological conditions, like severe infection or liver injury, macrophages recognize pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs) via toll-like receptors to promote creation of a large amount of pro-inflammatory cytokines, such as interleukin-1β (IL-1β), tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) [7]. Lipopolysaccharides (LPS) are the main PAMP in Gram-negative bacteria and a key inducer of inflammation. LPS is recognized by toll-like receptor 4 (TLR4) of cells [8] and actives innate immunity via the MyD88-dependent pathway [9]. In sepsis induced by Gram-negative bacteria, overdose of LPS was shown to stimulate Kupffer cells activation to produce a large volume of TNF-α, which

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induces TNF-R1-mediated hepatocytes apoptosis [10].

Myeloid differentiation factor 88 (MyD88) is an important adapter molecule of toll-like receptors (TLRs). Excepting for TLR3, all TLRs totally or partially, via MyD88, transduce signals into the cytoplasm including activation of downstream nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) pathways [9,11]. Studies have revealed that excessively activated TLR/MyD88 signaling is a key factor in the development of many immunity-mediated diseases, which provide us with a new therapeutic area targeting TLR/MyD88 signaling pathway to reduce the intensity of the immune response.

Previously, we synthesized a series of MyD88 inhibitors named TJ-M2010s (WIPO Patent Application Number: PCT/CN2012/070811), that bind to the TIR domain and inhibits MyD88 dimerization [12–14]. In our previous research, we found that TJ-M2010s had a therapeutic effect on a variety of diseases in mouse models: TJ-M2010-2 could protect renal ischemia reperfusion-induced progressive renal injury [12]; TJ-M2010-5 could ameliorate cardiac and skin graft rejection [13]; TJ-M2010-6 could cure non-obese diabetic (NOD) mice [14]. Based on these findings, we wondered if TJ-M2010-5 could become an effective drug for the treatment of inflammation-induced acute liver injury.

In this study, we established an acute liver inflammatory injury model in mice that was caused by D-gal and LPS. In this model, the synthetic function of hepatocytes was impaired by D-gal and of Kupffer cells aberrantly activated by LPS [15–17]. Over-activation of Kupffer cells led to secretion of a large number of pro-inflammatory factors. The over-production TNF-α especially, induced impaired hepatocyte fulminant apoptosis [18]. In addition, these apoptotic hepatocytes released large amounts of intracellular substances, such as High Mobility Group Box 1 (HMGB1), heat shock proteins (HSPs), hyaluronan and free DNA, which in turn combined with toll-like receptors and additionally activated more Kupffer cells [6]. We supposed that blockage of MyD88 function could inhibit this kind of aberrant activation and alleviate inflammation in liver, consequently protecting hepatocytes against apoptosis. In this study, we elucidated the role of MyD88 in D-gal/LPS-induced acute liver injury (ALI) MyD88 gene knock-out mice and investigated the effectiveness of TJ-M2010-5 for the treatment of ALI.

2. Materials and methods

2.1. Animals

Five-week-old male BALB/c WT mice was purchased from Hunan SJA Laboratory Animal Co. Ltd. (Hunan China). BALB/c MyD88$^{-/-}$ mice were generously provided by Dr. Maria-Luisa Alegre (University of Chicago, Chicago, IL, USA). All animals were housed under controlled conditions (21–23 °C, 55–60% humidity and 12 h day/night cycle) and in a pathogen-free facility. All animals were allowed to feed and drink freely; they were acclimatized to the environment for one week prior to experimentimentation. All animal experiments were performed according to the guidelines approved by the Institutional Animal Care and Use Committee at the Tongji Hospital, Wuhan, China.

2.2. Reagents

TJ-M2010-5, 3-(4-(4-benzylpiperazin-1-yl)-N-(4-phenylthiazol-2-yl)) propanamide, was synthesized at the Academy of Pharmacy, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China (WIPO Patent Application Number: PCT/CN2012/070811). The molecular structure of MyD88 and its interaction with MyD88 TIR domain were provided during previous study (Fig. 1) [13].

D-Galactosamine (D-gal) and lipopolysaccharides (LPS) were purchased from Sigma-Aldrich (Shanghai, China) and dissolved in saline before use. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Thermo Fisher Scientific (Shanghai, China). The enzyme-linked immunosorbent assay (ELISA) kits for IL-1β, TNF-α and IL-6 were purchased from Dakewe Biotech Co., Ltd. (Shenzhen, China). The ELISA kits for iNOS and COX2 were purchased from ELK Biotchnology CO., Ltd. (Wuhan, China). All antibodies used in western blot were purchased from Cell Signaling Technology and all antibodies used in flow cytometry were purchased from eBioscience.

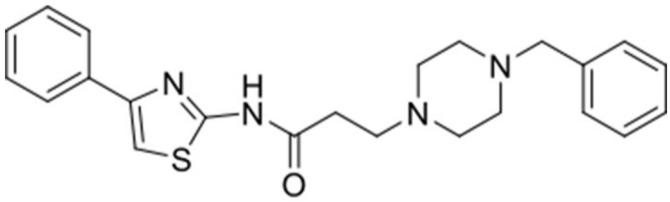

Fig. 1. The molecular structure of synthesized MyD88 inhibitor TJ-M2010-5.

2.3. Introduction of D-gal/LPS-induced acute liver injury model and treatment

Mice received intraperitoneal (i.p.) injection of D-gal and LPS at a dose of 800 mg/kg and 80 μg/kg body weight, respectively, to induce acute liver injury. Beginning two days before D-gal/LPS injection, TJ-M2010-5, dissolved in 0.5% carboxymethylcellulose (CMC), was administered as an i.p. injection to all mice at 50 mg/kg/day. The mice were then divided into four groups: (1) saline group: BALB/c mice were injected i.p. with saline as a control with an equal volume of D-gal/LPS; (2) D-gal/LPS group: BALB/c mice were treated with 0.5% CMC before D-gal/LPS injection; (3) TJ-M2010-5 group: BALB/c mice were pretreated with TJ-M2010-5 and then injected with D-gal/LPS; (4) MyD88 knock-out (KO) group: BALB/c MyD88$^{-/-}$ mice were injected D-gal/LPS without any other intervention.

2.4. Isolation of mononuclear cells from liver tissue

After mouse euthanasia, the liver was freed enough to expose the first hepatic portal and was perfused in situ with cold saline through the portal vein indwelling catheterization meanwhile breaking the inferior vena cava. Then the liver was excised for perfusion with collagenase type IV at 37 °C for 15 min and grinded on a 200-mesh sieve to create a cell suspension. Finally, mononuclear cells were collected using a Percoll gradient centrifugation method [19].

2.5. Bone marrow-derived macrophages (BMDMs) culture and intervention

We obtained tibial and femoral bone from 6- to 8-week-old male BALB/c mice in a bio-clean environment. Bone marrow cells were flushed and treated with red blood cell lysis solution (Beyotime, Shanghai, China), and then cultured in 6-well plates (10^6 cells/ml) in DMEM medium supplemented 10% FBS and 20 ng/ml macrophage colony-stimulating factor (M-CSF, Peprotech, London, UK) at 37 °C under 5% CO_2. On day 3, the culture medium was replaced with fresh cultures and the same dose of M-CSF was added as previously described. On the day 6, TJ-M2010-5 was added at different concentrations (0, 5, 10, 30 μM) for 2 h before stimulation with LPS (500 ng/ml). Twelve hours later, the cultural supernatants and BMDMs were harvested for subsequent experimentation.

2.6. Liver tissue hematoxylin-eosin staining, TUNEL assay and immunofluorescence

Fresh liver samples were collected from all mice. Each sample was fixed in 10% neutral formalin for 24–48 h and then embedded in paraffin. Sections, 5 μm in thickness, were stained with hematoxylin-eosin

(H&E) to assess histopathological liver damage using an optical microscope at different magnifications. Terminal-deoxynucleoitidyl Transferase Mediated Nick End Labeling (TUNEL) was used to detect hepatocyte apoptosis in acute liver injury mice according to the manufacturer's instructions. Liver tissues were also used to perform immunofluorescence with TNF-α antibody (Abcam) at 4 °C overnight. A Cy3-conjugated secondary antibody was then added at 37 °C for incubation 60 min in dark. The cell nuclei were labeled with 4′-6-diamidino-2-phenylindole (DAPI). We then observed images using a fluorescence microscope.

