

Basic Study

Fibrinogen-like protein 2 deficiency inhibits virus-induced fulminant hepatitis through abrogating inflammatory macrophage activation

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Abstract**BACKGROUND**

Heterogeneous macrophages play an important role in multiple liver diseases, including viral fulminant hepatitis (VFH). Fibrinogen-like protein 2 (FGL2) is expressed on macrophages and regulates VFH pathogenesis; however, the underlying mechanism remains unclear.

AIM

To explore how FGL2 regulates macrophage function and subsequent liver injury during VFH.

METHODS

Murine hepatitis virus strain 3 (MHV-3) was used to induce VFH in FGL2-deficient (*Fgl2*^{-/-}) and wild-type (WT) mice. The dynamic constitution of hepatic macrophages was examined. Adoptive transfer of *Fgl2*^{-/-} or WT bone marrow-derived macrophages (BMDMs) into WT recipients with macrophages depleted prior to infection was carried out and the consequent degree of liver damage was compared. The signaling cascades that may be regulated by FGL2 were detected in macrophages.

RESULTS

institutional Animal Care and Use Committee of Tongji Hospital (permit number: TJ-A20171008), and conducted in accordance with state guidelines from the Ministry of Science and Technology of China.

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Following MHV-3 infection, hepatic macrophages were largely replenished by proinflammatory monocyte-derived macrophages (MoMFs), which expressed high levels of FGL2. In *Fgl2*^{-/-} mice, the number of infiltrating inflammatory MoMFs was reduced compared with that in WT mice after viral infection. Macrophage depletion ameliorated liver damage in WT mice and further alleviated liver damage in *Fgl2*^{-/-} mice. Adoptive transfer of *Fgl2*^{-/-} BMDMs into macrophage-removed recipients significantly reduced the degree of liver damage. Inhibition of monocyte infiltration also significantly ameliorated liver damage. Functionally, *Fgl2* deletion impaired macrophage phagocytosis and the antigen presentation potential and attenuated the proinflammatory phenotype. At the molecular level, FGL2 deficiency impaired IRF3, IRF7, and p38 phosphorylation, along with NF- κ B activation in BMDMs in response to viral infection.

CONCLUSION

Infiltrated MoMFs represent a major source of hepatic inflammation during VFH progression, and FGL2 expression on MoMFs maintains the proinflammatory phenotype *via* p38-dependent positive feedback, contributing to VFH pathogenesis.

Key Words: Viral fulminant hepatitis; Fibrinogen-like protein 2; Proinflammatory macrophages; Infiltrating macrophages; P38

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Core Tip: In this study, we demonstrate that: (1) Monocytes infiltrating the liver represent a major source of hepatic inflammation, which has a decisive effect on the pathogenesis of viral fulminant hepatitis; (2) Viral infection induces FGL2 expression on macrophages, which is required for maintaining the inflammatory phenotype and cell function; and (3) FGL2 generates a positive feedback loop of an inflammatory cascade in macrophages in response to viral infection.

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INTRODUCTION

Viral fulminant hepatitis (VFH) is a type of acute liver failure (ALF) that can develop after viral infection in patients with hepatitis A, hepatitis B, and hepatitis E. VFH is a devastating syndrome characterized by severe liver injury with coagulation abnormalities and hepatic encephalopathy[1]. Pathologically, acute liver injury and subsequent massive hepatocyte loss are caused by hyperactivation of the innate immune response, followed by an excessive adaptive immune response to viral infection, leading to systemic inflammation. Innate immune cells, activated by pathogen-associated molecular patterns (PAMPs) from invading pathogens, damage-associated molecular patterns (DAMPs) released from necrotic cells, and afferent endotoxins from the portal vein, are dominant in conditions of sterile inflammation and non-virus-associated ALF[2,3]. Adaptive immunity activated by PAMPs seems to play a more important role in VFH than innate immunity because of the exposure to abundant viral antigens presented by antigen-presenting cells[4,5]. However, the extent of the contribution of innate immunity in generating viral antigens or DAMPs in VFH is not well defined.

Liver-resident macrophages, also known as Kupffer cells (KCs), are the largest population of hepatic immune cells that play an essential role in maintaining liver homeostasis and ensure rapid responses to hepatic insults[6]. Monocyte-derived macrophages (MoMFs) are an ontologically distinct subpopulation of hepatic

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macrophages, which are found in only small numbers under physiological conditions and then serve to replenish the macrophage population during liver injury[7]. Macrophages are generally classified into proinflammatory (M1) and anti-inflammatory (M2) macrophages (or alternatively activated macrophages), phenotypically corresponding to T helper (Th)1 and Th2 responses, respectively. However, the spectrum of macrophage classification remains obscure as the transition between macrophage subtypes occurs in response to microenvironment mediators[8,9]. Dynamic alterations in KC and MoMF populations were observed in the context of sterile inflammation in an experimental model of ALF induced by acetaminophen overdose, with replacement of KCs by MoMFs during disease progression[10]. Further analyses of this model revealed differential subsets of macrophages showing the protective and detrimental characteristics of KCs and MoMFs during the course of acetaminophen-induced ALF[10,11]. Moreover, an increase in the number of MoMFs with potent activity has been observed in non-pregnant patients with hepatitis E-associated ALF compared with that in pregnant patients. In a model of experimental VFH, hepatic leukocyte numbers were found to increase at an early stage of liver injury[12], suggesting the involvement of macrophages in disease progression. However, further investigation is needed to elucidate the precise roles of the distinct subpopulations of hepatic macrophages in VFH.

Fibrinogen-like protein 2 (FGL2), a membrane protein with prothrombinase at the N-terminal, is expressed in a variety of cells, such as macrophages, dendritic cells, and endothelial cells, and can be induced robustly and exclusively in macrophages in response to stimulation with cytokines [interferon (IFN)-gamma or tumor necrosis factor (TNF)- α], viral infection, and lipopolysaccharide (LPS)[7,13]. This suggests that FGL2 itself is a critical mediator of inflammation in that the interaction between inflammation and coagulation is reciprocally exacerbated in terms of inflammatory cytokine production and tissue factor secretion[11]. FGL2 deficiency was shown to prevent the development of VFH in mice following experimental murine hepatitis virus strain 3 (MHV-3) infection; this effect was speculated to be mainly dependent on its ability to regulate procoagulant activity, as evidenced by concomitant expression of FGL2 and fibrinogen production[14]. Emerging evidence suggests that FGL2 is a downstream effector molecule in the inflammatory cascades associated with VFH development and progression, which involve C5aR, TNF- α , and MSR1[7,12,15,16]. Interestingly, complement activation, a critical step for inflammation initiation, has also been observed in FGL2-deficient mice following MHV-3 infection[16], highlighting a complex regulatory network of inflammation involving FGL2 and other effectors. Therefore, we hypothesized that FGL2 plays a role in the reconstitution of hepatic macrophages to regulate proinflammatory macrophage activation that contributes to VFH progression and exacerbation. To test this hypothesis, we first explored the hepatic macrophage composition and expression pattern of FGL2 during the progression of VFH in patients with and without hepatitis B virus (HBV)-associated acute-on-chronic liver failure (ACLF), since the pathology of ACLF closely resembles that of ALF[17]. To explore the role of FGL2 in VFH progression and the underlying mechanism, we determined alterations in hepatic macrophage populations during progression of VFH in FGL2-deficient (*Fgl2*^{-/-}) and wild-type (WT) mice intraperitoneally injected with MHV-3 at different stages after infection.

MATERIALS AND METHODS

Ethics statement

All studies on human subjects were approved by the Ethics Committee of Tongji Medical College of Huazhong University of Science and Technology (permit number: TJ-C20170924), and all participants provided informed consent in compliance with the Helsinki Declaration revised in 1983.

The animal experiments were approved by the Institutional Animal Care and Use Committee of Tongji Hospital (permit number: TJ-A20171008) and were conducted in accordance with state guidelines from the Ministry of Science and Technology of China.

Patient samples

Seven patients with ACLF, as defined by the Asian Pacific Association for Study of the Liver (APASL) guidelines[18], who were undergoing liver transplantation were included in the study; liver samples were obtained during the surgery. The clinical features of the patients are summarized in Table 1. Control samples ($n = 3$) were

Table 1 Clinical characteristics of patients and controls

Characteristics	ACLF (n = 7)	Healthy controls (n = 3)
Age (year)	47.14 ± 3.3 ^a	30.33 ± 3.4
Sex (male:female)	6:1	3:0
Laboratory evaluation		
ALT (U/L)	642.9 ± 360.3	8.67 ± 1.76
Tbil (μmol/L)	429.8 ± 70.53 ^a	17 ± 2.34
PTA (%)	30.29 ± 3.3 ^a	118 ± 12.17
HBsAg (ng/mL)	3870 ± 1600	0
HBV-DNA (10 ⁶ copies/mL)	8.18 ± 6.78	0
Ascites and/or Encephalopathy	7	0

^aP < 0.5 vs controls.

