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Glycyrrhetinic acid alleviates hepatic inflammation injury in viral hepatitis disease via a HMGB1-TLR4 signaling pathway

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ABSTRACT

Various human disorders are cured by the use of licorice, a key ingredient of herbal remedies. Glycyrrhizic acid (GL), a triterpenoid glycoside, is the aqueous extract from licorice root. Glycyrrhetinic acid (GA) has been reported to be a major bioactive hydrolysis product of GL and has been regarded as an anti-inflammatory agent for the treatment of a variety of inflammatory diseases, including hepatitis. However, the mechanism by which GA inhibits viral hepatic inflammatory injury is not completely understood. In this study, we found that, by consecutively treating mice with a traditional herbal recipe, licorice plays an important role in the detoxification of mice. We also employed a murine hepatitis virus (MHV) infection model to illustrate that GA treatment inhibited activation of hepatic inflammatory responses by blocking high-mobility group box 1 (HMGB1) cytokine activity. Furthermore, decreased HMGB1 levels and downstream signaling triggered by injection of a neutralizing HMGB1 antibody or TLR4 gene deficiency, also significantly protected against MHV-induced severe hepatic injury. Thus, our findings characterize GA as a hepatoprotective therapy agent in hepatic infectious disease not only by suppressing HMGB1 release and blocking HMGB1 cytokine activity, but also via an underlying viral-induced HMGB1-TLR4 immunological regulation axis that occurs during the cytokine storm. The present study provides a new therapy strategy for the treatment of acute viral hepatitis in the clinical setting.

1. Introduction

In contrast to Western medicine which normally consists of a single compound designed for specific cellular targets, Traditional Chinese Medicine (TCM) incorporates a mixture of herbal remedies that have been developed through experience over thousand years and accepted conventionally in Eastern Medicine. However, the therapeutic basis and underlying molecular mechanism for the majority of the ingredients in TCM have yet to be elucidated [1,2]. Interestingly, a few herbs, including licorice, are present in the majority of conventional TCM recipes [3]. In addition, the major bioactive component of licorice, glycyrrhizic acid (GL), has been used to treat viral hepatitis and inflammatory disease [3,4]. The GL compound consists of two molecules glucuronic acid and one molecule glycyrrhetinic acid (GA) [5]. GA is also a key hydrolysis product of GL and is a triterpenoid saponins with a molecular weight of 470.69 Da (Fig. 1). GA has been shown to have anti-inflammatory effects in different systems through multiple mechanisms, such as inhibitory effects on reactive oxygen species (ROS) overproduction and NF-κB pathway activation [6,7]. However, little is

known about the role of GA in the immunological regulation during viral infection.

High-Mobility Group Box 1 (HMGB1) is a nuclear DNA-binding protein with highly conserved sequence, and plays an important role acting as an endogenous damage-associated molecular patterns (DAMPs) molecular [8]. HMGB1 also functions as an extracellular signaling molecule during inflammation, cell differentiation, cell migration, and tumor metastasis [9]. GL has been reported to interact with a motif within HMGB1 and inhibit its chemoattractant and mitogenic activities [6,10]. Additionally, GA can also bind selectively to HMGB1 protein and inhibit its cytokine activities by preventing accumulation of HMGB1 protein [11]. Some recent studies regarding the anti-inflammatory function of GA on hepatic injury have been performed. Severe liver disease induced by cytotoxic T lymphocyte (CTL) in a hepatitis B virus (HBV) transgenic mice model can be alleviated by treatment with GA, which can also inhibit the downstream HMGB1 signaling pathway in acetaminophen-induced acute liver injury [3,12]. HMGB1 is passively released from damaged or necrotic tissue cells and is actively secreted by inflammatory cells in response to stress, which
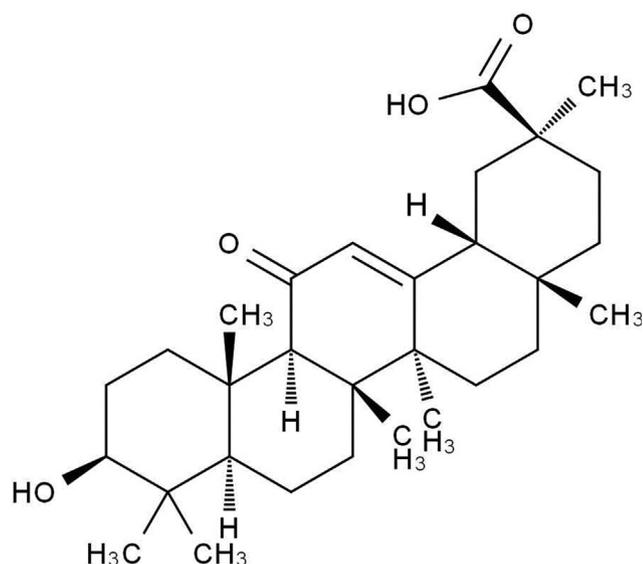
Fig. 1. Chemical structure of glycyrrhetinic acid (GA).

can activate innate immune receptors, such as toll-like receptors (i.e., TLR2 and TLR4), as well as the receptor for advanced glycation end-products (RAGE) [13]. Therefore, HMGB1 also acts as a therapeutic target for both sterile inflammation and infection [14]. However, the underlying mechanism for the effectiveness of GA on viral-induced liver injury is largely unclear.

In Asia, viral infections play an important role in acute and chronic liver failure with HBV being an important cause of acute-on-chronic liver failure (ACLF) in China [15,16]. Recently it has been reported that targeting the inhibition of NK cell activity can ameliorate liver damage in viral fulminant hepatitis [17]. Mouse hepatitis virus (MHV), a coronavirus with single-stranded RNA, causes several murine pathological illnesses, including enteritis, hepatitis, and encephalitis [18]. The outcome of viral infection depends on the virus strain and genetic background of the mouse. The MHV-A59 strain is a moderately hepatotropic virus, which may also cause moderate acute encephalitis and chronic demyelination [19,20]. In MHV-A59 infection animal models, adaptive immune cells have an unexpected role in tempering initial innate responses [21].

Although GL can exert anti-viral activity in SARS-associated coronavirus infection, the effect of its major metabolite, GA, on hepatic virus-induced tissue injury is not very clear [22]. Therefore, it was hypothesized that in a hepatotropic coronavirus model, GA may have different effects on virus replication and its protective function in tissue injury. To test this hypothesis, we examined whether the presence of licorice serves as a hepatoprotective agent against potentially toxic bioactive herbal ingredients. Then we characterized the effectiveness of GA in inhibiting HMGB1 cytokine activity and suppressing the induction of inflammatory genes known to be involved in hepatic injury while assessing the involvement of HMGB1/TLR4 signaling in mediating tissue injury during viral hepatitis. There is a pressing need for new approaches to prevent and manage viral hepatitis, and we hope that a better understanding of GA function will allow for emergence of new therapeutic approaches in Western medicine.

2. Materials and methods

2.1. Experimental animals and ethics statement

Experimental 8-week-old female C57BL/6 mice wild-type (WT) were purchased from Vital River Co., Ltd (Beijing, China). For the TLR4 knockout (KO) studies, 6-week-old C57BL/10ScNJ TLR4 KO and C57BL/10 J WT mice (18 g) were purchased from the Model Animal

Research Center of Nanjing University (Nanjing, China). Mice were housed and studied under specific pathogen-free conditions in the animal facilities at the Institute of Biophysics, Chinese Academy of Sciences (CAS) (Beijing, China).

Animal studies were carried out in accordance with the guidelines of the Animal Ethics and Experimentation Committee of the Institute of Biophysics. All animal experiments were approved by the Institutional Animal Care and Use Committees at the Institute of Biophysics, CAS (permit number: DWSWAQ-2012204).

