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LPS stimulation during HCV infection induces MMP/TIMP1 imbalance in macrophages

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Abstract

Introduction. During chronic hepatitis C virus (HCV) infections, HCV antigens establish cross-tolerance of endotoxins, but additional lipopolysaccharide (LPS) stimulation effects in this condition are poorly understood.

Aim. This study aims to investigate the effects of the upregulated LPS on MMP and TIMP expression during chronic hepatitis C infection.

Methodology. In the present study, we analysed the effect of HCV antigens and LPS stimulation on peripheral blood mononuclear cells (PBMCs) both *in vivo* and *in vitro*. Macrophages from HCV patients were isolated and their association with endotoxin tolerance was examined. MMP/TIMP1 expression and the related signalling pathways in macrophages were analysed. The macrophage and Huh7.5 cell co-culture model was used to analyse the effects of the cross-tolerance on collagen I deposition.

Results. LPS levels were found to be significantly higher in HCV patients, particularly in those with HCV-induced liver fibrosis. In addition, although LPS serum level was occasionally upregulated in the patients, it did not induce intense immune response in PBMCs due to endotoxin cross-tolerance, and this was measured according to the changes in IL-6 and TNF-α levels. However, TIMP1 expression increased significantly during stimulation, exhibiting a tolerance/resistance phenotype, which was associated with TGF-β/Erk activation in macrophages. However, MMP levels did not increase due to endotoxin tolerance, which ultimately led to MMP/TIMP imbalance and influenced the deposition of collagen I.

Conclusion. Increased LPS stimulation of macrophage during HCV antigen-induced endotoxin cross-tolerance contributes to MMP/TIMP1 imbalance and collagen I deposition.

INTRODUCTION

Currently, the human microbiota and its metabolites from the gastrointestinal tract are considered to be a very important organ in the immune system [1]. Previous experiments have demonstrated that gut-derived endotoxins are involved in the progression of many liver diseases [2, 3]. Endotoxins or lipopolysaccharides (LPS) are a major component of the Gram-negative bacterial cell wall, and act as an important pathogen-associated molecular pattern (PAMP) for Toll-like receptor 4 (TLR4) [4], inducing downstream signalling for the production of inflammatory cytokines such as TNF-α and IL-6 [5]. Increased plasma LPS levels have been detected in patients with chronic hepatitis, such as hepatitis C (HCV) infection [6]. However, the effects of upregulated LPS during HCV infection remain unclear.

During HCV infection, viral antigens such as HCV core and NS3 proteins trigger inflammatory pathways through TLR2 signalling and MyD88-dependent innate immune activation

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Keywords: endotoxin tolerance; liver fibrosis; HCV; Erk signal; TIMP1.

Abbreviations: ALT, alanine transaminase; ECM, extracellular matrix; ELISA, enzyme-linked immunosorbent assay; Erk, extracellular regulated protein kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HCV, hepatitis C virus; IRAK, IL-1R-associatedkinase; LPS, lipopolysaccharide; MMP, matrix metalloproteinase; NF-κB, nuclear factor kappa-B; PAMP, pathogen-associated molecular pattern; PBMCs, peripheral blood mononuclear cells; RT-PCR, Reverse Transcription-Polymerase Chain Reaction; SHIP1, SH2-containing inositol phosphatase-1; TGF-β, transforming growth factor-β; TIMP, MMPs and tissue inhibitor of metalloproteinase; TLR4, Toll-likereceptor 4; TRAF6, TNF receptor-associated factor 6.

Two supplementary tables and one supplementary figure are available with the online version of this article.

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[7]. MyD88 recruits IL-1R-associated kinase (IRAK) and induces the assembly of a complex composed of TNF receptor-associated factor 6 (TRAF6) and TRAF3, which leads to the activation of NF-κB-related gene transcription [2, 4]. Furthermore, NS3 protein has been reported to induce TGF-β signal activation by binding directly to the receptor [8]. Therefore, in chronic HCV infection, immune cells are consistently activated prior to HCV antigen stimulation, whereas LPS upregulation due to increased intestinal permeability or an impaired clearance mechanism could induce various signal transductions, which may establish unique endotoxin cross-tolerance [6].