Age, Serum alanine aminotransferase, total bilirubin, prothrombin time activity shown as mean ± SD. ACLF: Acute on chronic liver failure; ALT: Alanine aminotransferase; Tbil: Total bilirubin; PTA: Prothrombin time activity.

obtained from a healthy liver donor and from the paratumor tissues of a patient with hepatic hemangioma and a patient with hepatocellular carcinoma without cirrhosis.

Animals, viral infection, macrophage depletion, and inhibitor treatment

Female BALB/c mice (HFK Bioscience Company Ltd., Beijing, China) at 6-8 weeks of age were used in all animal experiments. The mice were housed in a controlled environment (specific pathogen-free, 12 h light/dark cycle, 21 ± 2 °C, humidity 50 ± 10%) and had free access to food and water. *Fgl2*^{-/-} mice were generated by introducing a deletion at the N-terminal of the open reading frame of the *Fgl2* locus using CRISPR/Cas9 technology. WT BALB/c mice were used as controls. To establish the fulminant hepatitis model, 100 plaque-forming unit (PFUs) of MHV-3 (American Type Culture Collection, Manassas, VA, USA) dissolved in 200 μL saline was injected into the WT and *Fgl2*^{-/-} mice intraperitoneally. To deplete macrophages, mice were intravenously injected with 200 μL clodronate liposomes or phosphate-buffered saline (PBS)-liposomes as a control (Liposome B.V., the Netherlands) 24 h prior to MHV-3 injection. To inhibit MoMF infiltration, a chemokine receptor-2 (CCR2) inhibitor (cenicriviroc; 10 mg/kg) was intraperitoneally injected 24 h prior to MHV-3 infection. WT or *Fgl2*^{-/-} mice were randomly selected to be sacrificed at 0, 24, 48, and 72 h following MHV-3 injection, and liver and blood samples were harvested for analysis.

Cell isolation

Hepatic mononuclear non-parenchymal cells from WT and *Fgl2*^{-/-} mice were isolated *via* liver perfusion, enzymatic digestion, and differential centrifugation, as described previously[19]. Peritoneal exudate macrophages (PEMs) were isolated from the mice 3 d after intraperitoneal injection of 3% starch broth (1 mL/mice). Bone marrow-derived macrophages (BMDMs) were differentiated from hematopoietic stem cells isolated from the femurs cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and macrophage-colony stimulating factor (30 μg/mL, Peprotech) for 7 days. BMDMs or PEMs were maintained in DMEM supplemented with 10% FBS and treated with LPS (100 ng/mL), interleukin (IL)-4 (20 ng/mL), or MHV-3 (5 × 10⁵ PFU/mL).

Macrophage adoptive transfer

KCs are the largest population of hepatic resident non-parenchymal cells and the first line of defense against pathogens and toxins[6]. The priming of KCs may therefore be involved in initiation of the hepatic immune response, a critical step for subsequent exacerbation of inflammatory accumulation. Accordingly, we also assessed liver damage following MHV-3 infection with depletion of macrophages by clodronate liposomes. WT mice were treated with 200 μL clodronate liposomes or PBS-liposomes as a control to deplete macrophages, which then served as the recipient mice for adoptive transfer. Harvested BMDMs from WT and *Fgl2*^{-/-} mice were intravenously injected into the recipient mice at 5 × 10⁵ cells/mouse. MHV-3 (100 PFU/mouse)

injection was performed 24 h later. Liver and blood samples were harvested at 48 h post MHV-3 infection for further analyses.

Flow cytometry

Single-cell suspensions were blocked with 2 µg/mL anti-CD16/CD32, followed by staining with fluorescence-conjugated antibody cocktails. For intracellular staining, surface-stained cells were fixed and permeabilized using Cytotfix/Cytoperm Fixation/Permeabilization Kit (No. 554714, BD Pharmingen) prior to incubation with target antibodies. Data were acquired on a BD FACS Canto II flow cytometer (BD Bioscience, San Jose, CA) and analyzed using FACS Diva software.

Detailed procedures and other materials are described in the supporting information.

Statistical analyses

Statistical testing was performed using GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA, United States). Comparisons between WT and *Fgl2*^{-/-} mice (or other situations where only two groups are compared) were performed using Student's unpaired t-test; one-way analysis of variance with Tukey's *post-hoc* test or Mann-Whitney *U* test was used for multiple comparisons (unless otherwise indicated). Data are presented as mean ± standard deviation; *P* < 0.05 was considered to indicate a statistically significant difference.

RESULTS

The number of proinflammatory macrophages with elevated FGL2 expression increases in patients with hepatitis virus-induced liver failure

Marked infiltration of leukocytes was observed in samples of the patients with ACLF, along with massive necrosis in the parenchymal lobules (Figure 1A). CD68+ macrophages markedly accumulated in the periportal areas (Figure 1B, Supplementary Figure 1), suggesting widespread liver damage[20]. Furthermore, macrophages were polarized to proinflammatory macrophages, as confirmed by the expression of S100A9 (Figure 1C), an alarmin molecule expressed in proinflammatory macrophages, activated neutrophils, and granulocytes, which is often used as a biomarker of inflammation[21]. Interestingly, the majority of S100A-positive activated proinflammatory macrophages also expressed FGL2 in consecutive sections of liver samples from patients with HBV-related liver failure (Figure 1D). These data suggest that a high expression level of FGL2 could be an important molecular event of hepatic macrophage polarization during viral liver failure.

Ly6C^{high} MoMFs dominate the hepatic macrophage population during VFH progression

In the experimental fulminant hepatitis mouse model, pathogenesis was divided into an early stage without global liver damage at 24 h post-infection of MHV-3, a progression phase with continuous enlargement of block necrosis and increased transaminase levels, and an end stage defined at 72 h post-infection when the animals were near death (Figure 2A and B). At the early stage of infection, hematoxylin and eosin staining showed that the hepatic architecture was intact and no obvious macrophages infiltration was noted (Figure 2A). Aggregating plots of the CD11b F4/80-expressing flow cytometry panel[22] showed a continuous increase in the number of hepatic MoMFs (CD11b^{high}F4/80^{int}) and a decline in the number of KCs (CD11b^{low}F4/80^{high}) over the course of infection, accompanied by enlarged lesions until confluent necrosis of the liver lobules occurred at the later stage (Figure 2A and C). Notably, KCs constituted the dominant hepatic macrophage population at the early stage of infection, with an increased M1 macrophage subset [induced nitric oxide synthase (iNOS+) KCs] (Figure 2D, Supplementary Figure 2A and C). However, MoMFs subsequently dominated the hepatic macrophage population, characterized by an increased ratio of Ly6C^{high} cells; however, the Ly6C^{high}/Ly6C^{low} ratio decreased at the end stage of the disease (Figure 2E, Supplementary Figure 2A), implying a transition from Ly6C^{high} MoMFs to Ly6C^{low} MoMFs. The dynamic alterations in hepatic macrophage populations were also revealed by an increase in the levels of proinflammatory cytokines TNF-α and IL-6 during progression, which then decreased at the end stage, along with a continued decrease in transforming growth factor (TGF)-β levels in macrophages (Supplementary Figure 2D). Consistent with the reconstitution of hepatic