2.7. Measurement of liver enzymes and ELISA assay

At 12 h after D-gal/LPS injection, blood samples were collected from mouse inferior vena cava and centrifuged (7500 *g*, 8 min) for serum collection. Plasma aspartate transaminase (ALT) and alanine transaminase (AST) concentrations were measured by the clinical laboratory of Tongji Hospital (Wuhan, China). The IL-1β, TNF-α, IL-6, iNOS and COX2 levels in sera and cultural supernatants were measured using ELISA kits according to the manufacturer's instructions.

2.8. Flow cytometry analysis

Mononuclear cells isolated from liver tissues and BMDMs were stained using PE-conjugated F4/80 antibody, FITC-conjugated CD80 antibody, and APC-conjugated CD86 antibody at 4 °C for 30 min in dark. Then cells were analyzed by flow cytometry (BD FACSCalibur).

2.9. Quantitative real-time PCR

Total RNA was extracted from mouse liver samples and BMDMs using the TRIzol® reagent (Beyotime, Shanghai, China) according to the manufacturer's instruction. cDNA templates were prepared using a PrimeScript™ RT reagent kit (Takara, Japan) and 500 ng total RNA was used for reverse transcription in 10 μl of the reaction mixture. Quantitative RT-PCR was performed on a StepOne System (Life Technologies) using a SYBR® Premix Ex TaqTM II (Tli RNaseH Plus) (Takara, Japan). Real-time PCR reactions were performed in triplicate and all samples were analyzed using the ΔΔCT value method. The following mouse sequence-specific primers were used (5′-3′): GAPDH forward, AGGTCGGTGTGAACGGATTTG; GAPDH reverse, TGTAGACC ATGTAGTTGAGGTCA; MyD88 forward, TTTA-TCTGCTACTGCCCCA ACG; MyD88 reverse, GCGGCGACACCTTTTCTCA; IL-1β forward, GCACTAC-AGGCTCCGAGATGAA; IL-1β reverse, GTCGTTGCTT-GGTT CTCCTTGT; TNF-α forward, ATGGCCTCCCTCTCATCAGT; TNF-α reverse, TGGTTTGCTACGAC-GTGGG; IL-6 forward, AGTGGCTAAGGA CCA-AGAC; IL-6 reverse, ATAACGCACTAGGTTTGCCGA; iNOS forward, ATTCAC-AGCTCATCCGGTACG; iNOS reverse, GGATCTTGACC ATCAGCTTGC; COX2 forward, GTGTATCCCCCCACAGTCAAA; COX2 reverse, ACACTCTGTTGTGC-TCCCGAA. Values were normalized to GAPDH mRNA.

2.10. Western blot analysis

Total protein, cytosolic protein and nuclear protein were extracted from liver tissue respectively according to manufacturer's instruction (Beyotime, Shanghai, China). Western blot was performed using a method previously described [12]. Protein concentration was measured using a BCA protein assay kit (Beyotime, Shanghai, China). The extracted proteins (50 μg) were separated on SDS-PAGE and then were transferred onto PVDF membranes and blocked with 5% nonfat milk at room temperature for 2 h. The membranes were washed with 1× tris-buffered saline with Tween 20 (TBST) for four times and incubated with primary antibodies overnight at 4 °C. The membranes were washed with 1× TBST again and incubated with HRP-conjugated secondary antibodies at room temperature for 1.5 h. The protein blots on the membranes were visualized using enhanced chemiluminescence (ECL, Beyotime). Total protein was used for determination of the expression levels of TLR4, MyD88, TNFR1, phosphorylated (p-) and total forms of p38, ERK and JNK. The NF-κB/P65 subunit was measured from cytosolic protein and nuclear protein. IκB-α was only determined from cytosolic protein.

2.11. Statistical analysis

All statistical analyses were performed using GraphPad Prism 6.02. All data are presented as mean ± standard deviation (s.d.). Survival rates were compared using a log-rank test. Paired comparisons were performed using a Student's *t*-test and multiple group comparisons were compared with one-way analysis of variance (ANOVA) followed by Dunnett's test. P values < 0.05 were considered statistically significant.

3. Results

3.1. MyD88 was significantly upregulated in D-gal/LPS-induced acute liver injury mice

To evaluate the expression of MyD88 in our D-gal/LPS-induced liver injury model we obtained hepatic tissue 12 h after D-gal/LPS stimulation and determined the MyD88 level via western blotting and real-time PCR. We found that the expression of MyD88 in D-gal/LPS group was higher than in the saline control group (Fig. 2A–C).

3.2. Inhibition or knock-out of MyD88 improves survival, protects liver function and attenuates liver damage after D-gal/LPS stimulation

Mice began to die 12 h after D-gal/LPS injection in the D-gal/LPS group and all died by 36 h after injection. However, pretreatment with TJ-M2010-5 and knock-out of MyD88 in mice dramatically improved their survival rate, which reached to 73.3% and 80.0% at 48 h, respectively (Fig. 3A). To evaluate the damage to liver function in each group, we determined plasma ALT and AST concentrations at 12 h post d-gal/LPS stimulation. As shown in Fig. 3B and C, ALT and AST levels were dramatically elevated in D-gal/LPS group rather than the saline group ($P < 0.001$). Strikingly, pretreatment with TJ-M2010-5 or knock-out of MyD88 efficiently prevented an increase in ALT and AST levels at 12 h ($P < 0.001$, $P < 0.001$). By analysizing pathologic slices of mice liver tissue, we found that D-gal/LPS group mice showed slight liver congestion, hepatocyte necrosis and inflammatory cells infiltration at 24 h after D-gal/LPS stimulation. However, these pathological alterations were significantly ameliorated, except for mild edema of hepatocytes, by TJ-M2010-5 pretreatment or knocking out MyD88 (Fig. 3D).

3.3. TJ-M2010-5 or knock-out MyD88 decreases hepatic apoptosis upon D-gal/LPS stimulation

TUNEL staining revealed a small amount of hepatocyte apoptosis at 12 h and numerous apoptotic hepatocytes at 24 h after D-gal/LPS stimulation. TJ-M2010-5 pretreatment or knock-out MyD88 could significantly reduce the number of apoptotic hepatocytes at 12 h and 24 h upon D-gal/LPS stimulation (Fig. 4A, B).

3.4. TJ-M2010-5 or knock-out of MyD88 inhibits Kupffer cell activation and attenuates inflammatory response

KCs represent a resident liver population of macrophages that have crucial functions in physiological and pathological processes. We isolated total mononuclear cells (MNCs) from mice livers 6 h after D-gal/LPS intraperitoneal injection and detected the activation of Kupffer cells in each group by flow cytometry (FCM). $F4/80^+$ cells were

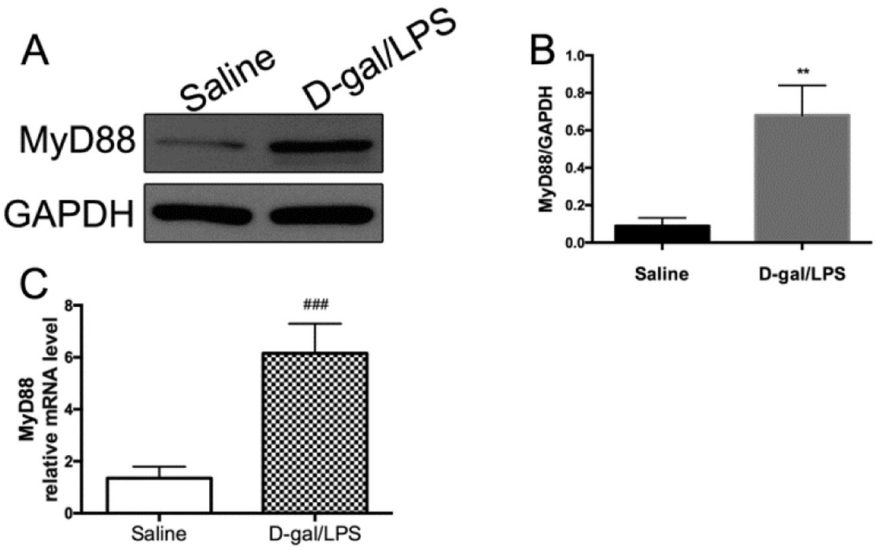

Fig. 2. MyD88 is significantly upregulated in D-gal/LPS-induced acute liver injury mice. Mice were exposed to D-gal/LPS for 12 h. **(A)** Total protein was extracted from the liver tissues and MyD88 expression were measured with western blot (one of three independent experiments shown). **(B)** Band densities were digitized and semi-quantitated. Each lane density of GAPDH was divided by that of MyD88 ($^{**}p < 0.01$ versus saline group, Student's *t*-test). Results are expressed as mean ± s.d. **(C)** Total RNA was extracted from liver tissues and MyD88 mRNA levels were measured by real-time PCR (one of three independent experiments shown) ($^{###}p < 0.001$ versus saline group, Student's *t*-test).