Fibrosis is a common pathological consequence of the accumulation of extracellular matrix (ECM). ECM changes in the liver depend on ECM synthesis and matrix metalloproteinase (MMP)-mediated ECM proteolytic degradation. The balance between MMPs and tissue inhibitor of metalloproteinase (TIMP) is particularly important for the development of liver fibrosis [9]. The most potent MMPs are collagenases, which act against collagens. These collagenases include MMP-1, MMP-8 and MMP-13 [10]. In contrast, TIMPs are specific endogenous inhibitors that bind to MMPs and block them from ECM substrates. In addition, TIMP1, TIMP2 and TIMP3 may function in liver fibrosis [10, 11].

In the current study, we investigated the effects of LPS stimulation during chronic HCV infection, analysed the effect of antigens on specific MMPs and TIMPs, and reported a novel mechanism of MMP/TIMP imbalance induced by HCV antigens and LPS signalling crosstalk.

METHODS

Subjects

The study subjects comprised 36 healthy donors, 33 normal HCV patients without progress and 25 HCV patients with cirrhosis. A no-progress patient had an Ishak score of less than F2, while an HCV cirrhosis patient had an Ishak score greater than F4. All samples were negative for HIV and hepatitis B virus. The protocol was approved by the Research and Ethical Committee of Tangdu Hospital of the Fourth Military Medical University. Informed consent was obtained from all donors.

Cells and stimulation

Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Hypaque density centrifugation (Sigma, St Louis, MO, USA). Macrophages were isolated from the PBMCs using CD14+isolation MicroBeads (Miltenyi Biotec, Inc., Auburn, CA, USA). Isolated macrophages were cultured with X-vivo 15 medium (Lonza, Walkersville, MD, USA). For LPS (Sigma, St Louis, MO, USA) single stimulation, macrophages from HCV patients or healthy donors were treated with 100 ng ml^{-1} LPS. For HCV antigen and LPS double stimulations, macrophages were first incubated with HCV supernatant (from pJFH1-transfected Huh7.5 cells, with 10^8 plaque-forming units (p.f.u.) of HCVcc [12]) for 5 days and then treated with LPS stimulation. A20 adenovirus overexpression vectors (ad-A20) were purchased from Genechem Co. Ltd (Shanghai, PR China). Signalling inhibitors, including U0126, JSH-23 and LY364947 (Abcam, Cambridge, MA, USA), were added to the culture medium.

ELISA and endotoxin measurement

IL-6 and TNF-α concentrations in the serum were determined by human IL-6 and TNF-α platinum ELISA kits (eBioscience, San Diego, CA, USA). Collagen I concentration was determined using the Human Pro-collagen I ELISA kit (Abcam, Cambridge, MA, USA). The results were detected using a microplate reader (BioTek, Shanghai, PR China). LPS level was measured by Limulus Amebocyte Lysate assay (Lonza, Basel, Switzerland).

RNA isolation and RT-PCR analysis

RNA was purified using an RNeasy Mini kit (Qiagen, Gaithersburg, MD, USA) and cDNA was synthesized using the RevertAid First Strand cDNA Synthesis kit (cat. no. K1622, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. Gene expression was measured by RT-PCR with SYBR Premix Ex *Taq* II (Takara, Mountain View, CA, USA) using a Bio-Rad iQ5 Real-time Thermocycler (Bio-Rad, Hercules, CA, USA). Gene expression level was determined relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primer sequences are shown in Table S1 (available in the online version of this article).