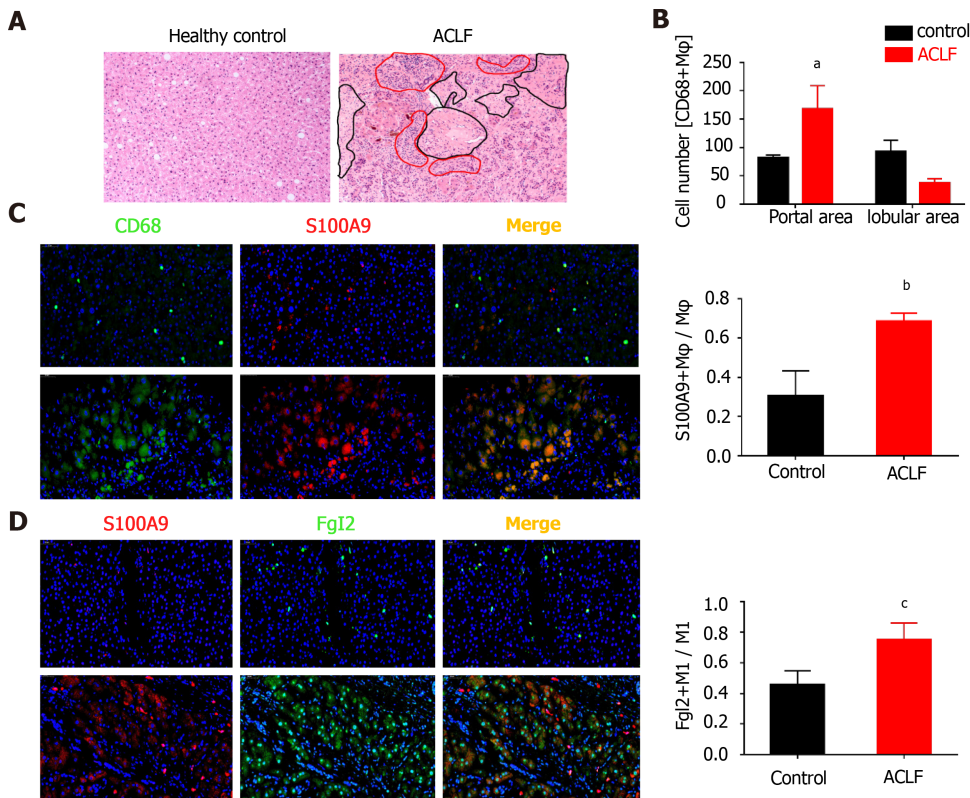


Figure 1 Fibrinogen-like protein 2 was expressed on proinflammatory macrophages in patients with hepatitis B virus-associated liver failure. A: Representative image of liver sections (200×) subjected to Hematoxylin and eosin staining (H&E); Black circles point to transparent necrosis, red circles point to infiltrating leukocytes; B: Calculation of CD68+ macrophages at periportal and lobular areas; C: Immunofluorescence against CD68, S100A9 on liver sections (200×) from patients of hepatitis B virus-associated acute chronic-on liver failure (acute-on-chronic liver failure (ACLF), $n = 7$) and healthy controls ($n = 3$); D: Immunofluorescence against S100A9 and fibrinogen-like protein 2 on liver sections (200×) from ACLF patients ($n = 7$) and healthy controls ($n = 3$), representative images on the left and statistical columns on the right. Data are expressed as mean ± SD.

macrophage populations in which Ly6C^{high} MoMFs are more inflammatory with an expanded spectrum of cytokine and chemokine expression compared with M1 macrophages[23], the serum MCP-1 level was remarkably increased during infection-induced liver failure progression (Supplementary Figure 2B).

Potent induction of FGL2 is associated with the proinflammatory phenotype of both KCs and MoMFs in response to viral infection

As shown in Figure 3A, over 90% of FGL2-positive KCs were found in iNOS+ macrophages based on flow cytometry. Moreover, Ly6C^{high} MoMFs constituted the major population of FGL2-positive MoMFs, and the expression level of FGL2 was remarkably higher in Ly6C^{high} MoMFs than in Ly6C^{low} MoMFs (Figure 3B). After viral infection, sequential induction of FGL2 expression was observed in both polarized M1 KCs and Ly6C^{high} MoMFs during disease progression, although a slight decrease in the expression level was observed when the KC and MoMF populations were somewhat exhausted at 48 and 72 h post viral infection, respectively (Figure 3C and D). However, FGL2 expression was maintained at low levels in Ly6C^{low} populations (Figure 3D). Together, these data suggested that FGL2 is markedly induced in proinflammatory macrophages upon viral infection.

FGL2 deficiency prevents proinflammatory macrophage activation after MHV-3 infection

In *Fgl2*^{-/-} mice (Supplementary Figure 3A), liver damage following MHV-3 infection was significantly attenuated, as revealed by narrowed necrosis foci and lower alanine transaminase (ALT) and aspartate transaminase (AST) levels compared with those in WT mice (Figure 4A and B). In addition, viral titers were reduced in *Fgl2*^{-/-} mice compared with those in their WT counterparts (Supplementary Figure 3B). Consistent with the pathology results, monocyte infiltration was significantly reduced in *Fgl2*^{-/-} mice, whereas a large number of KCs was maintained during VFH progression (Figure 4C). To determine whether *Fgl2* was required for proinflammatory

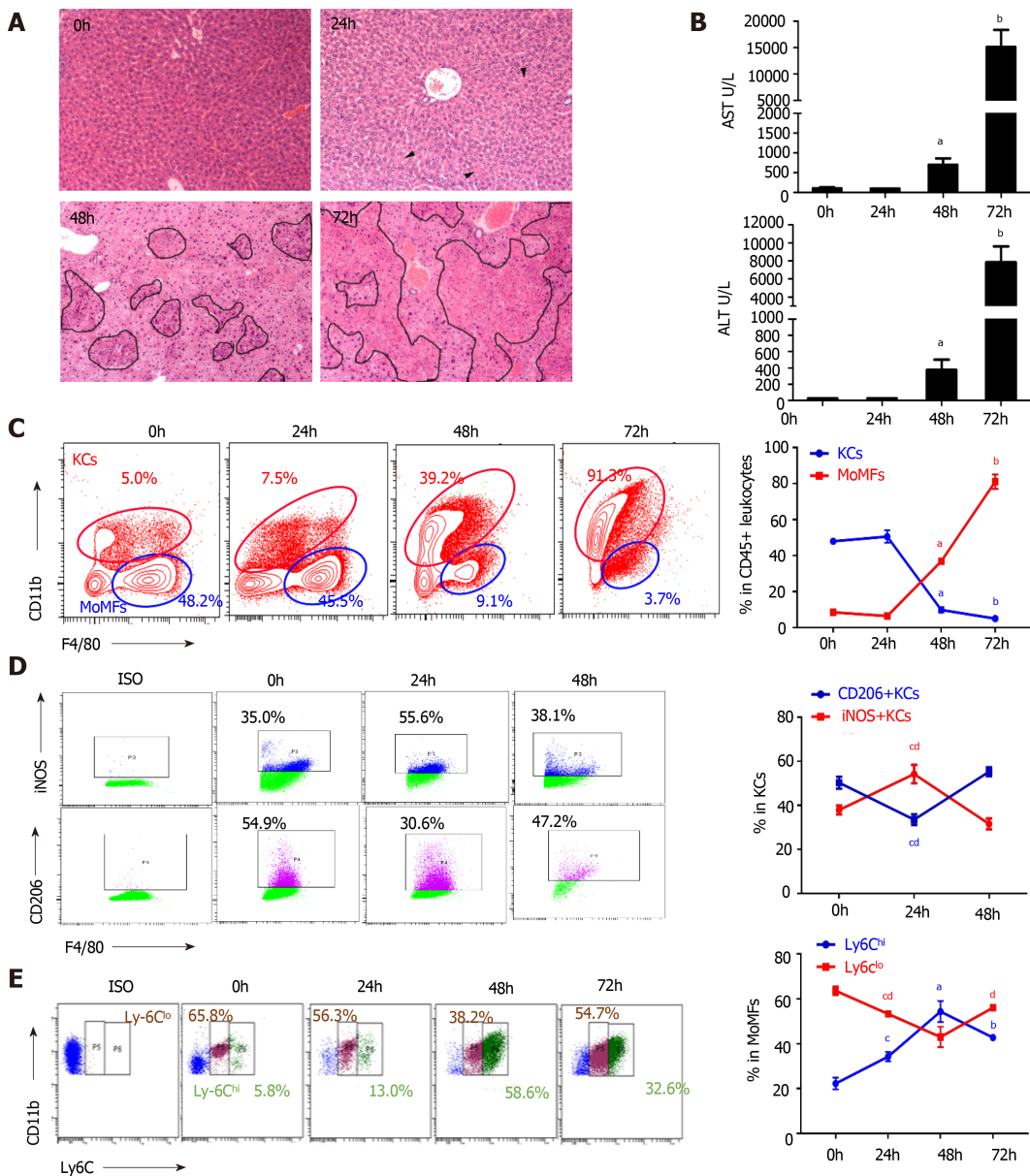


Figure 2 Dynamic alteration of macrophage subsets during viral fulminant hepatitis progression. A: Time-course H&E staining on liver sections of mice after murine hepatitis virus strain 3 infection. Arrowheads indicated hepatocyte cytoplasmic destruction, Circles pointed to necrosis; B: ALT and AST levels from serum of mice post-viral infection; C: Flow cytometry of KCs (CD45+ F4/80high), MoMFs (CD45+ Ly6C+ F4/80int) of cells (left), and their percentage in the liver at various time point after viral infection; D: Flow cytometry of KCs (left) and frequency of M1 (iNOS+), M2 (CD206+) macrophages in KCs (right); E: Representative image of MoMFs (left) and frequency of Ly6Chigh and Ly6Clow MoMFs at different time point after viral infection. Data are presented as mean ± SD (n = 5). These experiments were repeated at least three times. KCs: Kuffer cells; MoMFs: Monocyte derived macrophages.

macrophage activation, we investigated the polarization of KCs at the early stage of VFH when KC populations were reserved. We found a decrease in the M1 KC subset and an increase in the M2 KC numbers in FGL2-deficient mice before and after MHV-3 infection (Figure 4D), suggesting that FGL2 depletion impaired inflammatory macrophage activation and favored the M2 phenotype. As expected, a small Ly6C^{high} subset was also observed in the MoMF population in FGL2-deficient mice after viral infection, implying that *Fgl2* loss predisposed MoMFs to the Ly6C^{low} phenotype (Figure 4E). This interpretation was further supported by the reduced levels of TNF- α and IL-6 and the increased levels of TGF- β on KCs from *Fgl2*^{-/-} mice after viral infection compared with those in WT mice (Supplementary Figure 3C).