considered to represent Kupffer cells, and $CD80^+$ or $CD86^+$ represented markers for activated KCs. Our results showed that massive amounts of Kupffer cells were activated in the D-gal/LPS group and pretreatment with TJ-M2010-5 or knock-out of MyD88 significantly suppressed such activation (Fig. 5A–C). Further, to evaluate hepatic inflammation, we isolated total RNA from mouse liver 12 h after D-gal/LPS stimulation and measured the mRNA levels of several relevant pro-inflammatory cytokines (IL-1β, TNF-α, IL-6) and inflammatory mediators (iNOS, COX2) using real-time PCR. As shown in Fig. 5D–H, transcription of these pro-inflammatory factors was significantly increased in the D-gal/LPS group, and could be reduced by TJ-M2010-5 pretreatment or knocking out MyD88.

In addition, we tested mouse serum for IL-1β, TNF-α, IL-6, iNOS and COX2 expression by ELISA, which produced results consistent with the above results (Fig. 5I–M). Because TNF-α has a key role in inducing hepatocytes apoptosis, we further tested the content of TNF-α in liver tissues by immunofluorescence. Our results showed that levels of TNF-α were significantly increased in D-gal/LPS group, and application of TJ-M2010-5 or MyD88 knock-out minimized this increase at both 6 h and 12 h after D-gal/LPS injection (Fig. 6A, B).

3.5. TJ-M2010-5 or knock-out of MyD88 down-regulates the TLR/MyD88 signaling pathway

The abnormal activation of the TLR/MyD88 signaling pathway has been shown to contribute to liver inflammation, so we detected the

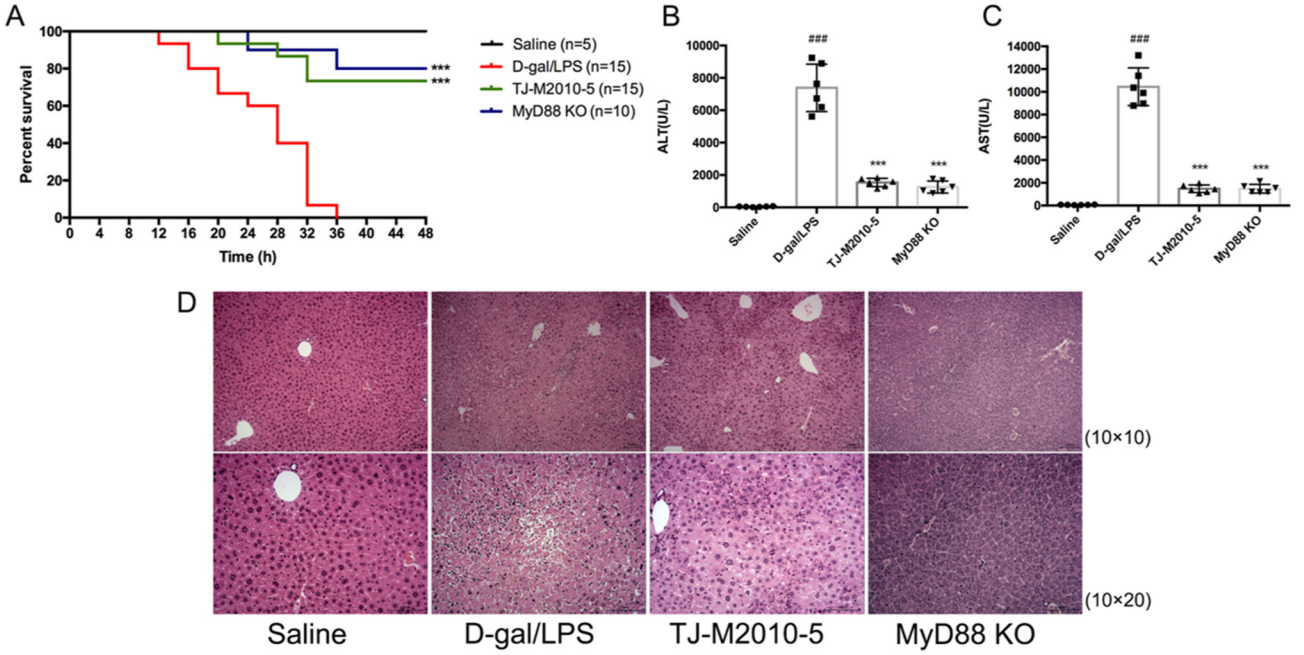

Fig. 3. TJ-M2010-5 or knock-out of MyD88 improves mouse survival and attenuates D-gal/LPS-induced liver damage. **(A)** After mice received intraperitoneal injection of D-gal/LPS, mouse survival was monitored for 48 h. The survival rate was 100% in the saline group, 0% in D-gal/LPS group, 73.3% in TJ-M2010-5 group, 80% in MyD88 KO group ($^{***}p < 0.001$ versus D-gal/LPS group, log-rank test). **(B, C)** Six mice were sacrificed in each group and blood samples were collected 12 h after injecting D-gal/LPS. Serum levels of ALT and AST were measured (representative results from one of three independent experiments shown) ($^{###}p < 0.001$ versus saline group; $^{***}p < 0.001$ versus D-gal/LPS group, one-way ANOVA with Dunnett's post-hoc-test.) Results are expressed as mean ± s.d. **(D)** Liver tissues were collected at 24 h after D-gal/LPS injection, and stained with hematoxylin & eosin. Images are presented from each group (original magnifications, ×100 and ×200) (representative results from one of three independent experiments shown).