Western blotting

Cells were harvested from lysis buffer (50 mM tris/HCl at pH 7.5, 150 mM NaCl, 2 mM EDTA) supplemented with protease inhibitor (Roche Applied Science, Indianapolis, IN, USA) and phosphatase inhibitor (cat. no. P2850, Sigma-Aldrich, St Louis, MO, USA). The lysate was run by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore, Billerica, MA, USA). Membranes were blocked and incubated with primary and secondary antibodies, including rabbit anti-NF-kB p-p65 Ab, rabbit anti-pSmad2 Ab, rabbit anti-ERK1/2 Ab, mouse anti-TNFAIP3 Ab, rabbit anti-IRAKM Ab, rabbit anti-SHIP-1 Ab, rabbit anti-TIMP1 Ab, rabbit anti-MMP1 Ab, rabbit anti-collagen I Ab and mouse anti-β-actin Ab (Abcam, Cambridge, MA, USA).

Macrophage and Huh7.5 co-culture system

Purified macrophage were resuspended at a concentration of 10^6 cells ml^{-1} and co-cultured with either Huh7.5 or JFH-1-transfected Huh7.5 cells at a ratio of 1:1. After 5 days of co-culture, LPS (100 ng ml^{-1}) was added to the medium, and the cells were analysed on the third day after LPS stimulation. Cells were cultured with X-vivo 15 medium (Lonza, Walkersville, MD, USA).

Statistical analysis

The Mann–Whitney U test and Student's *t*-test were used to determine statistically significant difference. Analyses were performed with Statistical Package for Social Science (SPSS