FGL2 induces KC polarization for initiation of inflammatory events after viral infection

Macrophage depletion by clodronate liposomes significantly relieved liver injury, as shown by decreased necrosis foci and ALT/AST levels, in both WT and FGL2-deficient

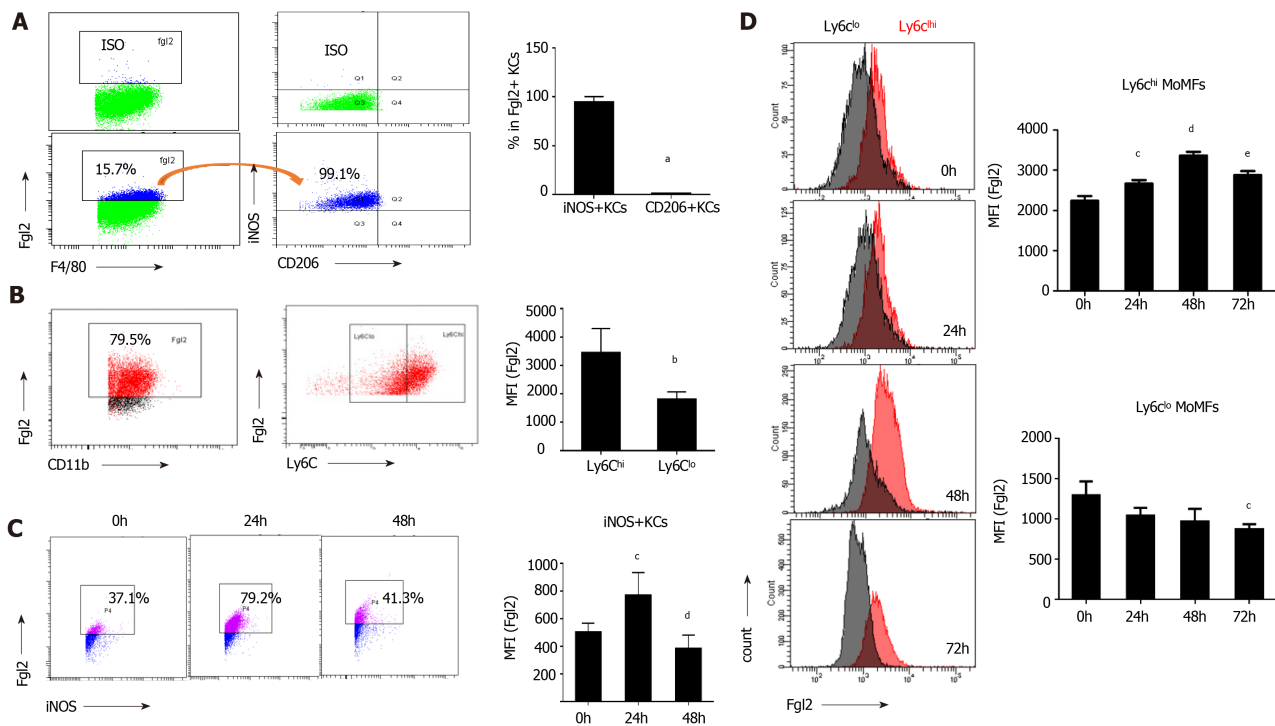


Figure 3 Fibrinogen-like protein 2 expression was robustly induced upon proinflammatory macrophage activation. A: Flow cytometry of fibrinogen-like protein 2 (Fgl2) +KCs expressing iNOS and CD206 under homeostatic conditions (left), and frequency of iNOS+ and CD206+ cells subsets (right); B: Representative image of Fgl2+MoMFs expressing Ly6C (left), and relative expression level of Fgl2 in Ly6ChiMoMFs and Ly6Clo MoMFs under physiological conditions (right); C: Time course-presentation of Fgl2 expression in M1 polarized KCs (left), and expression level of Fgl2 in Ly6ChiMoMFs and Ly6Clo MoMFs, respectively (right); D: Cell counts of Fgl2+ MoMFs (left), and relative level of Fgl2 expression on both MoMFs subset (right) following viral infection. Data are presented as mean \pm SD ($n = 5$). These experiments were repeated at least three times. MFI: Mean fluorescence intensity.

mice (Figure 5A and B). Notably, further reduction in liver ALT/AST levels and fewer necrotic hepatocytes were observed in FGL2-deficient mice after viral infection, suggesting the requirement of FGL2 for macrophage polarization and disease progression. To determine whether FGL2 regulates macrophage polarization directly, BMDMs from WT and *Fgl2*^{-/-} mice were transferred into WT recipient mice with self-BMDMs depleted in advance in the VFH model. Interestingly, *Fgl2*^{-/-} BMDMs exhibited reduced numbers of inflammatory polarized macrophages and increased numbers of anti-inflammatory macrophages (Figure 5D and E). Moreover, recipient mice adoptively transferred with *Fgl2*^{-/-} BMDMs exhibited reduced liver injury, as revealed by histological analysis (Figure 5A) and ALT/AST levels (Figure 5C), when compared with that in recipients transferred with WT BMDMs.

As depicted in Supplementary Figure 3D and E, smaller numbers of MPO^{high} myeloid cells, which are considered to be neutrophils, were observed in liver sections from mice with macrophage depletion or in FGL2-deficient mice compared with those from untreated WT mice following MHV-3 infection. However, no significant difference in neutrophil abundance was observed between WT and *Fgl2*^{-/-} mice with macrophage depletion.

Because MoMFs formed the largest component of myeloid cells during the acute stage and CCR2 is mainly expressed in proinflammatory monocytes (Ly6C^{high}) [24], we speculated that MoMF-derived inflammation is a major source of hepatic inflammation. Indeed, treatment with the CCR2 inhibitor cenicriviroc significantly reduced the virus-induced liver damage (Figure 5F and G). Collectively, these data suggest that FGL2 directly regulates proinflammatory macrophage polarization and that infiltrated MoMFs are the major source of hepatic inflammation during acute liver injury.

FGL2 deficiency attenuates macrophage M1 polarization *in vitro*

To further determine whether FGL2 regulates macrophage polarization directly *in vitro*, we examined its regulatory effect on BMDMs and PEMs by LPS stimulation or MHV-3 infection. As expected, in WT BMDMs, robust production of cytokines and chemokines, such as IL-1 β , TNF- α , IL-6, IL-12, and MCP-1, and reactive species, such as the NO-derived nitrite product (NO₂⁻), were observed in response to either LPS treatment or MHV-3 infection. In contrast, the levels of such products were markedly