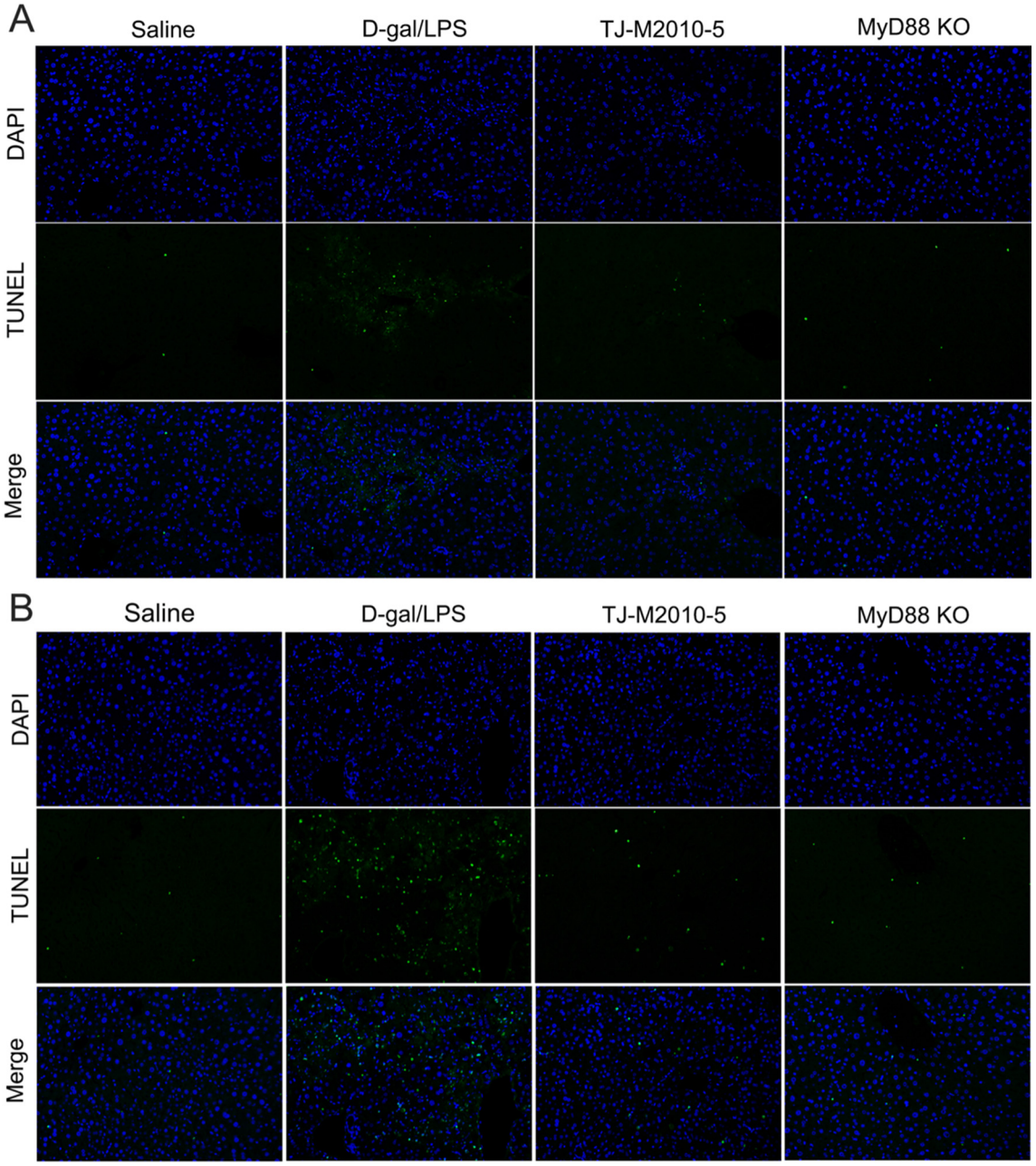

Fig. 4. TJ-M2010-5 or knock-out of MyD88 decreases hepatic apoptosis upon D-gal/LPS stimulation. Liver tissues were collected at **(A)** 12 h and **(B)** 24 h after D-gal/LPS injection. TUNEL stained apoptotic hepatocytes and DAPI stained nucleus in liver tissue sections from each group mice (original magnifications, ×200; representative results from one of three independent experiments shown).

expression of proteins related to this condition from mouse liver tissues 12 h after D-gal/LPS stimulation via western blot analysis. First, we measured TLR4, which was significantly increased in the D-gal/LPS group; this increase was attenuated in TJ-M2010-5 group and MyD88 KO group (Fig. 7A, E). Then we examined the nuclear translocation of NF-κB. We measured the content of the NF-κB/p65 subunit protein from cellular cytoplasm and nucleus respectively, and the content of IκB-α, an endogenous NF-κB inhibitor, from cytoplasm. Our results suggested that the level of cytoplasmic p65 was significantly decreased and the level of nuclear p65 from nuclear was significantly increased in the D-gal/LPS group. This indicated to us that the content of NF-κB nuclear translocation was increased. However, TJ-M2010-5 or knock-out of MyD88 significantly suppressed the increase in nuclear translocation of NF-κB. On the other hand, IκB-α content was decreased in the D-gal/LPS group and TJ-M2010-5 or MyD88 knock-out suppressed this decrease (Fig. 7B, F–H).

The MAPK signaling pathway plays a key role in cellular proliferation and apoptosis regulation. We tested the phosphorylation levels of p38, JNK, and ERK in our experimental models. Each level was increased in the D-gal/LPS group, and down-regulated by TJ-M2010-5 pre-treatment or MyD88 knock-out. Interestingly, the levels of the phosphorylation in TJ-M2010-5 group were lower than those of the MyD88 KO group (Fig. 7C, I–K). Finally, we measured one TNF-α receptor, TNFR1, which was expressed on the hepatocyte membrane. The activation of TNFR1 has been shown to contribute to hepatocytes apoptosis via the TNFR1-TRADD-FADD apoptotic pathway. Our results demonstrated that TNFR1 was highly expressed in D-gal/LPS group and its elevated expression was attenuated in the TJ-M2010–5 group and MyD88 KO group (Fig. 7D, L).