2.2. Drugs and reagents

The recipe for Xiaoyao Powder was first recorded in "Taiping Huimin Heji Jufang", one of the earlier pharmacopoeia from Song Dynasty in China [23]. In this TCM study, our modified recipe consists of Radix Bupleuri, Radix Angelicae Sinensis, Radix Paeoniae Alba, Rhizoma Atractylodis Macrocephalae, Poria, Rhizoma Zingiberis Recens, Radix Glycyrrhizae Preparata and Herba Menthae in a ratio of 5:5:5:5:5:5:4:1.

GA crystalline powder, in the form of 18β-glycyrrhetinic acid (97% purity) was kindly supplied by Huzhou R&D Center, institute for Nutritional Sciences, Shanghai Institute for Biological Sciences, CAS (Huzhou, China). The powder was dissolved in a PBS-buffered solution of 10% ethanol. After adding 0.1% dimethyl sulfoxide (DMSO), the solution was then heated to 55°Cand stirred evenly and continuously.

2.3. Experimental designs of the animal studies

The composition of the modified Xiaoyao Powder with licorice (TCM + L) included licorice (11.4% of total weight), while licorice was excluded from TCM-L mixtures. We divided the mice into three groups, which received a daily intragastric (i.g.) injection of PBS (control group), TCM + L PBS solution, or TCM-L PBS solution (100 mg/ml, 1 g/kg,) for 21 days. All mice were monitored for weight loss or mortality. Serum samples were collected on 5, 10, and 15 days after the first injection. Subsequently, animals were sacrificed, and liver tissue and serum samples were collected 21 days after the first injection.

In the MHV infection model, mice were infected by intraperitoneal (i.p.) injection of 8×10^5 plaque forming unit (PFU)/mouse (lethal) or 1×10^4 PFU/mouse (sublethal) of MHV-A59 in 0.2 ml Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, USA). Mice were monitored daily for weight loss or mortality. Animals were sacrificed and liver tissue and serum samples were collected 5 days post-infection (dpi).

2.4. Cell culture and virus titer assay

Murine fibroblast 17Cl-1 cells, murine dendritic D2SC cells, and mouse embryonic hepatic BNL.CL2 cells (ATCC) were grown in DMEM containing 10% fetal bovine serum (Gibco, USA), 100 IU/ml penicillin and 100 μg/ml streptomycin (Gibco, USA) in the presence of 5% CO2 at 37 °C. Murine macrophage RAW 264.7 cells (ATCC) were cultured in Roswell Park Memorial Institute (RPMI) medium 1640 (Gibco, USA) supplemented with 10% FBS, 100 IU/ml penicillin and 100 μg/ml streptomycin.

MHV-A59 virus stocks were produced in mouse 17Cl-1 cells. The titers in the livers of infected mice and supernatants of cells were determined by plaque assays in 17Cl-1 cell monolayers. After 24 h of incubation with serially diluted supernatants of tissue homogenates or supernatants, plaques were counted on the cell layers. All titrations were performed in duplicates, and the average PFU per gram of liver tissue or per milliliter of supernatant were calculated.

2.5. HMGB1 and antibody neutralizing assay

Two hours after viral infection, mice were administered GA solution (20 mg/kg, i.p.) or an equivalent volume of PBS as a control. Mice were

then i.p. injected every other day for total of three times. Purified anti-HMGB1 monoclonal neutralizing antibody 3E8 was prepared as previous described [24]. Two hours after MHV viral infection, mice were administered a diluted antibody solution (2.5 mg/kg, i.p.) or an equivalent volume of PBS as a control. Mice were then i.p. injected every other day for total of three times. To assess the effects of GA on HMGB1 cytokine activity, D2SC cells (5×10^5 cells/well) were co-incubated with HMGB1 (10 µg/ml HMGB1-FLAG protein) (Sigma-Aldrich, USA) and different amounts (10, 100, 1000 µg/ml) of GA for 6 h. The TNF-α and IL-6 levels in the supernatant were then assayed using an ELISA kit (eBioscience, USA).

Serum harvested from MHV-A59-infected mice was used for detecting the circulating levels of murine HMGB1 according to the manufacturer's instructions of ELISA kit (Chondrex Systems, USA).

2.6. Alanine aminotransferase (ALT) activity and liver histological analysis

Serum ALT levels were measured with a kit according to the manufacturer's instructions (Biosino Bio, Beijing, China) analysis. Liver specimens were fixed in 10% neutral-buffered formalin. Then paraffin-embedded liver sections (5 µm thick) were stained with Hematoxylin and Eosin (H&E) using standard techniques [17].

2.7. In vitro cytokine release assay

BNL.CL2 cells were infected with MHV-A59 at multiplicity of infection (MOI) = 0.5. Then one hour later, the cells were treated with GA (100 µg/ml) and anti-HMGB1 antibody (Ab) (10 µg/ml) for 15 min and then DMEM medium was added. Interferon-inducible protein-10 (IP-10) levels at different time points, in addition to virus titers, were measured 48 h after infection. The supernatant from MHV-infected BNL.CL2 cells (48 h) were UV-radiated for 30 min in order to inactivate the viruses. Anti-HMGB1 Ab (1 µg/ml) and GA solution (20 µg/ml) were incubated with the same volume of supernatant containing HMGB1 for 1 h at 37 °C, and then the mixture was added to the medium of RAW 264.7 cells. Eight hours after incubation, the total RNA of stimulated cells was isolated and IP-10 gene expression was assayed using real-time PCR. The TNF-α cytokine levels in the supernatants were also measured after stimulation.

Serum harvested from MHV-A59-infected mice was used for detecting the circulating levels of cytokine, including IL-1β, IP-10, IL-6, IL-10, IL-17A, and IL-22, according to the instructions of the ELISA kits (eBioscience, USA).

2.8. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from liver tissue or cultured cells using TRIzol (Invitrogen, USA) according to the manufacturer's instructions. The cDNA synthesized as described previously [21]. The reverse-transcribed mRNA expression of IP-10, IL-6, TNF-α, IL-1β, TLR4, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes were determined by qRT-PCR using SYBR Green Master Mix Kit (Applied Biosystems, Foster City, CA, USA). The primers for the following genes are listed in Table 1.

The StepOnePlus™ Real-Time PCR System (ABI, Foster City, CA,

USA) was used to analyze the samples quantitatively under the following conditions: 1 cycle at 95 °C (5 min), followed by 40 cycles of 95 °C (20 s), 55 °C (30 s), and 72 °C (10 s). The housekeeping gene expression of GAPDH was used as the endogenous control for sample normalization with the $2^{-\Delta\Delta}$CT method.

2.9. Statistical analysis

All results were expressed as the mean ± standard error of the mean. The difference between the two groups was examined using a Student's *t* test after analyzing the variance. Statistics were performed using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). A P-value of < 0.05 was considered statistically significant (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

3. Results

3.1. Licorice has a hepatoprotective role in the TCM recipe

As a well-known herb formulation, Xiaoyao Powder contains licorice as one of the ingredients of the recipe. This recipe is widely used to treat liver damage and mental disorders [1,3]. This Xiaoyao Powder solution was subjected to ultra-performance liquid chromatography coupled with mass spectrometry (UPLC-MS), and 16 common fingerprint peaks were defined and identified. Fourteen target compounds, such as saikosaponin c, albiflorin, paeoniflorin, quercetin, and ferulic acid, were characterized distinctly in the plasma from rats orally administrated the Xiaoyao Powder solution rat as determined by UPLC-MS/MS [23,25]. In this study, chronic hepatic toxicity investigation of a modified recipe solution was carried out by injecting the solution to mice. The difference in the formulation between mixtures with licorice (11.4%) and mixtures without licorice (including seven herbal ingredients) was tested. Interestingly, long-term daily administration of TCM (i.e., 1 g/kg) without licorice (TCM-L) led to body weight loss as compared to mice that received TCM + L (Fig. 2A). Furthermore, serum ALT and AST levels increased significantly 21 days after the first injection in the TCM-L group as compared to control mice and the TCM + L group (Fig. 2B-C). Additionally, we examined the induction of IP-10 and IL-1β, two inflammatory cytokines induced during liver injury. Removing licorice from the TCM formula resulted in increased IP-10 and IL-1β cytokine release (Fig. 2D-E) as well as the induction of other inflammatory genes, such as IP-10, TNF-α and IL-6 (Fig. 2F-H). These findings suggest that licorice could be a hepatoprotective agent in Xiaoyao Powder that alleviates the long-term toxic effects of the other bioactive ingredients in this formulation. Therefore, as the key metabolite of licorice, GA has the potential to alleviate viral-induced hepatic injury.