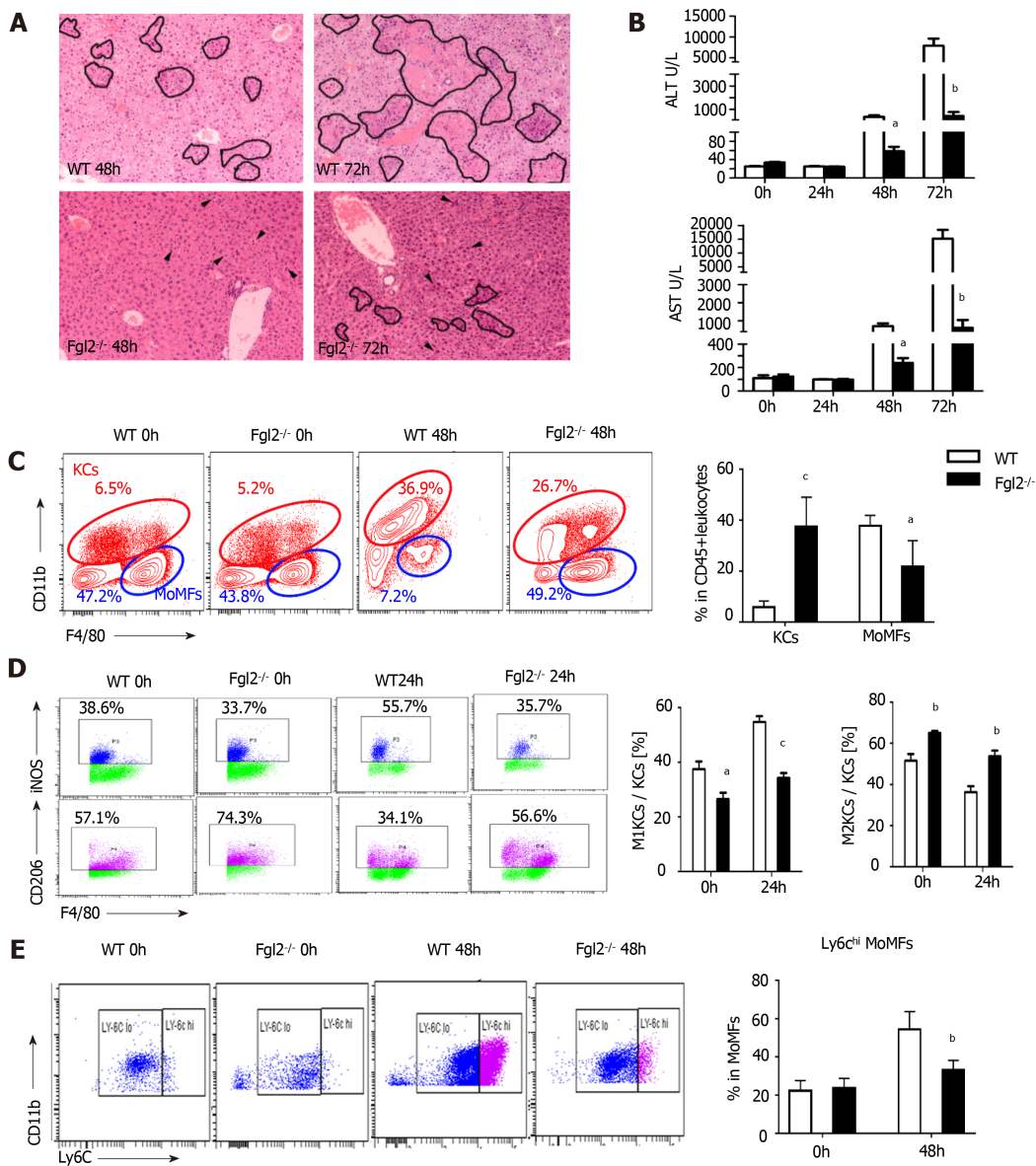


Figure 4 Fibrinogen-like protein 2 promotes pro-inflammatory macrophage polarization following murine hepatitis virus strain 3 infection.

A: H&E staining on liver sections from wild type (WT) and fibrinogen-like protein 2 (*Fgl2*^{-/-}) mice at 48 and 72 h post viral infection. Circled field represented bulk necrosis. Arrows point to necrotic cells; B: Serum alanine transaminase and aspartate transaminase levels at 0, 24, 48, and 72 h post viral infection in WT and *Fgl2*^{-/-} mice; C: Flow cytometry of KCs and MoMFs in hepatic CD45⁺ leukocytes at steady condition and 48 h following viral fulminant hepatitis in WT and *Fgl2*^{-/-} mice (left); and respective frequency at 48 h post infection; D: Frequency of polarized M1 (iNOS⁺), M2 (CD206⁺) KCs at 0 and 24 h post murine hepatitis virus strain 3 (MHV-3) infection; E: Frequency of Ly6Chi and Ly6Clow MoMFs at 0 and 48 h post MHV-3 infection. Data are presented as mean ± SD (n = 5). These experiments were repeated at least three times.

reduced in BMDMs from *Fgl2*^{-/-} mice under the same condition (Figure 6A). Consistently, the transcription levels of M1 indicators, such as NOS2, TNF- α , IL-6, IL-1 β , IL-12, and Marco, were significantly reduced in BMDMs of *Fgl2*^{-/-} mice compared with those in BMDMs from WT mice in response to either LPS stimulation or MHV-3 infection (Figure 6B and C). In contrast, both IL-10 production and the transcriptional levels of M2 markers were significantly higher in *Fgl2*^{-/-} BMDMs than in WT BMDMs in response to IL-4 treatment (Figure 6D and E). Similar results were also obtained in *Fgl2*^{-/-} PEMs in response to LPS treatment (Supplementary Figure 4). Taken together, these data suggest that FGL2 deficiency impairs the proinflammatory polarization of macrophages and promotes an alternative activated phenotype in response to IL-4 stimulation.

FGL2 deficiency reduces the antigen presentation and phagocytosis of macrophages

A critical function of hepatic macrophages during homeostasis maintenance is to engulf pathogens, endotoxins, and debris of dead cells *via* a process called

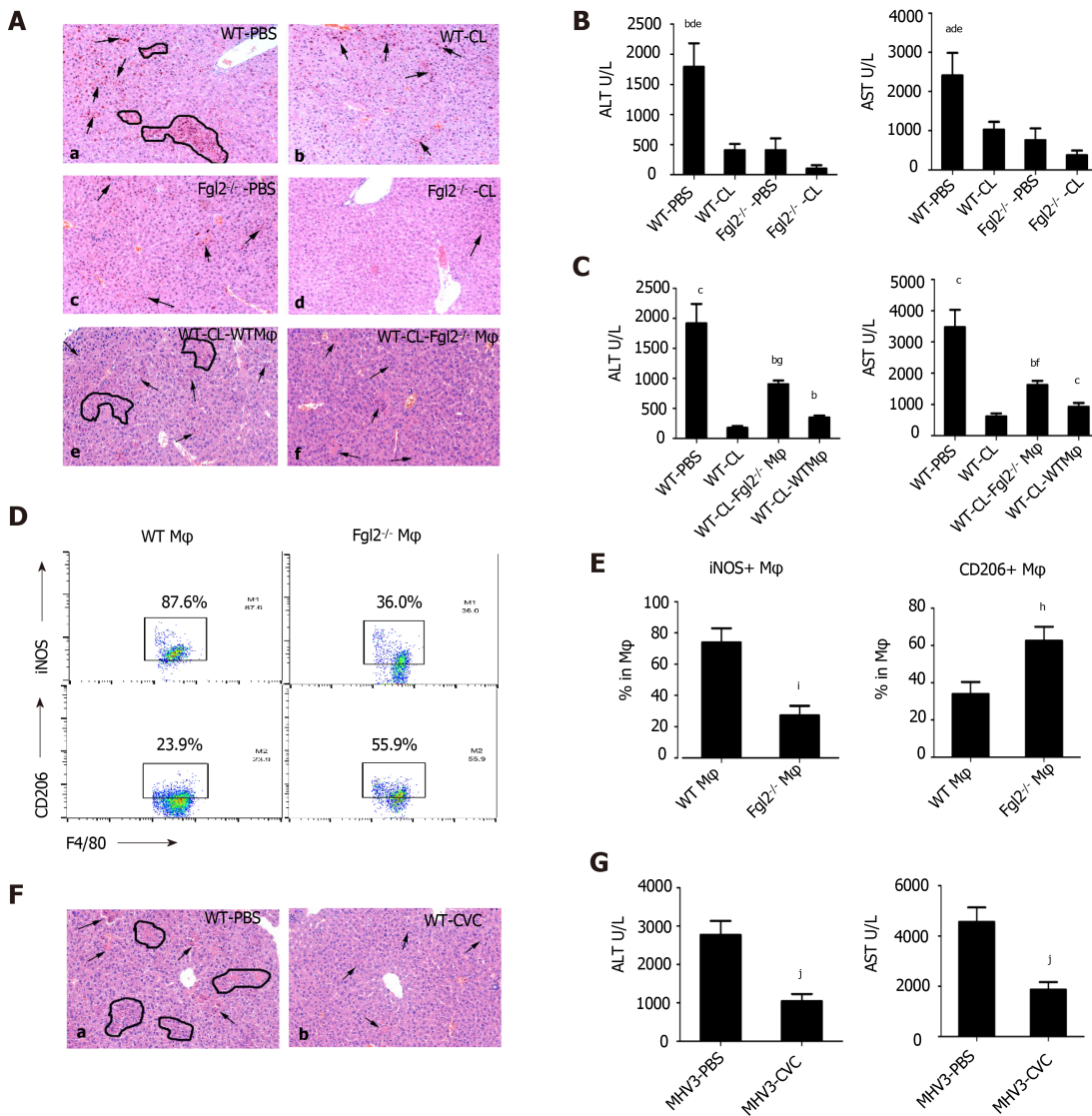


Figure 5 Depletion of macrophages in fibrinogen-like protein 2 synergically attenuated liver damage after viral infection. A: H&E staining on liver sections from mice administered with either clodronate liposomes (CL) (b and d on the right) or PBS-liposomes (PBS) (a and c on the left) at 48 h post murine hepatitis virus strain 3 (MHV-3) infection; e-f, liver sections from bone marrow-derived macrophages (BMDMs) adoptive transferred mice at 48 h post MHV-3 infection (e: WT BMDM donor; f: Fibrinogen-like protein 2 (*Fgl2*^{-/-}) BMDM donor); B: Serum ALT and AST levels from clodronate liposomes-treated WT and *Fgl2*^{-/-} mice at 48 h post MHV-3 infection; C: Serum AST and ALT levels from WT and *Fgl2*^{-/-} BMDM chimeric mice at 48 h post infection; D: representative F4/80+ iNOS+ and F4/80+ CD206+ donor macrophages from WT and *Fgl2*^{-/-} BMDM chimeric mice at 48 h post infection; E: statistical analysis of iNOS+ and CD206+ donor macrophages in chimeric mice at 48 h post infection; F: H&E-stained liver section from mice which were treated with Cenicriviroc (CVC) in advance and subjected to MHV-3 infection for 48 h. Arrows represented necrotic cells, circles represent areas of hepatocyte necrosis. Image magnificance: 200×; G: aminotransferase levels of CVC treated viral fulminant hepatitis mice and its control. Data were presented as mean ± SD (n = 5). These experiments were repeated at least three times. BMDM: Bone marrow derived macrophages.