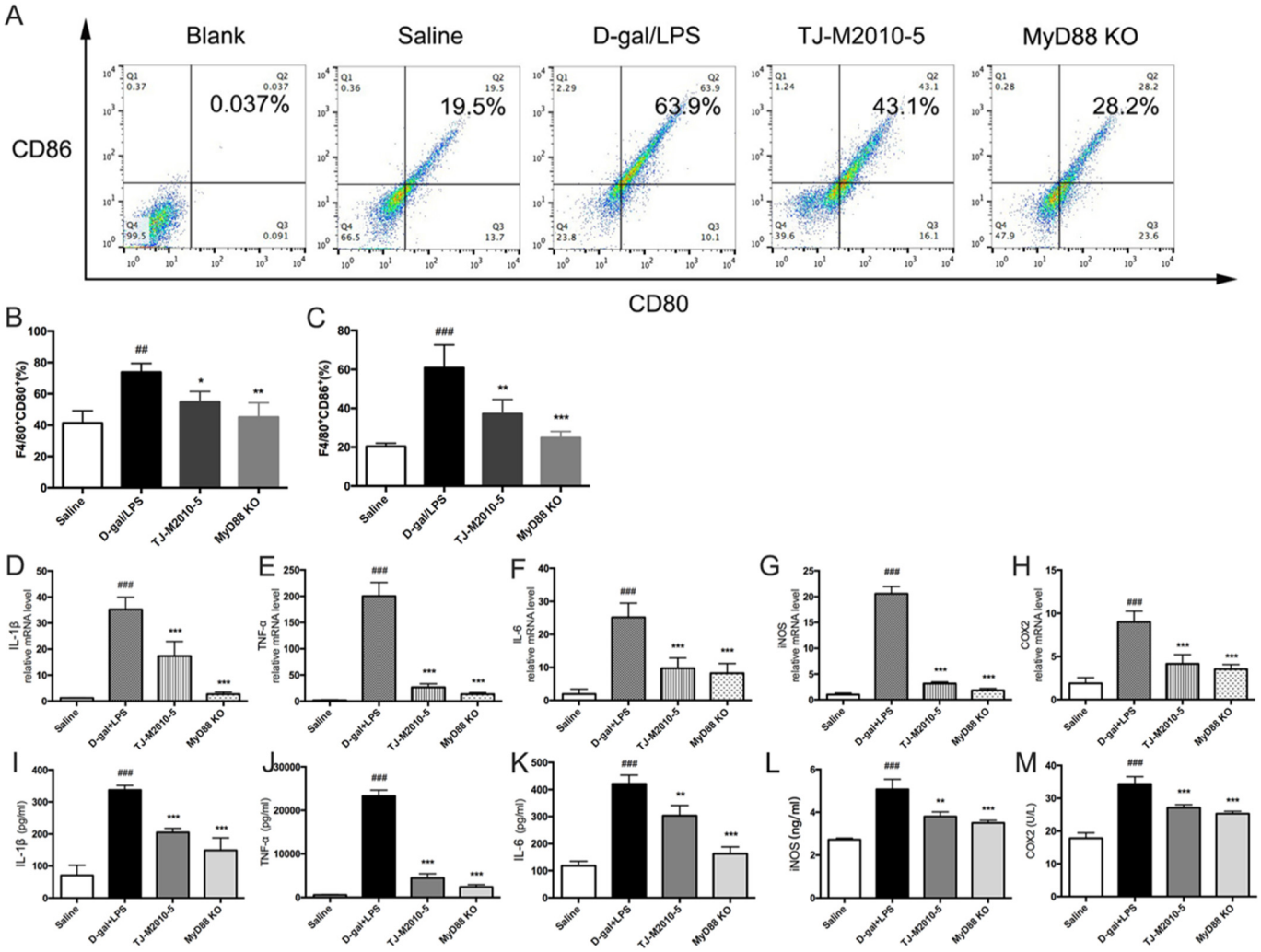

Fig. 5. TJ-M2010-5 or knock-out of MyD88 inhibits Kupffer cell activation and attenuates inflammatory responses in vivo. **(A)** Mononuclear cells were isolated from mouse liver tissues 6 h after D-gal/LPS injection and were stained with CD80, CD86 and F4/80 antibodies. The positivity ratio of CD80 or CD86 in F4/80$^+$ cells was measured by FCM (three mice were sacrificed for each group and one of three independent experiments shown). Blank: single stained F4/80 antibody and F4/80$^+$ cells. **(B, C)** Quantitative analysis of FCM results are shown. ($^{\#\#}p < 0.01$ versus saline group, $^{\#\#\#}p < 0.001$ versus saline group; $^{*}p < 0.05$ versus D-gal/LPS group, $^{**}p < 0.01$ versus D-gal/LPS group, $^{***}p < 0.001$ versus D-gal/LPS group; one-way ANOVA with Dunnett's post-hoc-test). Results are expressed as mean ± s.d. **(D–H)** RNA was extracted from liver tissues at 12 h after D-gal/LPS injection. IL-1β, TNF-α, IL-6, iNOS and COX2 mRNA levels were measured by real-time PCR ($^{\#\#\#}p < 0.001$ versus saline group; $^{***}p < 0.001$ versus D-gal/LPS group; one-way ANOVA with Dunnett's post-hoc-test). Results are expressed as mean ± s.d (representative data from one of three independent experiments shown). **(I–M)** Serum samples were collected at 12 h after D-gal/LPS injection (three mice were sacrificed from each group). IL-1β, TNF-α, IL-6, iNOS and COX2 serum levels were quantified by ELISA ($^{\#\#\#}p < 0.01$ versus saline group; $^{**}p < 0.01$, $^{***}p < 0.001$ versus D-gal/LPS group; one-way ANOVA with Dunnett's post hoc test). Results are expressed as mean ± s.d (representative data from one of three independent experiments shown).

3.6. TJ-M2010-5 inhibits LPS-induced macrophages activation and suppresses pro-inflammatory response in vitro

To further confirm whether TJ-M2010-5 directly affected macrophage activation, we obtained BMDMs from mice and detected the inhibitory effect of TJ-M2010-5 on LPS-induced macrophages activation in vitro. Our results showed that LPS stimulation increased BMDMs expression in CD80 and CD86. TJ-M2010-5 suppressed BMDM expression in a dose-dependent manner, especially 30 μM TJ-M2010-5 significantly inhibited BMDM activation (Fig. 8A–C). In addition, we examined the levels of IL-1β, TNF-α, IL-6, iNOS and COX2 at 12 h after LPS stimulation with or without TJ-M2010-5 pretreatment using real-time PCR and ELISA. The results also suggested the levels of these pro-inflammatory factors were inhibited by TJ-M2010-5 in a dose-dependent manner (Fig. 8D–M).

4. Discussion

The liver is an important organ which participates in metabolism, detoxification and energy conversion. Liver disease is often the result of multiple causes, such as viral infection, immunological factors, as well as abuse of toxic substances like alcohol, and so on [20–23]. If patients have a pre-existing liver disease, systemic infection can accelerate the progression of liver injury and result in acute liver failure (ALF). This condition has a high mortality rate because there is no satisfying therapy in conventional medicine [24]. There is a pressing need to confirm the underlying pathophysiological mechanism of ALI caused by infection and to develop a new therapeutic target to inhibit liver damage.

In this research, an ALI mice model was successfully established via intraperitoneal injection of D-gal and LPS, which simulates septicemia or endotoxemia-induced acute liver injury. D-gal is a kind of specific hepatocyte transcriptional inhibitor, which leads to major depletion of

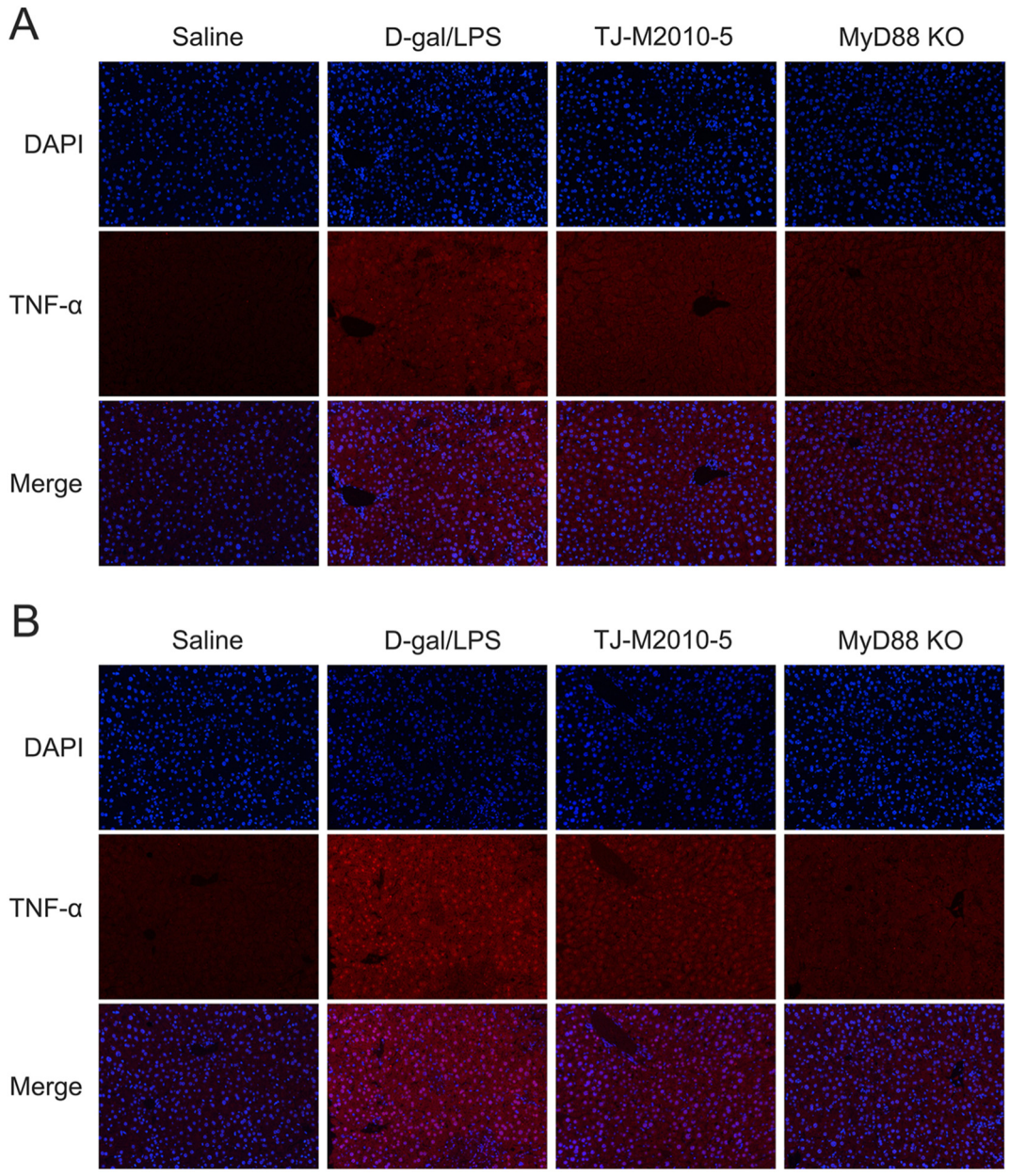

Fig. 6. TJ-M2010-5 or knock-out of MyD88 inhibits the production of TNF-α in liver tissues after D-gal/LPS injection. Liver tissues were collected at **(A)** 6 h and **(B)** 12 h after D-gal/LPS injection and stained with TNF-α (red) and nucleus (blue). (representative data from one of three independent experiments shown). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

uridine triphosphate (UTP) via irreversible binding. This depletion obstructs protein synthesis, inducing hepatocytes disfunction [25,26]. LPS is a major active component of endotoxins, derived from the outer membrane of Gram-negative bacteria cell wall, which activate some immunocytes to secrete pro-inflammatory factors, leading to inflammation-induced liver injury [27]. Due to the self-repair and regeneration capabilities of the liver and the resistance to infection in the body, low dose of LPS or D-gal alone is not enough to cause irreparable liver damage [28]. However, LPS in combination with D-gal in a low dose respectively can induce specific liver injury and mouse death. It is reported that D-gal could largely augment the sensitivity of hepatocytes to pro-inflammatory cytokines caused by LPS [29]. In this model, mouse liver macrophages played an important role in the process of hepatocyte apoptosis. They were activated by LPS, excreting a large number of pro-inflammatory cytokines [30]. The secreted TNF-α, combining with TNFR1 expressed on hepatocyte membrane, promoting hepatocytes apoptosis via the TNFR1-TRADD-FADD apoptotic pathway [31]. Depletion of these macrophages could directly alleviate liver inflammation [32] and indirectly protect liver after D-gal/LPS stimulation (supplementary results), but this method cannot be used in clinical practice. So inhibition of Kupffer cells activation may be a good remedial method to decrease inflammation-induced hepatocytes apoptosis.