3.2. GA treatment abates MHV-induced acute liver injury and mortality

In the following experiment, we set out to evaluate the effectiveness of GA in alleviating hepatic injury in a viral infection model. MHV causes hepatic and central nervous system diseases of varying severity, depending on the strain and is therefore used as a model for hepatitis, viral encephalitis, and demyelination. In our lab, we have established

Table 1
List of primers used for qRT-PCR.

Genes	Species	Forward sequence (5′-3′)	Reverse sequence (5′-3′)
IP-10	mouse	CCAGTGAGAATGAGGGCCATA	TCGTGGCAATGATCTCAACAC
IL-6	mouse	CACAGAGGATACCACTCCCAACA	TCCACGATTTCCCAGAGAACA
TNF-α	mouse	GGTGCCTATGTCTCAGCCTCTT	CGATCACCCCGAAGTTCAGTA
IL-1β	mouse	GAGCTGAAAGCTCTCCACCTCA	TCGTTGCTTGGTTCTCCTTGTAC
TLR4	mouse	TCAGAGCCGTTGGTGTATCTT	CCTCAGCAGGGACTTCTCAA
GAPDH	mouse	ACTCCACTCACGGCAAATTCA	CGCTCCTGGAAGATGGTG
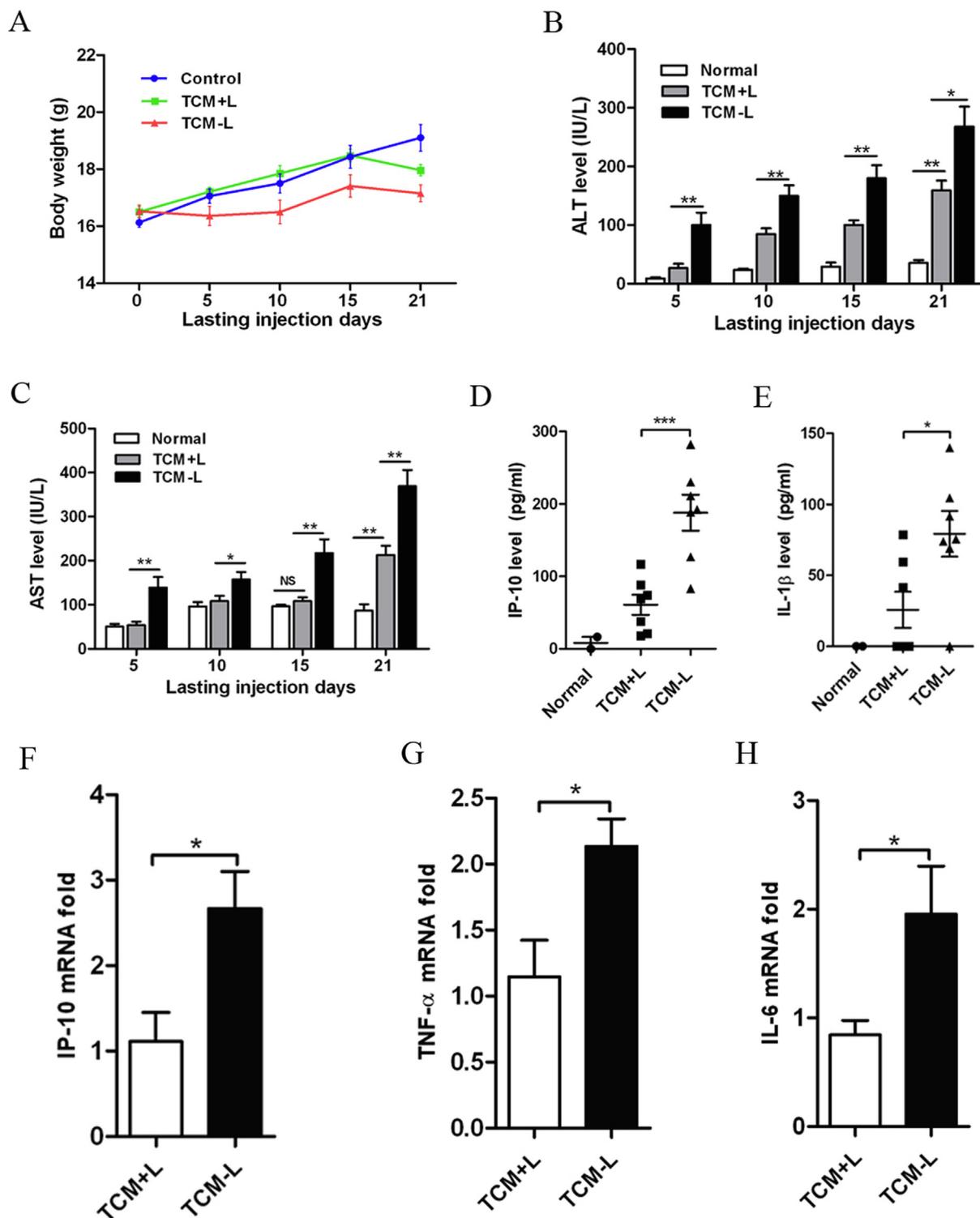
Fig. 2. Licorice in TCM formulas alleviates hepatic injury during long-term oral administration. C57BL/6 female mice were injected with PBS (control) or TCM + licorice solution (Xiaoyao Powder including licorice) or TCM-licorice solution (Xiaoyao Powder excluding licorice) every day (with dose of 1 g/kg, i. g. injection) for 21 days. (A) body weight, (B) serum ALT and (C) serum AST levels on days 5, 10, 15 and 21. (D-E) IP-10 and IL-1β cytokine levels in the serum of different groups on day 21. (F-H) RNA was isolated from liver samples on day 21 and levels of inflammatory genes IP-10, TNF-α and IL-6 were assessed using qPCR. The PCR data represents fold induction as compared to the control group (mean ± SEM, * P < 0.05). All other data are represented as the mean ± SEM; * indicates P < 0.05, ** indicates P < 0.01, and *** indicates P < 0.001.