phagocytosis, followed by antigen presentation by major histocompatibility complex (MHC) II for T cells. We therefore cultured *E. coli* labeled with red fluorescence together with peritoneal macrophages to examine their phagocytic capabilities. The number of engulfed bacteria was notably smaller in macrophages from FGL2-deficient mice than in macrophages from WT mice, although more macrophages were detected in *Fgl2*^{-/-} mice (Figure 7A and B). Furthermore, surface expression of MHC II was more severely impaired in FGL2-deficient macrophages than in WT macrophages under normal conditions or after viral stimulation (Figure 7C), whereas expression of CD80, CD86, or MHC I was not altered (data not shown). These data suggest that FGL2 deficiency attenuates both the phagocytic capacity and antigen presentation potential of macrophages.

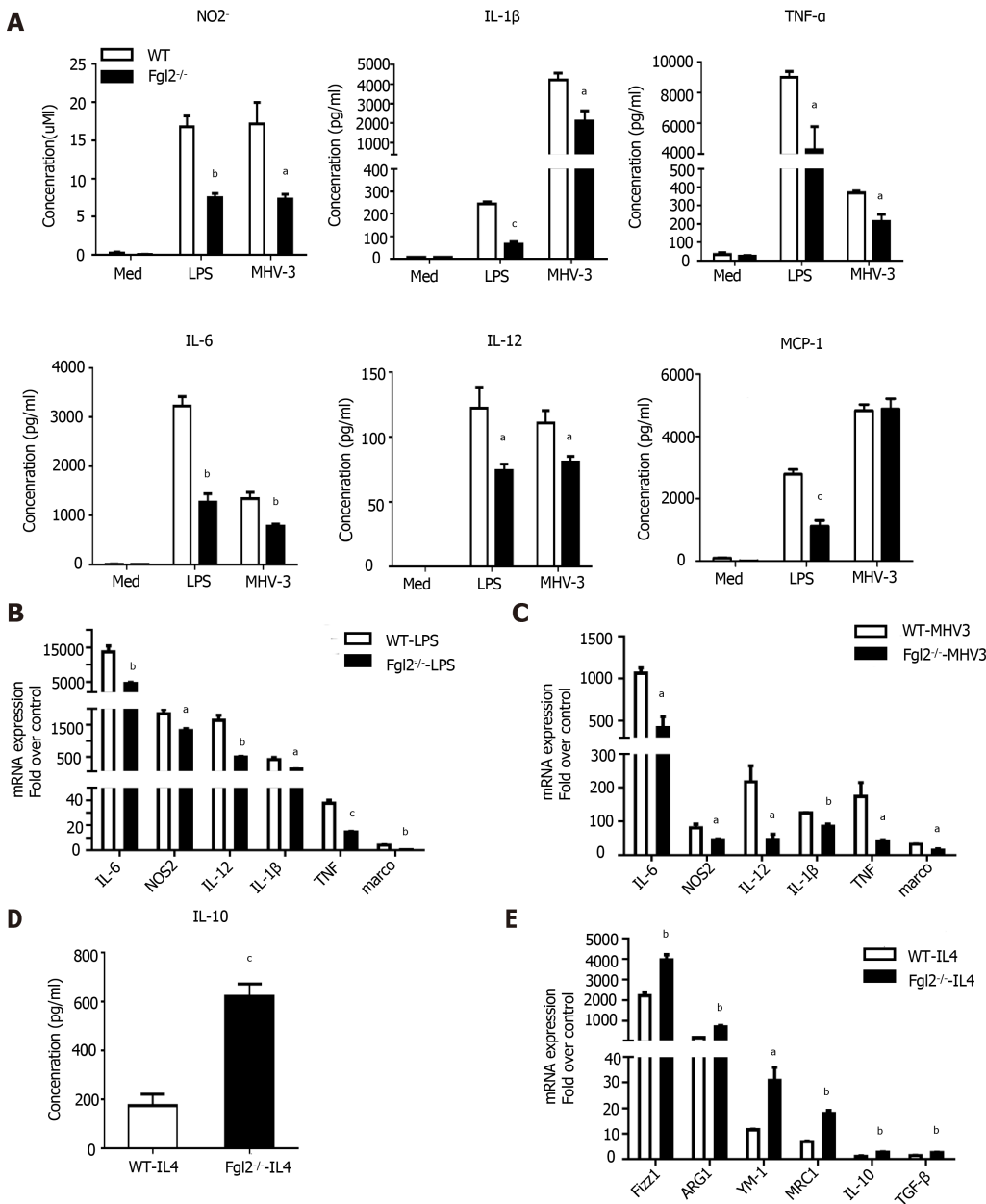


Figure 6 Fibrinogen-like protein 2 promoted bone marrow-derived macrophages M1 polarization *in vitro*. A: Supernatant concentration of NO₂⁻, IL-1 β , TNF- α , IL -6, IL-12 and MCP-1 released by bone marrow-derived macrophages (BMDMs) after LPS (100 ng/mL) treatment or murine hepatitis virus strain 3 (MHV-3) infection for 24 h; B: Relative transcriptional level of M1 markers (NOS2, IL-6, IL-12, IL-1 β , TNF- α and marco) of WT and fibrinogen-like protein 2 (*Fgl2*^{-/-}) BMDMs after the stimulation of LPS for 6 hours; C: Relative transcriptional level of M1 markers of WT and *Fgl2*^{-/-} BMDMs after MHV-3 infection for 8 hours; D: Supernatant IL-10 concentration of BMDMs culture after the IL-4 stimulation (20 ng/mL) for 72 h; E: mRNA expression of M2 markers (Fizz1, ARG1, Ym-1, MRC1, IL-10 and TGF- β) of WT and *Fgl2*^{-/-} BMDMs after of IL-4 treatment for 72 h. All data are presented as mean \pm SD ($n > 3$). These experiments were repeated at least three times.

FGL2 modulates the inflammatory signaling cascade by activating NF- κ B, IRF3, IRF7, and p38

To explore how FGL2 modulates inflammatory cascades, we examined the serial components of inflammatory signaling pathways in BMDMs by mimicking invading pathogen infections *in vitro*. Consistent with the results from MoMFs in the VFH model, FGL2 was robustly induced in BMDMs following LPS treatment and MHV-3 infection (Figure 8A). In addition, phosphorylation of I κ B α , p65, IRF3, and IRF7, which are critical modulators of NF- κ B activation and IFN expression, was remarkably impaired in *Fgl2*^{-/-} BMDMs in response to LPS treatment and viral infection (Figure 8B, Supplementary Figure 5). However, the expression of adaptor proteins such as TRIF and MyD88 was not altered under the same conditions (Figure 8B). Among mitogen-activated protein kinases (MAPKs), the level of phosphorylated p38 was reduced, whereas the levels of activated (phosphorylated) JNK and ERK were not