Toll-like receptors (TLRs) are widely expressed on the cell membrane of various cells [33]. In the liver, LPS is first combined with LPS-binding protein (LBP) and then transferred to toll like receptor 4 (TLR4) to be expressed on the surface of Kupffer cells. During the interaction with MD2 and CD14, myeloid differentiation factor 88 (MyD88) is activated and induces cytoplasmic signaling cascades that lead to the activation of NF-κB and MAPK signaling, resulting excessive macrophage activation [34]. It had been reported that knocking out TLR4 genes no longer induces hepatocyte apoptosis after LPS/D-gal

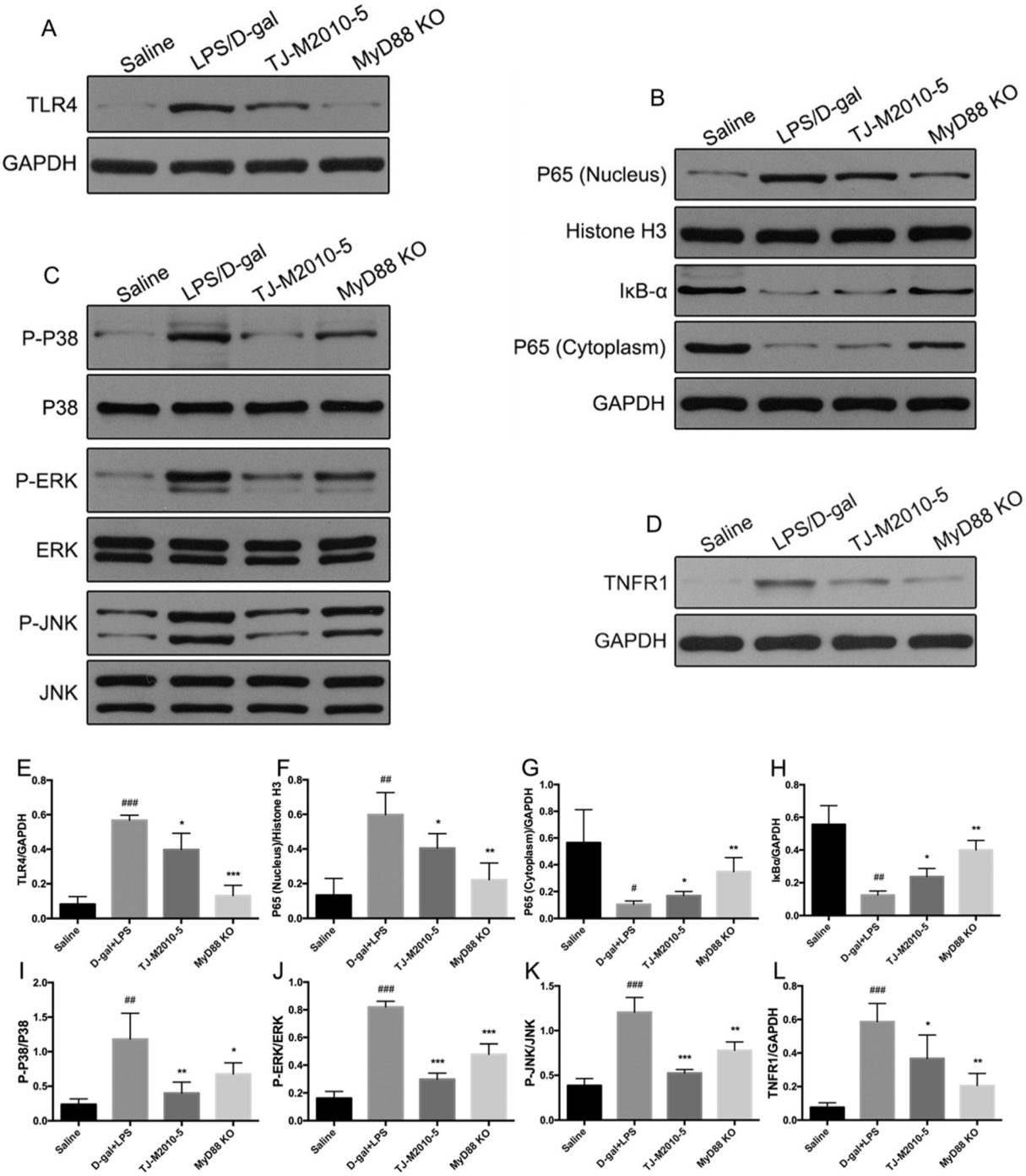

Fig. 7. TJ-M2010-5 or knock-out of MyD88 down-regulates the TLR/MyD88 signaling pathway and TNF-α receptor in vivo. Mice were exposed to D-gal/LPS for 12 h. **(A, C, D)** Total proteins were extracted from liver tissues. TLR4, p-p38, p-ERK, p-JNK and TNFR1 protein levels were analyzed by western blot (representative data from one of three independent experiments shown). **(B)** Cytosolic proteins and nuclear proteins were extracted from liver tissues respectively. Plasma proteins were used to detect NF-κB/P65 and IκB-α, and nuclear proteins were used to detect NF-κB P65 (representative data from one of three independent experiments shown). **(E–L)** Densitometric analysis of western blot results. The density of each internal reference band was divided by that of their target protein ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ versus saline group; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus D-gal/LPS group; one-way ANOVA with Dunnett's post-hoc-test). Results are expressed as mean ± s.d.

stimulation [35].

MyD88 adaptor protein is involved in all TLR signaling pathways except for TLR3, and so plays a dominant role in Kupffer cells activation. Therefore, we hypothesized that blocking MyD88 could also alleviate liver damage in this mouse model. In our previous work, we synthesized small molecules named TJ-M2010s, which targeted MyD88 at the TIR domain and blocked homodimerization of MyD88. We observed that TJ-M2010s could reduce LPS-induced dendritic cells (DCs) activation and further inhibited T-cell proliferation. This inhibition has been shown to help resolve diseases related to a strengthened immune system, such as cardiac or skin graft rejection, type 1 diabetes and renal ischemia reperfusion injury [12–14]. In this study, we presented a key role of MyD88 in D-gal/LPS-induced ALI by knocking out the MyD88 gene and demonstrating the effectiveness of TJ-M2010-5