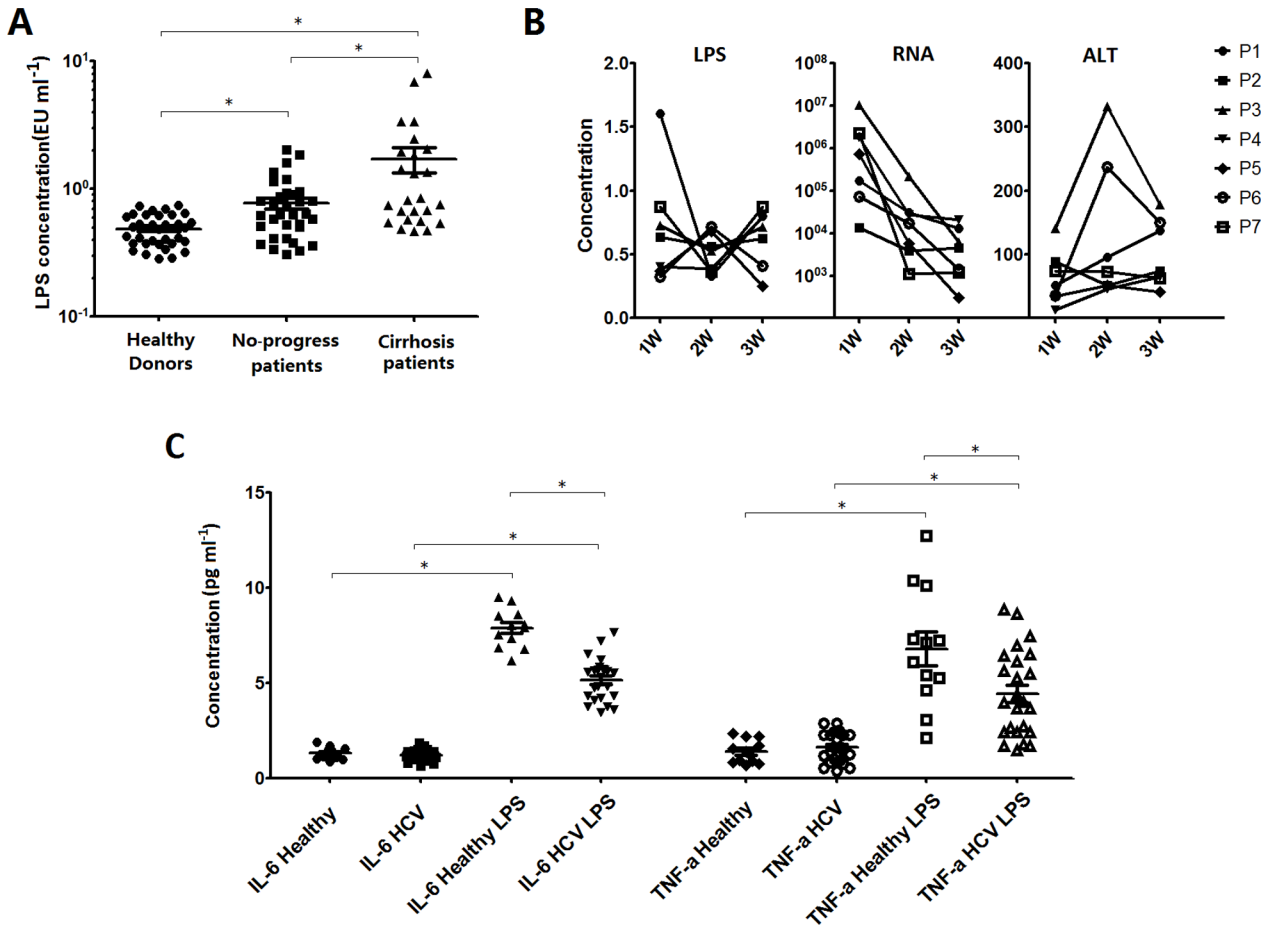

Fig. 1. LPS changes in serum and cytokine expression in LPS-stimulated PBMCs from HCV patients. (a) LPS expression was upregulated in HCV infection and cirrhosis. LPS levels were lower in healthy volunteers (0.49 EU ml^{-1}) than in HCV (0.77 EU ml^{-1}, P=0.001) and HCV-induced cirrhosis patients (1.71 EU ml^{-1} P<0.0001). (b) LPS, HCV-RNA and ALT levels of seven HCV patients were tested at weeks 1, 2 and 3. LPS level fluctuated in each person at different time points. (c) Cytokine responses of PBMCs were analysed between healthy volunteers and no-progress patients. PBMCs were isolated and stimulated with LPS *in vitro*. IL-6 and TNF-α levels were measured by ELISA. *, P<0.05.

Statistics 21.0, IBM Corp., Armonk, NY, USA) and GraphPad Prism version 5.0 (La Jolla, CA, USA). A P-value <0.05 was considered statistically significant.

RESULTS

PBMCs from HCV patients exhibit an endotoxin tolerance phenotype

No-progress HCV patients, HCV fibrosis patients and uninfected volunteers were included in the serum LPS evaluation (Table S2). Serum LPS was found to be significantly lower in healthy volunteers than in the no-progress (P=0.001) and cirrhosis patients (P<0.0001). Additionally, a significant difference in LPS levels between no-progress and cirrhosis patients was also observed (P=0.02) (Fig. 1a). Furthermore, to determine LPS variations in the patients at different time points, he LPS levels of seven HCV patients were tested at weeks 1, 2 and 3, and LPS levels were found to fluctuate in each person at different time points (Fig. 1b). However, LPS levels were not correlated with ALT or HCV RNA levels, which may have been due to the complicated effects associated with increased intestinal permeability or the impaired clearance mechanism caused by the hepatitis.

It is well established that LPS as a TLR4 activator can induce endotoxin tolerance [13]. The experiment compared the IL-6 and TNF-α response of PBMCs between healthy volunteers and HCV patients before and after LPS stimulation, respectively. PBMCs were isolated and then stimulated with LPS *in vitro*. The IL-6 and TNF-α levels were measured by enzyme-linked immunosorbent assay (ELISA) (Fig. 1c). Accordingly, significant differences could be found between non-LPS stimulation and LPS stimulation conditions, either in healthy donors or in HCV patients. However, there were also significant differences