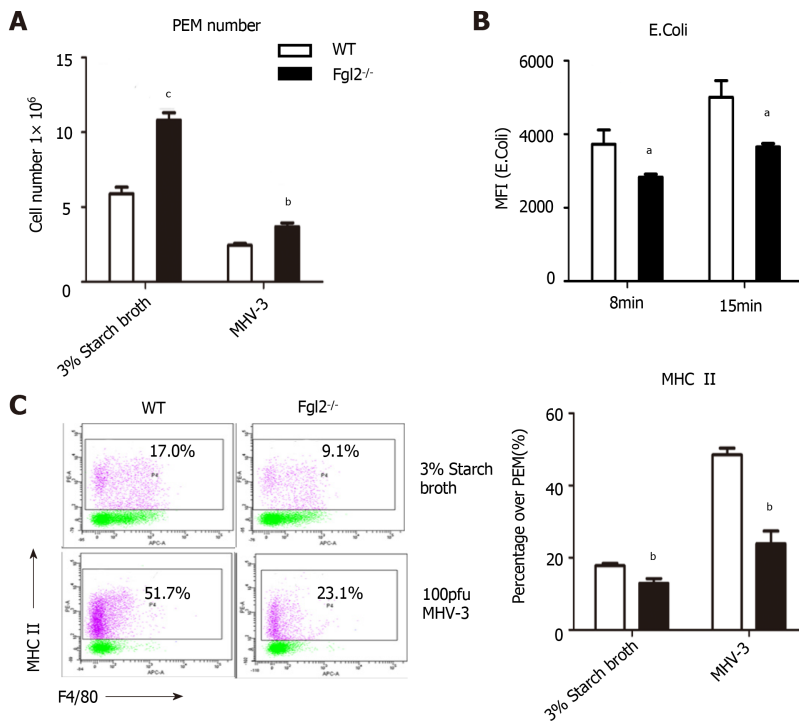


Figure 7 Fibrinogen-like protein 2 deficiency resulted in low expression of major histocompatibility complex II and impaired phagocytosis of macrophage. A: Absolute number of PEMs under the stimulation of 3% starch broth or murine hepatitis virus strain 3 (MHV-3) extracted from wild-type (WT) and fibrinogen-like protein 2 (*Fgl2*^{-/-}) mice; B: MFI of FITC+ phagocytosed *E.Coli* by PEMs from WT and *Fgl2*^{-/-} mice at different time points; C: Expression of major histocompatibility complex II on WT and *Fgl2*^{-/-} PEMs under the stimulation of 3% starch broth or MHV-3 infection. All data are presented as mean \pm SD. These experiments were repeated at least three times. PEMs: Peritoneal exudate macrophages.

affected in *Fgl2*^{-/-} BMDMs compared with those in WT BMDMs following LPS treatment and viral infection (Figure 8C, Supplementary Figure 5), suggesting that FGL2 induces a positive feedback proinflammatory loop through p38 activation. Phosphorylation of p65 at Ser276 by c-Raf1 enables the recruitment of acetyltransferase CREB-binding protein (CBP) and p300, which leads to the acetylation of p65 and thus an enhanced NF- κ B transcription rate[16]. Interestingly, phosphorylation of c-Raf1 and p65 (Ser276) and acetylation of p65 were reduced in *Fgl2*^{-/-} BMDMs compared with those in WT BMDMs following either LPS stimulation or viral infection (Figure 8D). Phosphorylation of TBK, a modulator downstream of SYK that is involved in C-type lectin receptor (CLR)/Toll-like receptor (TLR) signaling-mediated innate immunity [25], did not differ between WT and *Fgl2*^{-/-} BMDMs following LPS and MHV-3 challenge (Figure 8E). Taken together, our data suggest that induction of FGL2 in macrophages regulates inflammatory signaling by modulating p38, IRF3, IRF7, and p65 phosphorylation.

DISCUSSION

Fulminant hepatitis, the most severe form of acute viral hepatitis, is a type of ALF induced by viral infection. Mounting evidence suggests that leukocyte infiltration initiates inflammation and liver damage in chronic viral hepatitis and alcoholic and non-alcoholic hepatitis with acute liver injury. However, there is no ideal experimental model to completely mimic the specific clinical manifestations of acute viral hepatitis. Most studies to date have focused on acute hepatitis B, in which virus-specific cytotoxic T lymphocytes (CTLs) mediate liver damage subsequent to viremia[26,27]. Similarly, non-virus-specific CD8⁺ T cells with innate-like cytolytic activity promote liver damage in patients with acute hepatitis A[28]. The innate immune response is involved in the early stage of viral clearance. Among innate immune cells, natural killer (NK) cells are preferentially studied because of their potent capacity to direct viral clearance as well as liver injury. Evidence from experimental VFH models and patients with acute viral hepatitis has revealed the detrimental contribution of NK cells to disease outcome[29,30]. Macrophages are the largest population of non-parenchymal immune cells in the liver, which proliferate and are activated in patients

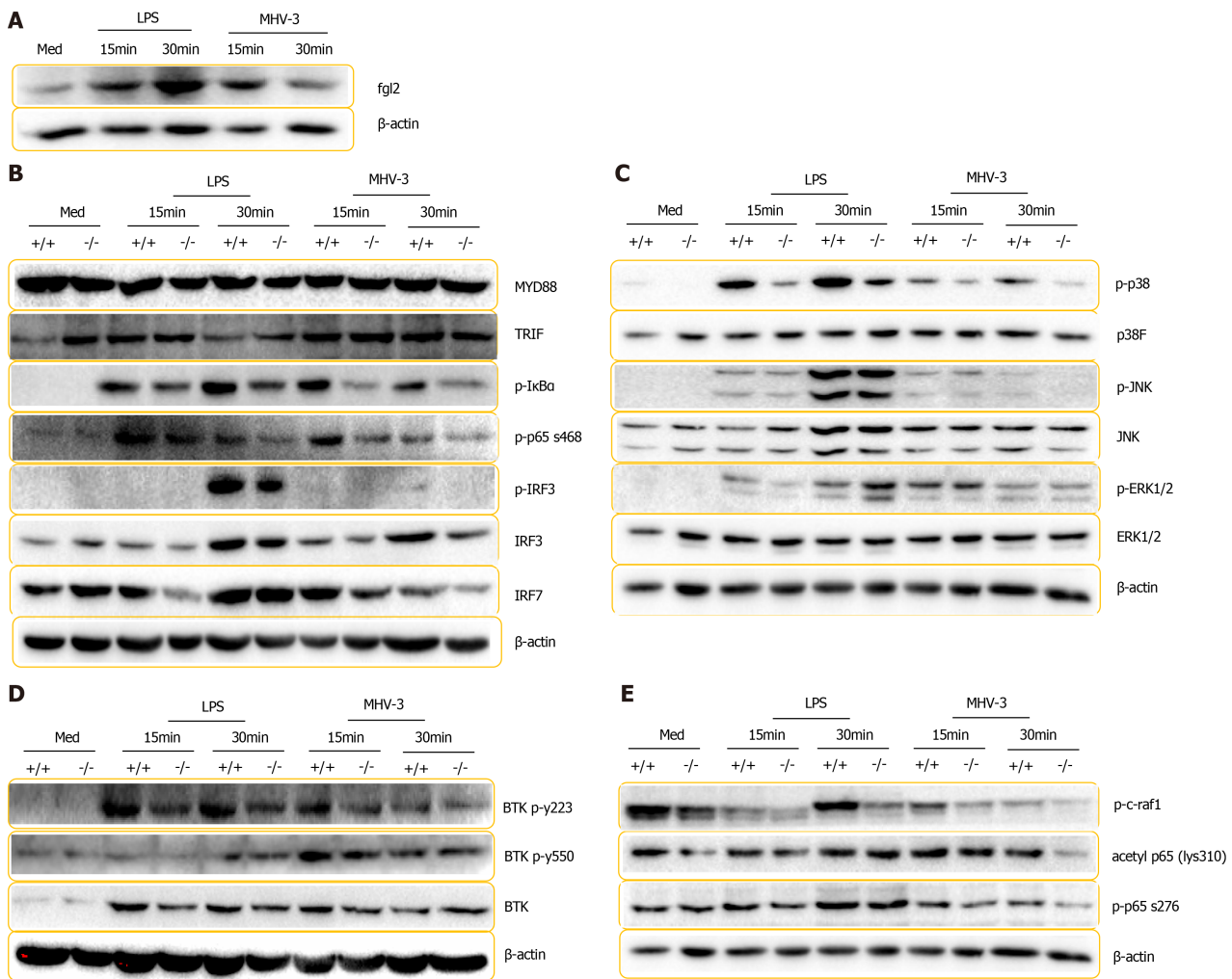


Figure 8 Fibrinogen-like protein 2 deficiency impaired inflammatory cascade in response to lipopolysaccharide treatment of viral infection. A: Immunoblot for fibrinogen-like protein 2 (Fgl2) and β -actin expression in wild-type (WT) BMDMs in response lipopolysaccharide (LPS) stimulation and murine hepatitis virus strain 3 (MHV-3) infection. B-E: Immunoblot for proteins panels from lysates of WT and *Fgl2*^{-/-} BMDMs in response to LPS treatment and MHV-3 infection; B: MyD88, TRIF, $\text{I}\kappa\text{B}\alpha$, phosphorylated $\text{I}\kappa\text{B}\alpha$, phosphorylated p65 (S468) and IRF3, phosphorylated IRF3, and IRF7; C: p38, JNK, ERK and phosphorylated p38, JNK, ERK; D: Phosphorylated c-Raf, phosphorylated p65 (S276), acetyl p65 (lys310); E: BTK, and phosphorylated BTK (Y223), BTK (Ty550). These experiments were repeated at least three times.

with acute hepatitis E virus infection and associated ALF, although their function is somewhat impaired[31]. The development of VFH is associated with hepatic macrophage replenishment by infiltrating macrophages, suggesting that macrophage infiltration is a fundamental event in VFH progression[32]. However, the pathological involvement of innate immunity in the liver damage occurring during acute viral hepatitis is not fully understood.