acute lethal and sub-lethal MHV infection models using the MHV-A59 virus strain, which is a moderately hepatotropic and neurotropic virus (Fig. S1). Murine survival studies suggested that administration of GA (20 mg/kg, i.p.) on the day of infection and every other day after infection leads to an increased survival rate in MHV-infected mice as compared to the control group (Fig. 3A). In addition, the virus titer assay showed that GA treatment significantly inhibited the viral proliferation in the liver (Fig. 3B). As the marker of hepatotoxicity, ALT
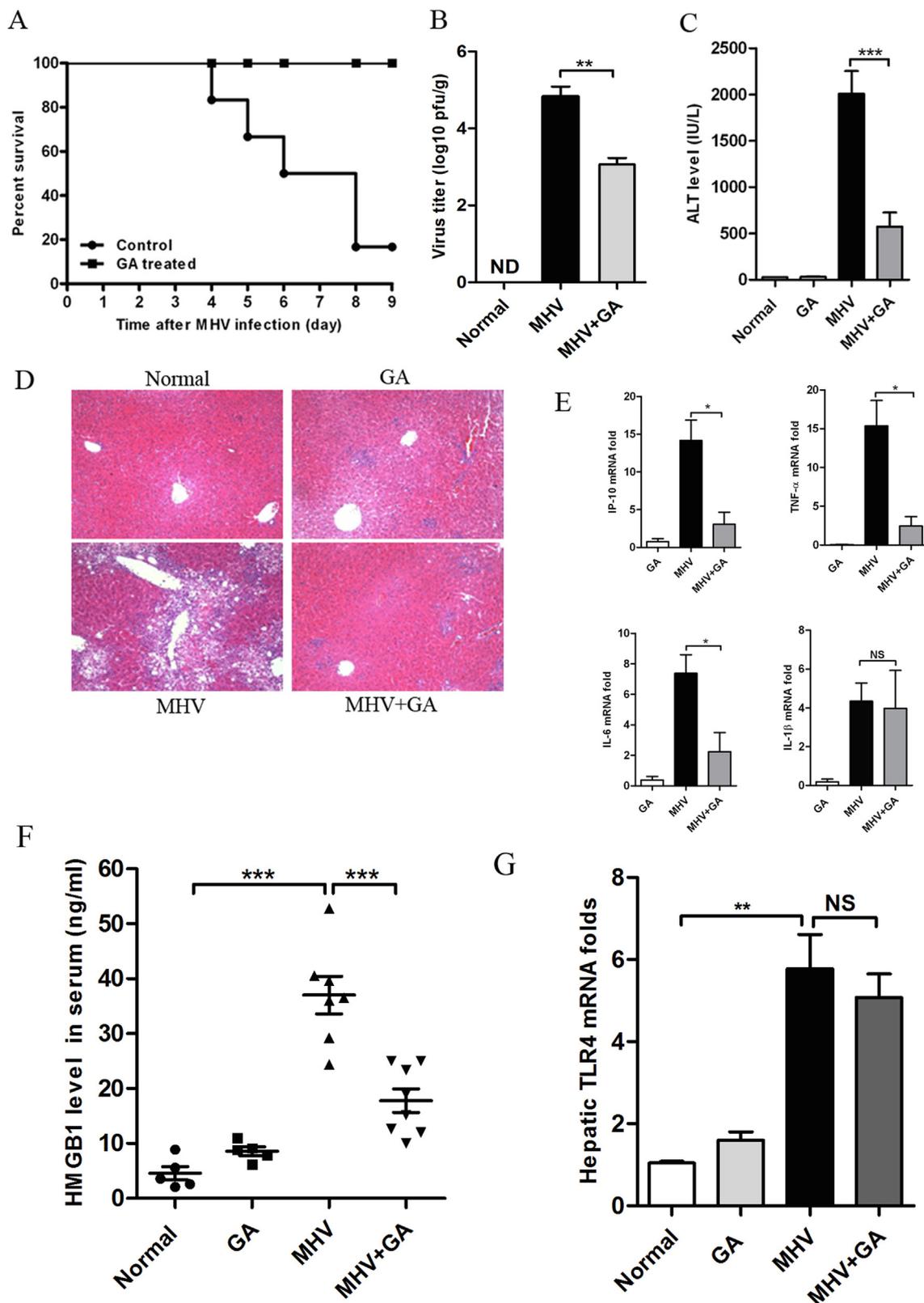
Fig. 3. Protective effects of GA against MHV infection in mice by suppressing induction of inflammatory cytokines. C57BL/6 mice were infected with 8×10^5 pfu/mouse (i.p.) for survival studies and 1×10^4 pfu/mouse (i.p.) for acute infections. Two hours later, mice were injected with GA (20 mg/kg, i.p.) every other day (total three times). Five days post infection (dpi), mice were sacrificed and serum and liver samples were collected. (A) Effects of GA administration on the survival of MHV-infected mice. (B) Virus titer in liver homogenates. (C) Serum ALT levels at 5 dpi. (D) Representative H&E staining of liver tissues at 5 dpi (magnification $\times 100$). (E) RNA was isolated from liver samples at 5 dpi and levels of inflammatory genes IP-10, TNF-α, IL-6 and IL-1β were assessed using qPCR. (F) HMGB1 release assay was performed using the serum from GA treated mice infected by MHV at 5 dpi. (G) Expression of the TLR4 gene in hepatic tissue from infected mice at 5 dpi. The data show fold inductions as compared to the control group (mean ± SEM, * indicates P < 0.05, ** indicates P < 0.01, and *** indicates P < 0.001).

exhibited a decreasing trend until the level of 530 IU/ml (Fig. 3C). Furthermore, GA treatment decreased MHV-induced hepatic injury and exhibited fewer necrotic foci upon liver histological analysis (Fig. 3D). In some sterile hepatic injury diseases, GA attenuates HMGB1-mediated inflammatory by inhibiting TNF-α, IL-1β and IL-6 production [7,26,27]. Pro-inflammatory chemokine IP-10 is a predictive marker of hepatitis C virus (HCV) and HBV infection [28,29]. We then determined the gene expression of these four pro-inflammatory cytokines, which have been shown to be key triggers in the innate immune response in hepatic injury in mouse models (Fig. 3E). Our qPCR data demonstrated that GA treatment during viral infection in mice can suppress the induction of several pro-inflammatory cytokine genes, except IL-1β (Fig. 3E), which has some different expression in sterile inflammation disease [26].

In line with previous reports about sterile hepatic injury, it was found that GA treatment also significantly inhibited HMGB1 levels in the serum of infected mice as compared to control mice (Fig. 3F) [11]. Surprisingly, the high level of TLR4 gene expression in hepatic tissue from MHV-infected mice was not affected by GA treatment, which is in accordance with sterile hepatic injury (Fig. 3G) [26,27]. Thus, studies regarding HMGB1 cytokine activity induced by GA needs to be explored further.

3.3. HMGB1 cytokine activity and secondary inflammatory responses in MHV-infected cells is inhibited by GA in vitro

It has been reported that GL has a variety of pharmacological properties, including anti-inflammatory and anti-viral activities [22,30]. However, it is not clear if GA, as the secondary metabolite of GL, could act as anti-viral factor in hepatic cells in vitro. Since GA treatment affected MHV replication in vivo, we explored the possibility and mechanisms by which GA can affect cellular injury. Because of viral infectivity, a significant amount of HMGB1 was released by BNL.CL2 cells infected with MHV (MOI = 0.5) as well as MEF and 17Cl-1 cells (Fig. 4A and Fig. S2). In order to determine whether GA is able to inhibit the HMGB1-induced expression of inflammatory genes, D2SC cells were co-incubated with HMGB1 solution and different doses of GA for 6 h. Analysis of the culture supernatants suggested that HMGB1 stimulation leads to the release of IL-6 and TNF-α, which were inhibited by GA treatment in a dose-dependent manner (Fig. 4B-C). The supernatant solution from infected BNL.CL2 cells was also exposed to UV radiation for virus inactivation and then added to RAW 264.7 cells, after incubating with anti-HMGB1 antibody or GA for 8 h, qPCR analysis showed that IP-10 expression in macrophages was able to be inhibited by both HMGB1 antibody and GA treatment (Fig. 4D). TNF-α cytokine levels released from RAW 264.7 cells, which was triggered by MHV-infected hepatocyte supernatant, showed the same trend as IP-10 (Fig. 4E). Similar to our in vivo data, GA treatment in vitro was able to inhibit the induction of IP-10 in MHV-infected BNL.CL2 cells (Fig. 4F). Therefore, the above results suggested that GA exerts regulation functions in hepatic cells and immune cells. Interestingly, GA and HMB1 antibody treatment did not affect the viral proliferation in BNL.CL2 cell (Fig. 4G). This was not in accordance with the data from other murine cells. This difference may be due to indirect and immunological viral inhibition mechanisms of GA in hepatic infection model.