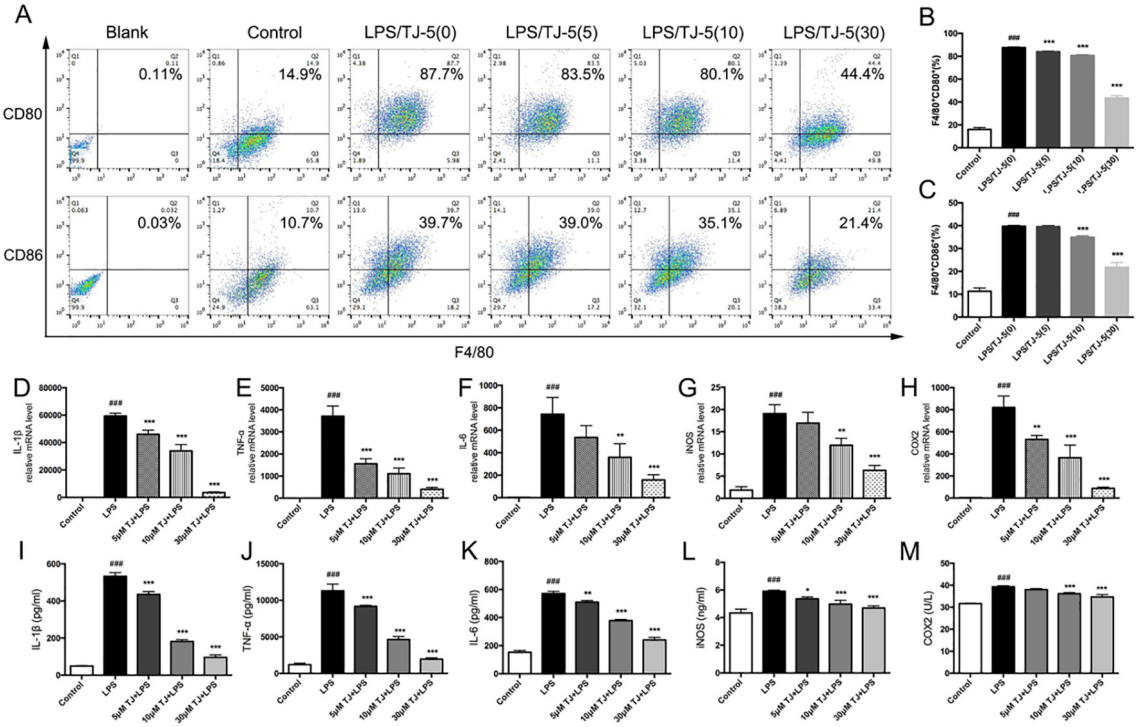

Fig. 8. TJ-M2010-5 interferes with BMDM activation and reduces the level of inflammatory factors in vitro. BMDMs were incubated with TJ-M2010-5 for 2 h and then stimulated with LPS for 12 h. **(A)** BMDMs were collected to measure CD80 and CD86 levels by FCM. TJ-M2010-5 inhibited CD80 and CD86 levels in a dose-dependent manner (one of three independent experiments is shown). Blank: Unstained BMDMs; Control: BMDMs stained with CD80, CD86 and F4/80 antibodies in the absence of LPS intervention. **(B, C)** Quantitative analysis of the FCM results. ($^{\#\#\#}p < 0.001$ versus control group; $^{***}p < 0.001$ versus LPS group; one-way ANOVA with Dunnett's post-hoc-test). Results are expressed as mean ± s.d. **(D–H)** RNA was extracted from BMDMs 12 h after LPS stimulation. IL-1β, TNF-α, IL-6, iNOS and COX2 mRNA levels were measured by real-time PCR. ($^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ versus control group; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus LPS group; one-way ANOVA with Dunnett's post-hoc-test). Results are expressed as mean ± s.d (representative data from one of three independent experiments shown). (I–M) BMDMs culture supernatant was collected 12 h after LPS stimulation. The levels of IL-1β, TNF-α, IL-6, iNOS and COX2 were measured by ELISA. ($^{\#\#\#}p < 0.001$ versus control group; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus LPS group; one-way ANOVA with Dunnett's post-hoc-test). Results are expressed as mean ± s.d (representative data from one of three independent experiments shown).

(one of TJ-M2010 series) in the treatment of ALI.

We observed that MyD88 protein was significantly upregulated in liver tissue after D-gal/LPS stimulation. Knocking out MyD88 or inhibiting MyD88 activation by TJ-M2010-5 decreased mouse mortality. Conversely, mice treated with D-gal/LPS started to die at 12 h post-treatment, and mortality reached 100% at 48 h. However, when MyD88 was knocked-out or there was pretreatment with TJ-M2010-5, the survival rate 48 h after D-gal/LPS injection reached 80% and 73.3% respectively. In addition, the activities of serum ALT and AST increased after D-gal/LPS injection. This increase was attenuated by knocking out MyD88 or pretreatment with TJ-M2010-5. Results of H&E liver tissue staining strongly support our conclusions about the hepato-protective effects of TJ-M2010-5. When exposed to D-gal/LPS, liver tissue showed large areas of hemorrhage and putrescence, combined with increasing inflammatory cell infiltration. However, knocking out MyD88 or pretreatment with TJ-M2010-5 attenuated these changes except for mild edema of hepatocytes. Thus all the phenomena suggested that inhibition of MyD88 activation could suppress the lethal liver damage and that TJ-M2010-5 has a great potential of clinical application in liver protection.

Based on the above results, we further investigated the protective mechanism of TJ-M2010-5 against D-gal/LPS-induced ALI in mice. NF-κB and MAPK activation are related to the regulation of macrophage activation and pro-inflammatory factor secretion [36]. TJ-M2010-5 treatment effectively inhibited the nuclear translocation of NF-κB and the phosphorylation of p38, JNK, and ERK in the liver after D-gal/LPS stimulation. The expression of TLR4 was also reduced. We concluded that due to a reduction in hepatocytes apoptosis, DAMPs released from hepatocytes were decreased and induced lower expression of TLR4 [37–39]. Then we tested the activation level of macrophages isolated from mice liver tissues. We found that TJ-M2010-5 could effectively reduce the proportion of activated macrophages. In line with in vivo results, co-culture with TJ-M2010-5 markedly inhibit the activation of BMDMs after LPS stimulation in a dose-dependent manner in vitro. Pro-inflammatory factors were secreted from macrophages, which directly or indirectly damaged hepatocytes. They were also efficiently reduced by TJ-M2010-5 treatment in vivo and in vitro.

Taken together, these findings suggest that TJ-M2010-5 treatment could effectively reduce the inflammatory response of the liver protecting this critical organ against damage from pro-inflammatory factors. Accordingly, this research provides a new therapeutic insight into the inhibition of MyD88 via inhibition with TJ-M2010-5 as a way to protect the liver from septicemia or endotoxemia in vulnerable patients.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2018.11.051.