Experimental VFH established by either the coronavirus MHV-3 or infection with other hepatitis-causing viruses induces acute liver injury followed by ALF in 3-5 d in different strains of mice[12,14]. This suggests that adaptive immunity may not be the central event in disease progression, because of the rapid mortality despite infiltration of T cells to the liver[33]. In addition to monocytes, dendritic cells and neutrophils are also recruited to the liver during the progression of VFH, although the characteristics of the infiltrating leukocytes and their respective contributions to inflammation are unclear[12]. Neutrophils are a small subset of leukocytes that are required for monocyte infiltration during liver injury in mouse models of non-alcoholic steatohepatitis, acetaminophen overdose, and hepatitis virus infection[9,12,24]. In this study, we found that MoMFs dominated the population of infiltrating leukocytes, with a proinflammatory $\text{Ly6C}^{\text{high}}$ MoMF subset comprising the majority of the population, suggesting that targeting the transition from $\text{Ly6C}^{\text{high}}$ MoMFs to Ly6C^{low} MoMFs may be a promising strategy for treating VFH. Notably, the continued decrease in KC numbers during VFH suggests that necrosis occurred not only in hepatocytes but also in non-parenchymal cells.

FGL2 deficiency has been shown to prevent fulminant hepatitis following MHV-3 infection, which otherwise causes ALF with 100% mortality[14]. Moreover, FGL2 expression is largely distributed in and robustly induced by macrophages, in spite of moderate expression in dendritic cells and endothelial cells following viral infection [7]. The mechanism by which FGL2 contributes to VFH is generally considered to involve its procoagulant activity[14,34]. However, reciprocal activation between inflammation mediators or cytokines and tissue factor-mediated fibrin deposition and thrombin generation, the major contributor to inflammation-initiated coagulation, is largely dependent on IL-6[11,35]. It is therefore difficult to determine whether colocalization between fibrin deposition and FGL2 expression solely results from the prothrombinase activity of FGL2. In addition, inflammation may be induced by tissue factors secreted by local endothelial and immune cells[36]. Proinflammatory macrophage polarization is a process resulting from the Th1 response and pattern recognition receptor (PRR)-mediated signaling events initiated by PAMPs or DAMPs [37]. Unlike soluble FGL2 expressed by regulatory T cells and Th2-like immune cells with immunosuppressive activity, membrane FGL2 can amplify the classical inflammatory cascades and may act as a co-receptor to cooperate with TLRs or other PPRs for signal transduction. Our results suggest that FGL2 deficiency may foster a default transition from proinflammatory macrophages to an alternative activated phenotype because more M2 macrophages and Ly6C^{lo} MoMFs were observed under normal conditions. The inflammatory cytokines secreted by endothelial and dendritic cells in which FGL2 is expressed may also play a role; however, compared to macrophages, these cells are in relatively low abundance[38].

The innate immune responses triggered by viral infection are largely regulated by PPR-mediated signaling, which depends on cytosolic adaptors (*e.g.*, TRIF and MyD88) and kinase-dependent factors[39]. Macrophage M1 polarization promotes the activation and translocation of NF- κ B and the IRF3/IRF7 complex to initiate the transcription of Th1-associated gene targets[40]. p38 MAPKs are known to modulate PPR signaling by collaborating with NF- κ B-mediated transcription[33]. CLRs are another type of PPR that modulate TLR-mediated signaling in the defense against invading pathogens[14]. In invertebrates, numerous molecules containing fibrinogen-related domains participate in immune response transcription and have been shown to act as PPRs in defense processes, such as agglutination, and to cooperate with CLRs to synergistically clear pathogens[41]. Thus, we questioned whether FGL2 regulated TLR signaling in a CTL-like manner. MyD88-dependent PPR signaling involves activation of members of the IRAK family, which in turn stimulates the E3 ligase activity of TRAF6, enabling activation of the downstream ubiquitin-dependent kinase TAK1[42]. Upon activation, TAK1 activates MAPK and its downstream kinase IKK, which in turn phosphorylates the NF- κ B inhibitor I κ B α , leading to ubiquitin-dependent I κ B α degradation and subsequent NF- κ B activation[42]. Thus, TRIF-dependent PPR signaling involves TRAF3 recruitment and activation of TBK1 and IKK β , which stimulate IRF3 phosphorylation and NF- κ B activation, thereby leading to the transcriptional induction of type I IFNs and inflammatory cytokines[43]. Membrane proteins that regulate membrane PPR-mediated signaling function as co-receptors or factors involved in TLR assembly, internalization, or trafficking[44-47]. Accordingly, FGL2 may act as an assembly factor, could interact with PPRs for ligand-receptor binding, or even mediate the trafficking between the membrane and endosome, thereby facilitating inflammatory signaling transduction. Previous data showed that MHV-3-induced FGL2 expression in macrophages relies on p38 activation[48]. In this work, we found that FGL2 is needed for p38 phosphorylation, suggesting that FGL2 expression provides a positive feedback loop for proinflammatory aggregation in macrophages.

The exact role of resident macrophages and MoMFs in VFH is obscure. We speculate that resident macrophages may initiate liver inflammation during the early stage of viral infection because MoMFs exacerbate liver damage during the acute phase. Resident macrophages represent the first line of defense against viral infection. Once primed, resident macrophages produce chemotaxins and cytokines that recruit and activate the MoMFs, and our results suggest that FGL2 expression on macrophages prompts and enhances their inflammatory phenotype.

CONCLUSION

Our data revealed, for the first time, that Ly6C^{high} MoMF infiltration is a critical event for hepatic inflammatory accumulation and subsequent liver damage during virus-

induced hepatitis progression. FGL2 expression is required for maintaining the proinflammatory Ly6C^{high} MoMF phenotype by mediating IRF3, p65, and p38 phosphorylation, thereby forming a positive feedback loop of inflammatory accumulation in the liver.

ARTICLE HIGHLIGHTS

Research background

Viral fulminant hepatitis (VFH) is a devastating syndrome that pathologically caused by excessive activation of both innate and adaptive immunity. However, the extent of contribution of innate immunity in VFH is not well defined. Macrophage polarization have been implicated in host defense and the pathogenesis of various hepatic diseases. Fibrinogen-like protein 2 (FGL2) can be induced robustly and exclusively in macrophages in response to cytokines or viral infection. Exploring their roles in VFH can greatly improve our understanding of the disease and thus seek the therapeutic approaches.

Research motivation

Hepatic macrophages are attractive therapeutic targets because their functions can be inhibited or augmented to alter disease outcomes. A better understanding of their biological properties and immunologic function in liver homeostasis and pathology may pave the way for new diagnostic and therapeutic approaches for liver failure or other liver diseases.

Research objectives

To evaluate the role of Fgl2 in the reconstitution of hepatic macrophages during VFH progression by the VFH mouse model.

Research methods

Liver sections of liver failure patients and controls were immuno-stained for macrophages examination. Murine hepatitis virus strain 3 (MHV-3) was used to induce VFH experimental model in wild type and *Fgl2*^{-/-} mice. Adoptive transfer or depletion of macrophages were employed to assess liver damage and hepatic macrophages alteration. Signal cascade induced by LPS or MHV-3 were detected in macrophages.

Research results

Infiltrated MoMFs is a major source of hepatic inflammation during VFH progression. Fgl2 expression on macrophages prompts and enhances the inflammatory phenotype of hepatic macrophages, which breaks the previous understanding that the mechanism of Fgl2 during VFH is generally considered to be procoagulant activity.

Research conclusions

We revealed for the first time that pro-inflammatory monocyte-derived macrophages (MoMFs) infiltration is critical event for hepatic inflammatory accumulation and subsequent liver damage during virus-induced hepatitis progression and Fgl2 is required for maintaining the pro-inflammatory hepatic macrophages phenotype.

Research perspectives

Macrophages are 'keystones' of liver architecture in both homeostasis and disease. The development of potential therapies highly depends on a fundamental knowledge about the mechanisms that trigger the polarization and control the fate of hepatic macrophages. We believe that a better understanding of the specific mechanisms underlying macrophages participation in diseases will definitely result in the increased efficacy of these therapies.

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