3.4. HMGB1 neutralizing antibody can suppress mouse mortality and severity of MHV-induced liver injury

MHV-infected mice showed high levels of HMGB1 (67 ng/ml) in their serum at 5 dpi, suggesting that MHV infection causes release of HMGB1 into the serum either through active release by inflammatory cells or due to hepatocellular injury caused by viral replication (Fig. 5A). In order to examine whether HMGB1 mediates the pathogenesis of MHV-induced lethality, mice were injected with a HMGB1 neutralizing antibody after infection and then every other day. During MHV infection with a lethal dose (8×10^5 PFU/mouse), injection of the

blocking antibody completely rescued mice as compared to the PBS control (Fig. 5B). Interestingly, the virus titers on 5 dpi in the infected livers of the antibody-treated group and control group were not significantly different (Fig. 5C), suggesting that HMGB1 antibody treatment does not affect viral replication in the liver. However, the neutralizing antibody-treated group expressed fewer indicators of liver injury, such as decreased release of ALT and lack of histological necrotic foci and inflammatory cell infiltration (Fig. 5D-E). These results indicate that HMGB1 plays an important role in mediating liver injury in a MHV infection model. Furthermore, inflammatory cytokine expression data illustrated that a HMGB1 neutralizing antibody significantly suppresses the transcriptional induction of IP-10, TNF-α and IL-6 genes in infected liver tissue (Fig. 5F-H), which is similar to the effect in the GA treatment group.

3.5. MHV infection mediates hepatic injury through a TLR4-dependent signaling pathway

TLR4 signaling has been reported to contribute to tissue damage in both pathogenic hepatic injury and sterile inflammatory hepatitis [31–33]. As a ligand of TLR4, HMGB1 plays an important role in many viral infection models [34–36]. Therefore, we examined whether TLR4 signaling is also critical in a MHV infection model. Survival curve data showed that TLR4 KO mice are protected against lethal MHV infection as compared to infected WT mice (Fig. 6A). Interestingly, there was no distinct difference in virus titer between TLR4 KO and WT infected-mice, which suggests that virus inhibition is not the major cause underlying the protective role during lethal dose infection in TLR4 KO mice (Fig. 6B). Furthermore, TLR4 KO mice exhibited decreased signs of liver injury as compared to control mice, such as lower ALT levels in serum and fewer of necrotic foci in histological analysis (Fig. 6C-D). Additionally, IP-10, TNF-α and IL-6 gene were not strongly induced in the TLR4 KO mice infected with MHV, further suggesting that TLR4 is an important immune receptor in mediating inflammation during MHV infection (Fig. 6E-G). Although TLR4 KO mice may not affect MHV proliferation in vivo, the TLR4 gene could play a key role in determining the level of HMGB1 release in infected mouse serum, as well as the GA treatment effect (Fig. 6H). TLR4 is one of downstream receptors of the HMGB1, but TLR4 mediates HMGB1 release in viral infection similar to its regulation in hepatic sterile inflammation disease [37,38].

3.6. GA treatment or TLR4 deficiency alleviates cytokine production stimulated by MHV in vivo

In accordance with our survival curve data, body weight loss in MHV-infected TLR4 KO and GA treated mice groups was rescued at 5 dpi (Fig. 7A). Assay results of IL-1β, IP-10, IL-6 suggested that TLR4 deficiency significantly inhibits the production of pro-inflammatory cytokines in the serum of infected mice, which is similar to GA treatment effect (Fig. 7B-D). Viral-induced fulminant hepatitis can cause acute liver failure involved with IL-17 and IFN-γ release and Th17 cell responses [39]. Viral infection triggers early production of IL-22 from γδT cells in the liver, and IL-22 plays an important role in host immunity and tissue homeostasis induced by infectious and inflammatory diseases [40]. Our findings illustrated that IL-17A and associated cytokine IL-22 in infected TLR4 KO mice decreased significantly, similar to mice treated with GA (Fig. 7E-F). The production of these cytokines are usually from the liver, spleen, and thymus stimulated by viral infection.

Unlike above pro-inflammatory cytokines, IL-10 was stimulated and reached the concentration of 2432 pg/ml in the serum of GA-treated mice, which suggests that GA may function as positive stimulator to IL-10 cellular secretion. Furthermore, IL-10 production in MHV-infected mice depended, to a great extent, on the expression of the TLR4 gene (Fig. 7G). Although derived from the same interleukin family, IL-10 and IL-22 exhibited differing trends in GA-treated mice (Fig. 7F-G).
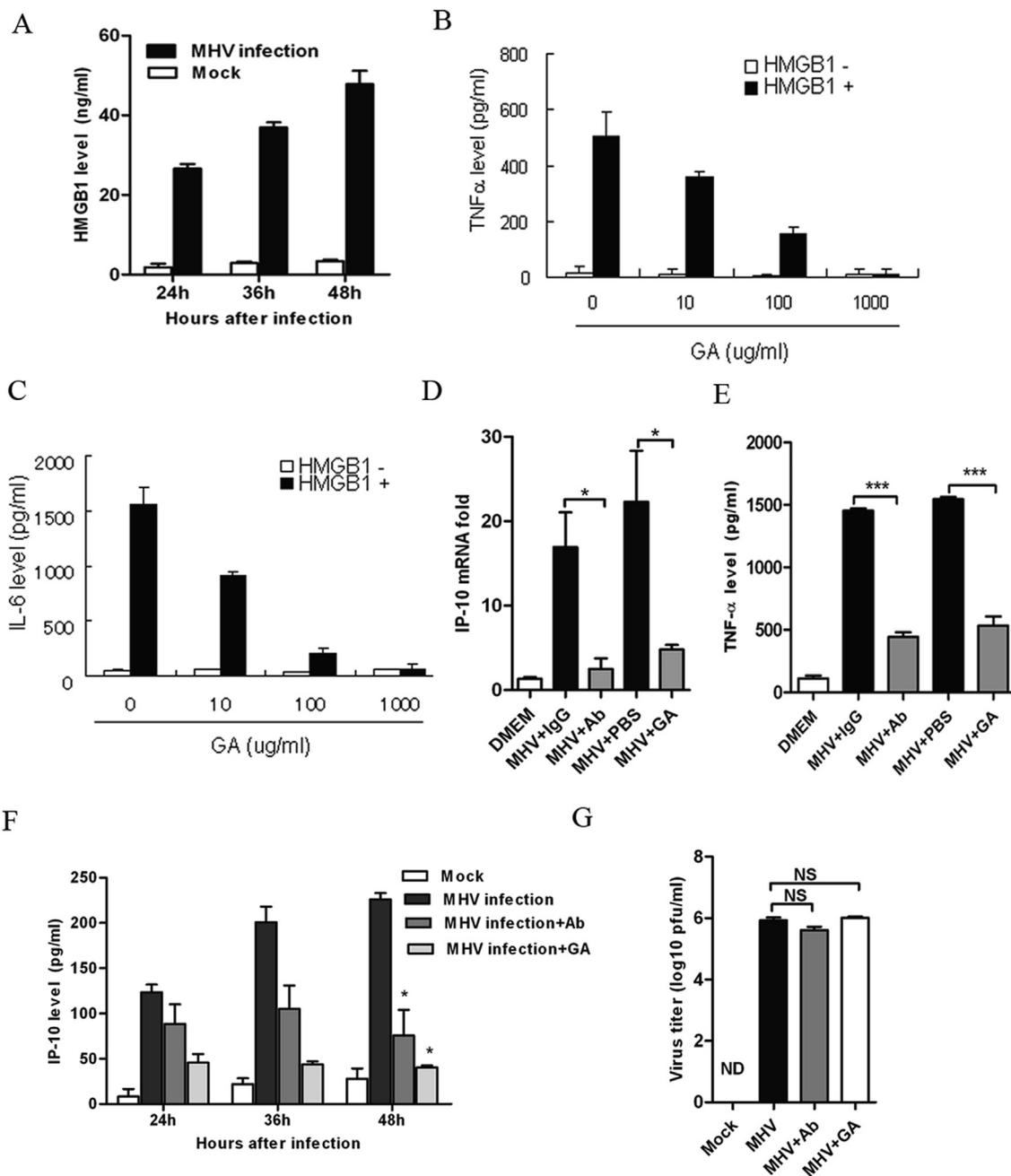
Fig. 4. GA inhibits immune activation triggered by HMGB1 *in vitro*. (A) HMGB1 release assay in the media of BNL.CL2 hepatocyte cells after MHV infection (MOI = 0.5). D2SC cells were co-incubated with HMGB1 (10 μg/ml, HMGB1-Flag protein was purified using anti-Flag M2 Affinity Gel) and different amounts of GA for 6 h. The culture supernatants were subsequently collected and analyzed for TNF-α (B) and IL-6 (C) production. (D) IP-10 mRNA expression analysis and (E) secreted TNF-α protein levels assay in RAW 264.7 cells stimulated by supernatant from infected BNL.CL2 and treated with GA (100 μg/ml) or anti-HMGB1 Ab (10 μg/ml) for 8 h. (F) IP-10 secretion analysis in the infected BNL.CL2 cellular supernatants in the presence of GA (100 μg/ml) or HMGB1 neutralizing antibody (10 μg/ml). (G) Virus titer of infected BNL.CL2 treated with control, GA (100 μg/ml) or anti-HMGB1 Ab (10 μg/ml). The data are represented as the mean ± SEM. * indicates P < 0.05, and ** indicates P < 0.01.