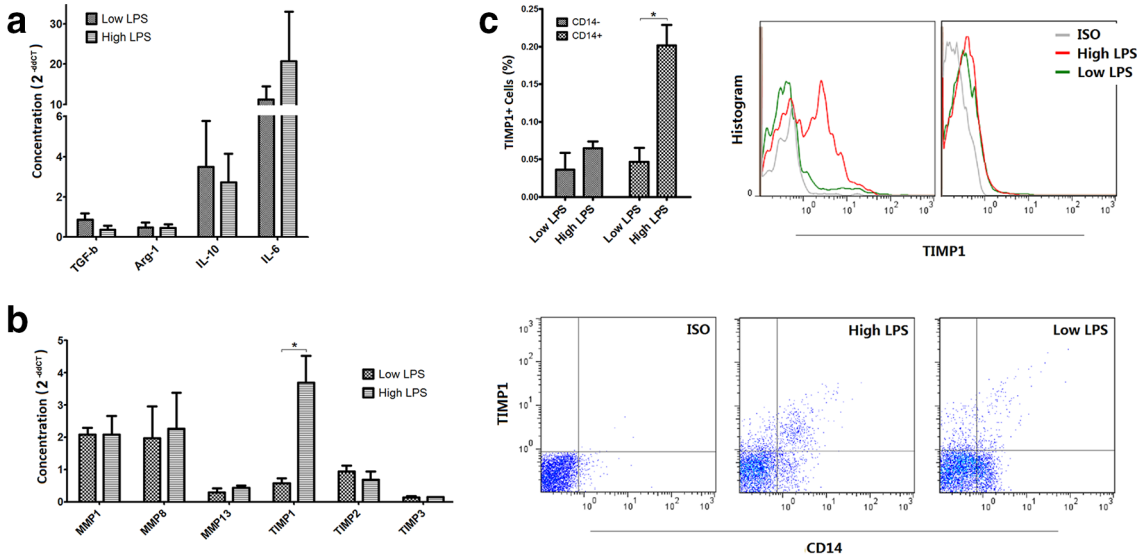

Fig. 2. MMP and TIMP expression in PBMCs from patients with different LPS levels. (a) PBMCs from patients with high LPS levels and low LPS levels were analysed for TGF-*β*, Arg-1, IL-10 and IL-6. (b) MMP and TIMP expression in PBMCs from patients with different LPS levels were tested. TIMP1 expression increased in high-LPS patients, while MMPs and TIMPs showed no significant changes. (c) Flow cytometry analysis of TIMP1 expression in macrophages from patients with different LPS levels. *, $P<0.05$.

in the cytokine response level between healthy donors and the no-progress patients after LPS stimulation. Therefore, PBMCs from the patient group showed weaker cytokine response (IL-6 and TNF-α expression) to LPS than those from the healthy control group, suggesting that endotoxin tolerance was induced by HCV antigens and LPS stimulation.

TIMP1 in PBMCs differed between high- and low-LPS patients

To further explore the effects of LPS variation, PBMCs from six no-progress patients with the highest serum LPS and six patients with the lowest serum LPS levels were analysed for TGF-*β*1, Arg-1, IL-10, IL-6, MMP and TIMP expression using RT-PCR (Fig. 2a, b). No significant changes were observed in TGF-*β*1, Arg-1, IL-10 and IL-6 expression, which indicate that LPS variation did not induce polarization changes in PBMCs (Fig. 2a). However, TIMP1 expression significantly increased in patients with high serum LPS compared to patients with low serum LPS, while MMP levels did not change significantly (Fig. 2b). Therefore, LPS may lead to MMP/TIMP imbalance. The PBMCs were also analysed by flow cytometry. The flow cytometry results confirmed that TIMP1 expression was upregulated more in high LPS patients than in low LPS patients. Furthermore, it was found that increased TIMP1 expression was mainly contributed by the macrophages; however, TIMP1 expression levels did not change in other cells (Fig. 2c).