References

[1] W.M. Lee, R.H. Squires Jr., S.L. Nyberg, E. Doo, J.H. Hoofnagle, Acute liver failure: summary of a workshop, Hepatology 47 (4) (2008) 1401–1415.
[2] W. Bernal, G. Auzinger, A. Dhawan, J. Wendon, Acute liver failure, Lancet 376 (9736) (2010) 190–201.
[3] T. Lescot, C. Karvellas, M. Beaussier, S. Magder, Acquired liver injury in the intensive care unit, Anesthesiology 117 (4) (2012) 898–904.
[4] J. Yan, S. Li, S. Li, The role of the liver in sepsis, Int. Rev. Immunol. 33 (6) (2014) 498–510.
[5] L.E.B. Savio, P. de Andrade Mello, V.R. Figliuolo, T.F. de Avelar Almeida, P.T. Santana, S.D.S. Oliveira, C.L.M. Silva, L. Feldbrugge, E. Csizmadia, R.D. Minshall, M.S. Longhi, Y. Wu, S.C. Robson, R. Coutinho-Silva, CD39 limits P2X7 receptor inflammatory signaling and attenuates sepsis-induced liver injury, J. Hepatol. 67 (4) (2017) 716–726.
[6] O. Krenkel, F. Tacke, Liver macrophages in tissue homeostasis and disease, Nat. Rev. Immunol. 17 (5) (2017) 306–321.
[7] F. Tacke, Targeting hepatic macrophages to treat liver diseases, J. Hepatol. 66 (6) (2017) 1300–1312.
[8] T. Takayashiki, H. Yoshidome, F. Kimura, M. Ohtsuka, Y. Shimizu, A. Kato, H. Ito, H. Shimizu, S. Ambiru, A. Togawa, M. Miyazaki, Increased expression of toll-like receptor 4 enhances endotoxin-induced hepatic failure in partially hepatectomized mice, J. Hepatol. 41 (4) (2004) 621–628.
[9] J. Deguine, G.M. Barton, "MyD88: A Central Player in Innate Immune Signaling." F1000Prime Rep, 6 (2014), p. 97.
[10] M. Nowak, G.C. Gaines, J. Rosenberg, R. Minter, F.R. Bahjat, J. Rectenwald, S.L. MacKay, C.K. Edwards 3rd, L.L. Moldawer, LPS-induced liver injury in D-galactosamine-sensitized mice requires secreted TNF-alpha and the TNF-p55 receptor, Am. J. Physiol. Regul. Integr. Comp. Physiol. 278 (5) (2000) R1202–R1209.
[11] J. Siednienko, T. Gajanayake, K.A. Fitzgerald, P. Moynagh, S.M. Miggin, Absence of MyD88 results in enhanced TLR3-dependent phosphorylation of IRF3 and increased IFN-beta and RANTES production, J. Immunol. 186 (4) (2011) 2514–2522.
[12] L.M. Zhang, J.H. Liu, C.B. Xue, M.Q. Li, S. Xing, X. Zhang, W.T. He, F.C. Jiang, X. Lu, P. Zhou, Pharmacological inhibition of MyD88 homodimerization counteracts renal ischemia reperfusion-induced progressive renal injury *in vivo* and *in vitro*, Sci. Rep. 6 (2016) 26954.
[13] C. Li, L.M. Zhang, X. Zhang, X. Huang, Y. Liu, M.Q. Li, S. Xing, T. Yang, L. Xie, F.C. Jiang, H.Y. Jiang, W.T. He, P. Zhou, Short-term pharmacological inhibition of MyD88 homodimerization by a novel inhibitor promotes robust allograft tolerance in mouse cardiac and skin transplantation, Transplantation 101 (2) (2017) 284–293.
[14] X. Zhang, S. Xing, M. Li, L. Zhang, L. Xie, W. He, J. Liu, S. Chang, F. Jiang, P. Zhou, Beyond knockout: a novel homodimerization-targeting MyD88 inhibitor prevents and cures type 1 diabetes in NOD mice, Metabolism 65 (9) (2016) 1267–1277.
[15] T. Fukuda, A. Mogami, H. Tanaka, T. Yoshikawa, M. Hisadome, H. Komatsu, Y-40138, a multiple cytokine production modulator, protects against D-galactosamine and lipopolysaccharide-induced hepatitis, Life Sci. 79 (9) (2006) 822–827.
[16] S.A. Sheriff, T. Devaki, Lycopene stabilizes lipoprotein levels during D-galactosamine/lipopolysaccharide induced hepatitis in experimental rats, Asian Pac. J. Trop. Biomed. 2 (12) (2012) 975–980.
[17] L. Ma, X. Gong, G. Kuang, R. Jiang, R. Chen, J. Wan, Sesamin ameliorates lipopolysaccharide/D-galactosamine-induced fulminant hepatic failure by suppression of toll-like receptor 4 signaling in mice, Biochem. Biophys. Res. Commun. 461 (2) (2015) 230–236.
[18] A. Mignon, N. Rouquet, M. Fabre, S. Martin, J.C. Pages, J.F. Dhainaut, A. Kahn, P. Briand, V. Joulin, LPS challenge in D-galactosamine-sensitized mice accounts for caspase-dependent fulminant hepatitis, not for septic shock, Am. J. Respir. Crit. Care Med. 159 (4 Pt 1) (1999) 1308–1315.
[19] Q. Yang, Y. Liu, Y. Shi, M. Zheng, J. He, Z. Chen, The role of intracellular high-mobility group box 1 in the early activation of Kupffer cells and the development of Con A-induced acute liver failure, Immunobiology 218 (10) (2013) 1284–1292.
[20] L.G. Guidotti, F.V. Chisari, Immunobiology and pathogenesis of viral hepatitis, Annu. Rev. Pathol. 1 (2006) 23–61.
[21] H. Saithanyamurthi, A.J. Faust, Drug-induced liver disease: clinical course, Clin. Liver Dis. 21 (1) (2017) 21–34.
[22] S. Bala, T. Csak, K. Kodys, D. Catalano, A. Ambade, I. Furi, P. Lowe, Y. Cho, A. Iracheta-Vellve, G. Szabo, Alcohol-induced miR-155 and HDAC11 inhibit negative regulators of the TLR4 pathway and lead to increased LPS responsiveness of Kupffer cells in alcoholic liver disease, J. Leukoc. Biol. 102 (2) (2017) 487–498.
[23] N. Nedelkopoulou, B. Vadamalayan, D. Vergani, G. Mieli-Vergani, Anti-TNFalpha treatment in children and adolescents with combined inflammatory bowel disease and autoimmune liver disease, J. Pediatr. Gastroenterol. Nutr. 66 (1) (2018) 100–105.
[24] J.M. Canabal, D.J. Kramer, Management of sepsis in patients with liver failure, Curr. Opin. Crit. Care 14 (2) (2008) 189–197.
[25] D. Keppler, R. Lesch, W. Reutter, K. Decker, Experimental hepatitis induced by D-galactosamine, Exp. Mol. Pathol. 9 (2) (1968) 279–290.
[26] K. Decker, D. Keppler, Galactosamine hepatitis: key role of the nucleotide deficiency period in the pathogenesis of cell injury and cell death, Rev. Physiol. Biochem. Pharmacol. 71 (1974) 77–106.
[27] D. Liu, C. Li, Y. Chen, C. Burnett, X.Y. Liu, S. Downs, R.D. Collins, J. Hawiger, Nuclear import of proinflammatory transcription factors is required for massive liver apoptosis induced by bacterial lipopolysaccharide, J. Biol. Chem. 279 (46) (2004) 48434–48442.
[28] J. Best, P. Manka, W.K. Syn, L. Dolle, L.A. van Grunsven, A. Canbay, Role of liver progenitors in liver regeneration, Hepatobiliary Surg. Nutr. 4 (1) (2015) 48–58.
[29] L. Dejager, C. Libert, Tumor necrosis factor alpha mediates the lethal hepatotoxic effects of poly(I:C) in D-galactosamine-sensitized mice, Cytokine 42 (1) (2008) 55–61.
[30] H. Tsutsui, S. Nishiguchi, Importance of Kupffer cells in the development of acute liver injuries in mice, Int. J. Mol. Sci. 15 (5) (2014) 7711–7730.
[31] O. Micheau, J. Tschopp, Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes, Cell 114 (2) (2003) 181–190.
[32] W. Huang, A. Metlakunta, N. Dedousis, P. Zhang, I. Sipula, J.J. Dube, D.K. Scott, R.M. O'Doherty, Depletion of liver Kupffer cells prevents the development of diet-induced hepatic steatosis and insulin resistance, Diabetes 59 (2) (2010) 347–357.
[33] Y. Chen, R. Sun, Toll-like receptors in acute liver injury and regeneration, Int. Immunopharmacol. 11 (10) (2011) 1433–1441.
[34] Y.C. Lu, W.C. Yeh, P.S. Ohashi, LPS/TLR4 signal transduction pathway, Cytokine 42 (2) (2008) 145–151.
[35] Z. Ben Ari, O. Avlas, O. Pappo, V. Zilbermints, Y. Cheporko, L. Bachmetov, R. Zemel, A. Shainberg, E. Sharon, F. Grief, E. Hochhauser, Reduced hepatic injury in toll-like receptor 4-deficient mice following D-galactosamine/lipopolysaccharide-induced fulminant hepatic failure, Cell. Physiol. Biochem. 29 (1–2) (2012) 41–50.
[36] Y.S. Kim, C.B. Ahn, J.Y. Je, Anti-inflammatory action of high molecular weight *Mytilus edulis* hydrolysates fraction in LPS-induced RAW264.7 macrophage via NF-kappaB and MAPK pathways, Food Chem. 202 (2016) 9–14.
[37] Z. Yang, Y. Deng, D. Su, J. Tian, Y. Gao, Z. He, X. Wang, TLR4 as receptor for HMGB1-mediated acute lung injury after liver ischemia/reperfusion injury, Lab. Investig. 93 (7) (2013) 792–800.
[38] S. Jovanovic Stojanov, V. Martinovic, D. Bogojevic, G. Poznanovic, A. Petrovic, S. Ivanovic-Matic, I. Grigorov, Modulation of diabetes-related liver injury by the HMGB1/TLR4 inflammatory pathway, J. Physiol. Biochem. 74 (2) (2018) 345–358.
[39] S.Y. Tsai, J.A. Segovia, T.H. Chang, I.R. Morris, M.T. Berton, P.A. Tessier, M.R. Tardif, A. Cesaro, S. Bose, DAMP molecule S100A9 acts as a molecular pattern to enhance inflammation during influenza A virus infection: role of DDX21-TRIF-TLR4-MyD88 pathway, PLoS Pathog. 10 (1) (2014) e1003848.