Therefore, the detailed molecular and cellular mechanisms of the secretion of these cytokines need to be explored further.

4. Discussion

In this study, we first demonstrated that licorice, a common component in the majority of herbal remedies, may be included as hepatoprotective agent to alleviate potential toxic adverse effects of ingredients in TCM. Next, we investigated if GA, as the active metabolic ingredient of licorice, could influence viral-induced hepatic injury. Our data demonstrated that GA inhibits MHV-A59-induced hepatitis by

suppressing HMGB1 release and cytokine activity *in vivo* and *in vitro*, which was consistent with the results from using HMGB1 neutralizing antibody. Additionally, our findings further demonstrated that the HMGB1-TLR4 axes is also involved in mediating tissue injury during hepatic viral infections, as blocking this pathway can effectively hinder the inflammatory injury in MHV-infected mice.

Licorice root extract contains various bioactive compounds and has potent hepatoprotective activity, which makes it a key detoxification ingredient in a wide variety of traditional herbal formulations [3]. However, it is unclear what molecular actions make this herb an effective ingredient. Xiaoyao Powder usage has been proven to be safe
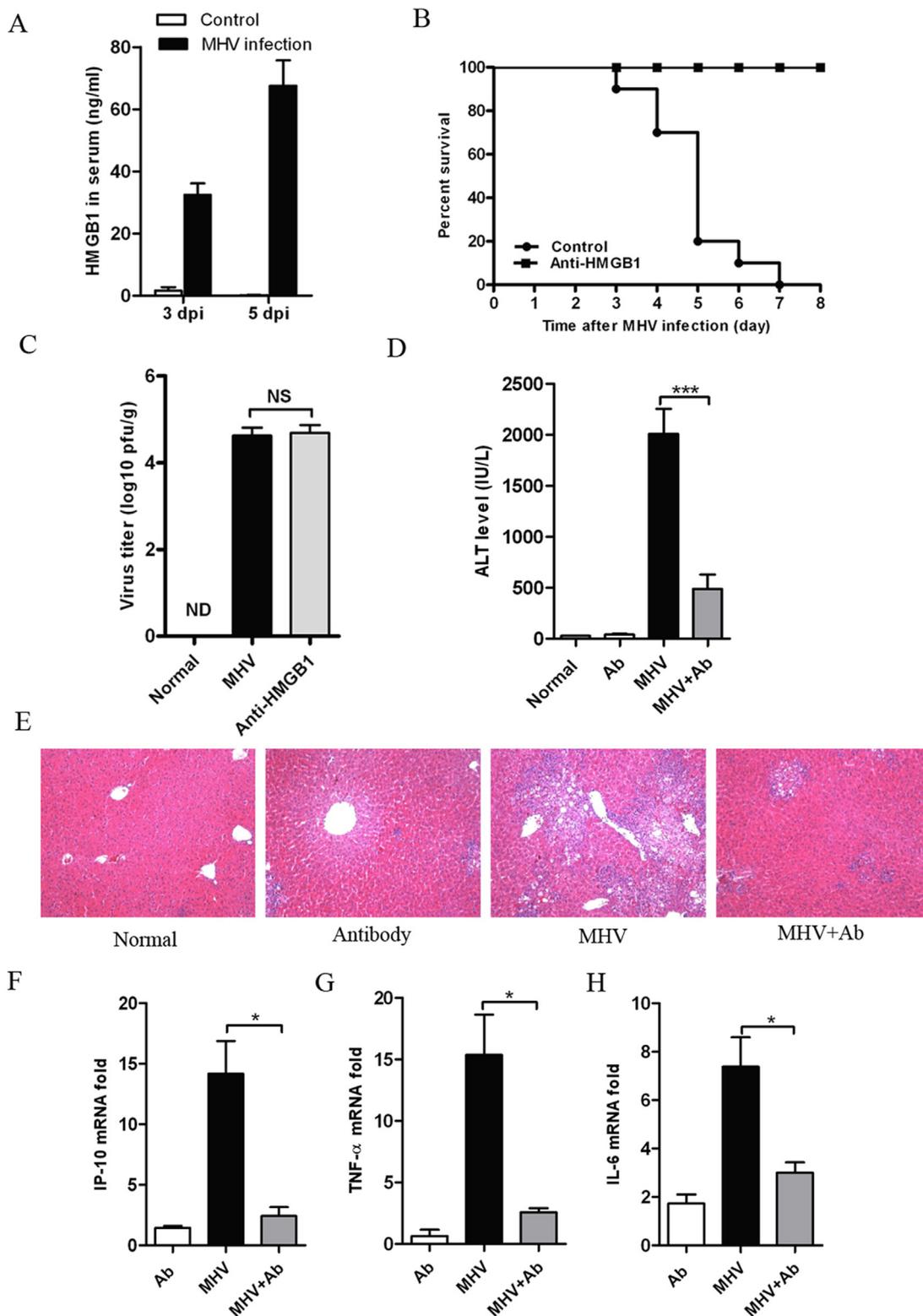
Fig. 5. Neutralizing HMGB1 antibody effectively alleviates liver injury induced by MHV-A59 infection. C57BL/6 mice were infected i.p. with 8×10^5 pfu/mouse for survival studies and 1×10^4 pfu/mouse for acute infections. Two hours after infection, the animals were administered a HMGB1 neutralizing antibody (2.5 mg/kg, i.p.) every other day (total three times). (A) Serum HMGB1 levels following infection at 3 and 5 dpi. (B) Effects of a HMGB1 neutralizing antibody on the survival of MHV infected mice. (C) Virus titers in liver homogenates of infected mice. (D) Serum ALT levels at 5 dpi; (E) Representative H&E staining of liver tissues on 5 dpi (magnification, ×100). (F-H) RNA was isolated from the liver samples levels, and the expression of inflammatory genes IP-10, TNF-α and IL-6 was assessed using qPCR. The data are represented as the mean ± SEM, * indicates P < 0.05, and *** indicates P < 0.001.