LPS induced MMP/TIMP1 imbalance in HCV antigen-related endotoxin tolerance

To identify the signal transduction involved in TIMP1 expression during HCV-antigen/LPS stimulation, macrophages were isolated from PBMCs of healthy donors. The cells were first incubated with HCVcc supernatant for 5 days followed by additional LPS stimulation. According to RT-PCR and Western blot analysis, TIMP1 expression was upregulated significantly 2 days after LPS stimulation; however, MMP1 and MMP8 did not show changes even during the following days (Fig. 3a, b). Although MMP-1, MMP-8 and TIMP-1 are all considered to be NF-κB controlling genes [13–16], TIMP-1 is also predicted to be a TGF-*β* target gene [17, 18], which is involved in MAPK/Erk signal transduction [19, 20]. Therefore, TIMP1 expression would be probably regulated by a signalling response network. As a result, we examined the signalling activities of NF-κB, TGF-*β* and Erk regarding p65, Smad2 and Erk1/2 phosphorylation. NF-κB signalling activity was slightly upregulated on the first day and downregulated on the second day after LPS stimulation, while TGF-*β* and Erk signalling activities were enhanced on the first and second days after LPS stimulation (Fig. 3c).

During endotoxin tolerance, negative regulators function to inhibit NF-κB response [21–23] and these regulators, including A20, IRAK-M and SHIP1, were examined (Fig. 3c). Accordingly, A20, IRAK-M and SHIP1 expression could be detected after continuous HCV antigen stimulation. Interestingly, while IRAK-M and SHIP1 were further upregulated, A20 declined after additional LPS stimulation. Therefore, it is supposed that NF-κB activity was restrained to a certain level by A20, IRAK-M and SHIP1 expression in continuous HCV antigen stimulation. Moreover, after additional LPS stimulation, NF-κB was further suppressed by increased IRAK-M and SHIP1 on the second day, while A20, as an NF-κB-regulated gene [24], was downregulated during NF-κB inhibition.