and is commonly used to treat hepatic stagnation and splenic deficiency without any reports of significant hepatotoxicity. However, the formula without licorice led to body weight loss and increased serum aminotransferase levels in mice, which are both indicators of hepatotoxicity. Therefore, our studies have suggested that the inclusion of licorice protects against hepatotoxicity induced by other ingredients
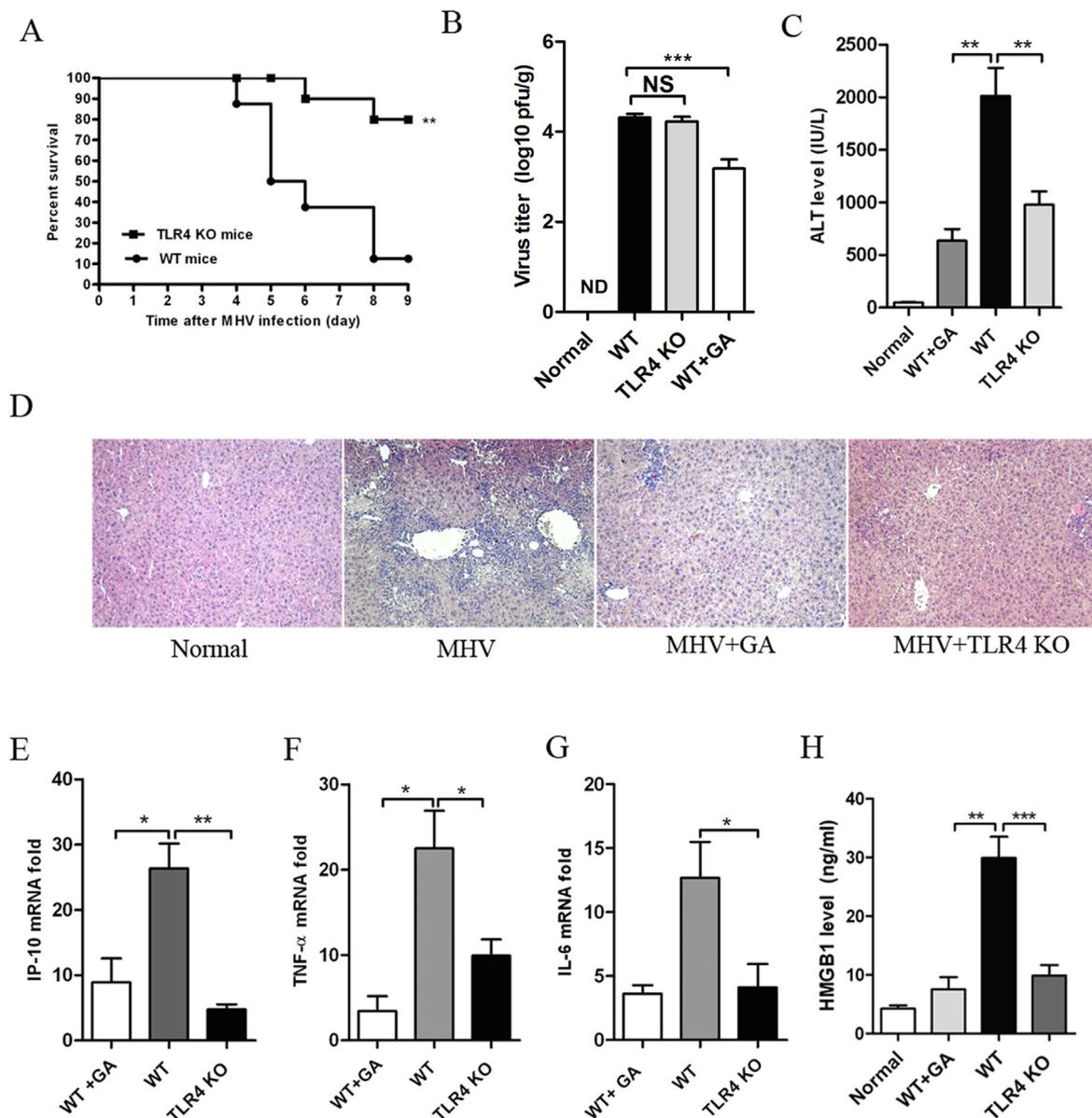
Fig. 6. TLR4 signaling plays a key role in MHV-induced hepatic injury. C57BL/6 wild type (WT) and TLR4 knockout (KO) mice were infected as described previously. Two hours later, WT mice were injected with GA (20 mg/kg, i.p.) every other day (total three times). At 5 days post infection (dpi), mice were sacrificed and serum and liver samples were collected. (A) Effects of TLR4 gene deficiency on the survival of MHV-infected mice. (B) Virus titer of liver homogenates from infected mice. (C) Serum ALT levels at 5 dpi. (D) Representative H&E staining of liver tissues at 5 dpi (magnification, ×100); (E-G) IP-10, TNF-α, and IL-6 mRNA expression in the liver of infected mice. (H) Levels of HMGB1 released in the serum in MHV-infected TLR4 KO and GA-treated mice at 5 dpi. The data represent the mean ± SEM, * indicates P < 0.05, ** indicates P < 0.01, and *** indicates P < 0.001.

(such as Radix Bupleuri) in the herbal formula.

GA is a hydrolysate of glycyrrhizic acid in the aqueous extract of licorice root, which is known to have various immune-modulating and antivirus activities [41]. For example, GA can attenuate LPS-induced fulminant hepatic failure through the deactivation of MAPKs and NF-κB pathway, resulting in the inhibition of TNF-α production [42]. GA also inhibits viral proliferation directly and indirectly *in vitro* and *in vivo* [30,43]. Our GA treatment results demonstrated that GA can not only rescue mortality of virus-infected mice and alleviate liver injury, but GA injection can also significantly inhibit viral proliferation in the liver. GL and GA both have been reported to exert antiviral effects, but there is a difference between the antiviral profiles of GL and GA [44,45]. Although GL exerts antiviral activity in SARS-associated coronavirus infection *in vitro*, solid evidence of the antiviral activity of GA in the MHV infection animal model is absent [30]. In this study, we first demonstrated the antiviral activity of GA in a murine coronavirus infection

model *in vivo*. To further investigate the mechanism of GA, we performed a cellular assay *in vitro* and found that it was involved with HMGB1 release and activity. Interestingly, in both hepatic and inflammatory cells, GA treatment or HMGB1 neutralizing antibody treatment significantly inhibited HMGB1 cytokine-inducing activity. Surprisingly, GA did not affect viral proliferation *in vitro*, which was different from previous reports [30]. This may be because we used mouse embryonic liver cells in our study, whereas previous studies have used Vero cells. The response of mouse embryonic liver cells to MHV infection may differ from Vero cells, and as such, a distinct cellular signaling pathway may be activated in mouse embryonic liver cells *in vitro*. Furthermore, it is well known that the virus titer in infected mice is determined directly by drug inhibition and indirectly by host immune-regulation [45,46]. Therefore, the antiviral activity of GA in MHV infection may depend on the activation of cytotoxic T lymphocytes, which protect against infected hepatic cells.
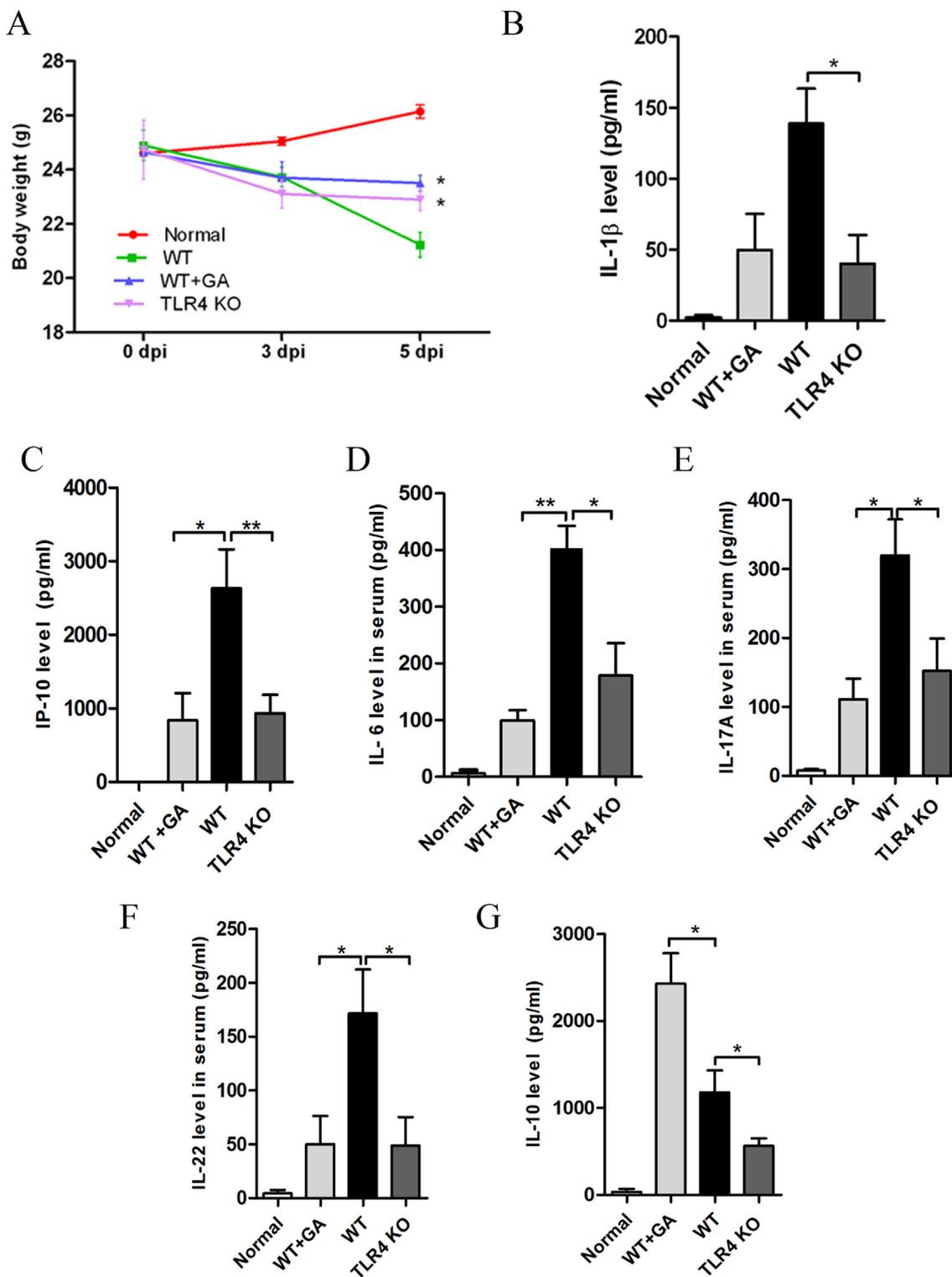
Fig. 7. GA regulates pro-inflammatory and anti-inflammatory cytokine release in MHV-infected mice. At 5 days post infection (dpi), mice were sacrificed, serum samples were collected, and cytokines concentration was assayed with an ELISA kit. (A) Body weight change curve of the different infected mouse groups. (B-G) IL-1β, IP-10, IL-6, IL-17, IL-22, and IL-10 cytokine measurement in the serum of infected mice. The data are represented as the mean ± SEM; * indicates P < 0.05, and ** indicates P < 0.01.

Many viruses cause cytopathologic effects indirectly by inducing host cells to release inflammatory factors and cause an inflammatory response [47]. When the amount of inflammatory factor is moderate, the host can resist viral invasion. If an excessive amount is produced, it will cause serious pathological damage [47]. During respiratory viral infection, serum HMGB1 acts as a biomarker and a therapeutic target

[38]. However, in viral infection disease, the effect of HMGB1 on virus replication is multifactorial. For example, it has been reported that HMGB1 released from virus-infected cells can block HCV infection. In contrast, the A-box of HMGB1 protein is the functional domain that interacts with HCV RNA and enhances viral replication [36,48]. To reduce viral inflammatory tissue injury, HMGB1 neutralizing antibody

combined with a conventional anti-proliferation drug might be more useful against severe influenza A virus infection [49]. Based on our neutralization experiment, HMGB1 antibody exerted therapeutic effect during MHV-A59 infection, but blocking HMGB1 did not significantly affect viral replication *in vivo*, which is consistent with the previous study on influenza virus [50].

TLRs are sensors for pathogen-associated molecular patterns (PAMPs) and play important roles in immune responses. It has been reported that TLR4 is key receptor of innate immune signaling responses to influenza virus and other respiratory viruses [51]. Although host HMGB1 and RAGE interaction is also a major mechanism driving serious liver injury, the TLR4 pathway has recently been demonstrated to be more involved in respiratory syncytial virus and human papilloma virus (HPV) infection [52–54]. Previously, the expression of TLR3, TLR4, TLR7, TLR9 and TLR10 genes from hepatic tissue was shown to be significantly upregulated in some viral infection models [32,55]. Although GA did not significantly affect TLR4 gene expression during viral infection, expression of TLR4 gene mediated the MHV-induced hepatic inflammation damage and determined HMGB1 release levels in the serum (Fig. 3G and 6H). In fact, pretreatment with a TLR4 blocking agent decreased the HMGB1 levels from virus-infected cells via the TLR4-NFκB pathway, and the inactivation of NFκB resulted in a decreased release of various pro-inflammatory cytokines, including IL-1β, IL-6, TNF-α and HMGB1 [37,54]. Furthermore, reduced HMGB1 levels led to the inhibition of NFκB pathway in various immune cells, and the inflammation response was alleviated significantly. However, the involvement of TLR4 signaling in virus-induced HMGB1 secretion remains elusive and needs to be investigated further.

Interestingly, TLR4 gene deficiency did not lead to the down-regulation of virus titer in the liver, which suggests that in MHV-A59 infection, the HMGB1-TLR4 axis exerts pro-inflammatory function without directly affecting virus replication. This result was not consistent with HCV and human adenovirus infection, which may due to the different viral pathological and replication mechanism [36,56,57]. Acute viral hepatitis caused by MHV is characterized by acute necrosis of hepatocytes, inflammation and production of TNF-α, IFN-γ, IL-2, and IL-17A [58,59]. In acute hepatitis B virus infection, the anti-inflammatory cytokine IL-35 can suppress liver inflammation by reducing IL-17 and IL-22 production, which results in regulating HBV peptides-inducing Th17 cells [60]. In this study, we show for the first time that IL-17A, IL-6, and IL-22 cytokines were released into the serum in response to MHV-A59 infection and are inhibited by GA treatment or TLR4 deficiency. However, there were different variations of IL-10 levels under the two intervening pathways. Although the anti-inflammatory cytokine IL-10 has been shown to have efficacy in reducing inflammatory injury in the liver, its effect on viral titers in some types of hepatitis remains unclear [61]. Based on our MHV infection data, IL-10 was up-regulated by GA treatment or mediated in a TLR4-dependent manner, which might be a new GA regulatory mechanism of cytokine storms resulting from viral infection, excluding the HMGB1-TLR4-IL-17 axis.

5. Conclusions

In summary, we demonstrated here for the first time that GA significantly ameliorates MHV-induced hepatic inflammatory damage via HMGB1-TLR4 signaling pathway, which is similar to treatment with a HMGB1 neutralizing antibody. Furthermore, this protective effect of GA was specifically associated with impaired IL-17 and IL-22 levels, and was not due to the direct inhibition of intracellular viral replication. Future studies are warranted to investigate the molecular mechanisms underlying how GA inactivates the HMGB1-TLR4 axis and which immune cell plays a major role in the cytokine storm induced by hepatic virus infection. Conclusively, this present study provides a new strategy for the immunological therapy of acute viral hepatitis in the clinical setting.

CRediT authorship contribution statement

Xiaodong Shi: Conceptualization, Methodology, Investigation, Data curation, Writing - original draft, Writing - review & editing, Project administration. **Lijia Yu:** Methodology, Validation, Data curation, Visualization. **Yinglin Zhang:** Formal analysis, Investigation, Data curation. **Zequan Liu:** Validation, Formal analysis. **Huawei Zhang:** Software, Investigation. **Yansong Zhang:** Supervision, Validation. **Ping Liu:** Methodology, Data curation. **Peishuang Du:** Resources, Supervision.

Declaration of Competing Interest

All authors have no conflict of interest to disclose.

Acknowledgements

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2020.106578.

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