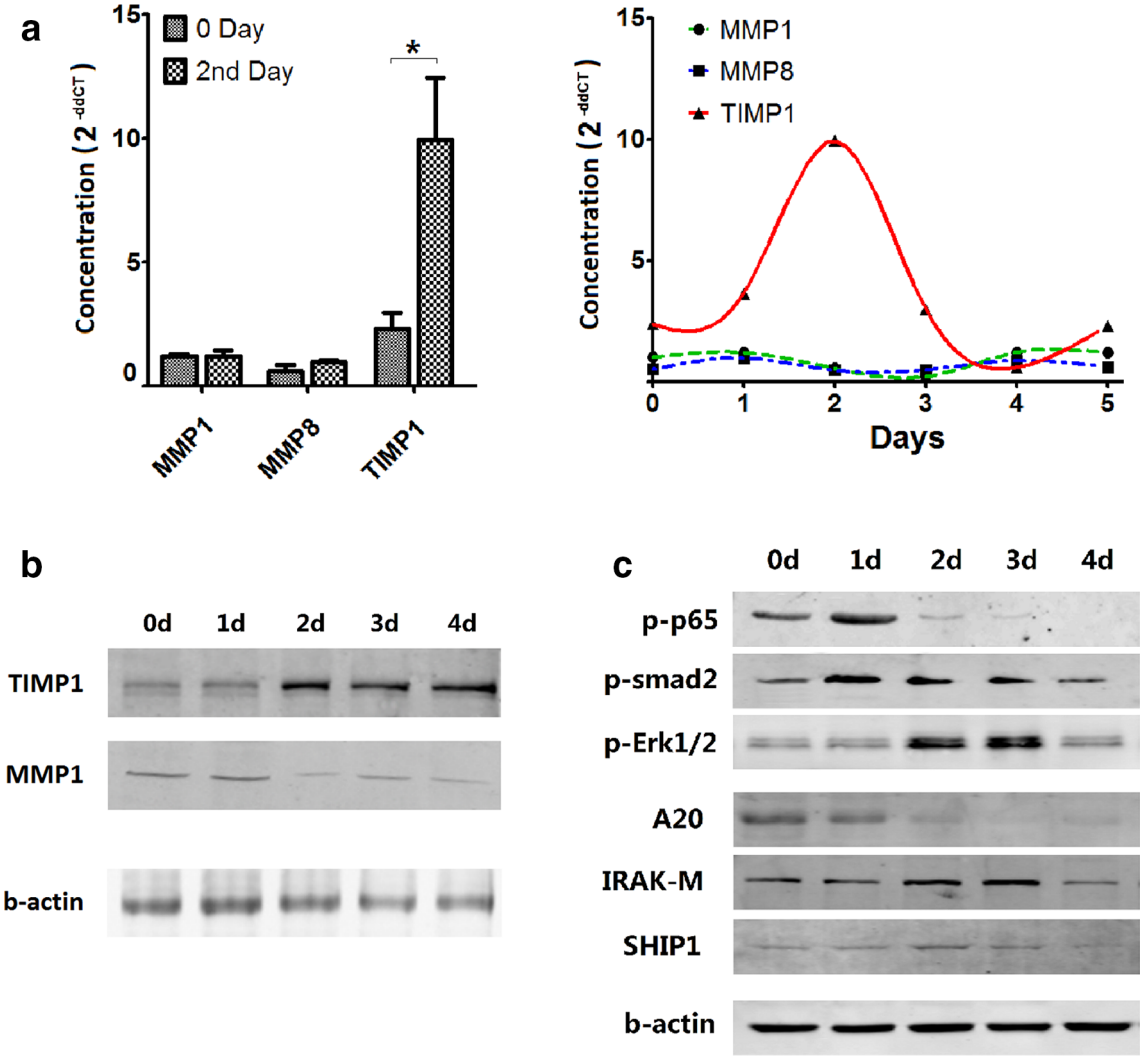

Fig. 3. NF-κB and TGF-*β* signal activation during TIMP1 upregulation. Macrophages from healthy donors were isolated. Cells were preincubated with HCVcc supernatant and then stimulated with LPS. (a) MMP1, MMP8 and TIMP1 expression was analysed by RT-PCR at different time points. (b) TIMP1 and MMP1 expression was analysed by Western blot. (c) The activities of NF-κB, TGF-*β* and Erk signalling were tested regarding p65, Smad2 and Erk1/2 phosphorylation; additionally, the inhibitors, including A20, IRAK-M and SHIP1, were examined. *, *P*<0.05.

TIMP1 expression was negatively regulated by A20 through the TGF -*β*/Erk pathway

To confirm that increased TIMP1 expression was induced by TGF-*β* activity, isolated macrophages were incubated with NF-κB inhibitor, TGF-*β* inhibitor, or Erk inhibitor besides HCV antigen/LPS stimulation (Fig. 4a). TIMP1 expression and signalling activity were tested by RT-PCR and Western blotting, respectively. Consequently, TGF-*β*1 and Erk inhibitors suppress TIMP1 expression, while NF-κB had little effect (Fig. 4b). This suggests that TIMP1 expression is controlled through TGF-*β* and Erk signal transduction. Because TGF-*β*-induced Erk activation requires TRAF6 recruitment [25–27] and A20 appears to move polyubiquitin chains for TRAF6 degradation [28, 29], it is assumed that A20 may suppress Erk activation by TRAF6 ubiquitylation. Stimulated cells overexpressed A20, while TIMP1 and Erk activity were modulated. Accordingly, A20 overexpression, but not NF-κB signal inhibition, could suppress TIMP1 expression and Erk activity (Fig. 4c, d) . Collectively, these findings suggested that TIMP1 was upregulated through TGF-*β*/Erk signal transduction and was suppressed through A20 expression.

TGF-*β* pathway was activated by NS3 during TIMP1 upregulation

Because the HCVcc supernatant contains HCV core and NS3 antigens (Fig. S1), the effects of these antigens on TIMP1 expression were tested. HCV core and NS3 neutralizing antibodies were incubated in the system, respectively. As a result, core antibody suppressed MMP1 expression (Fig. 5a), which may reflect HCV core activation influencing TLR2

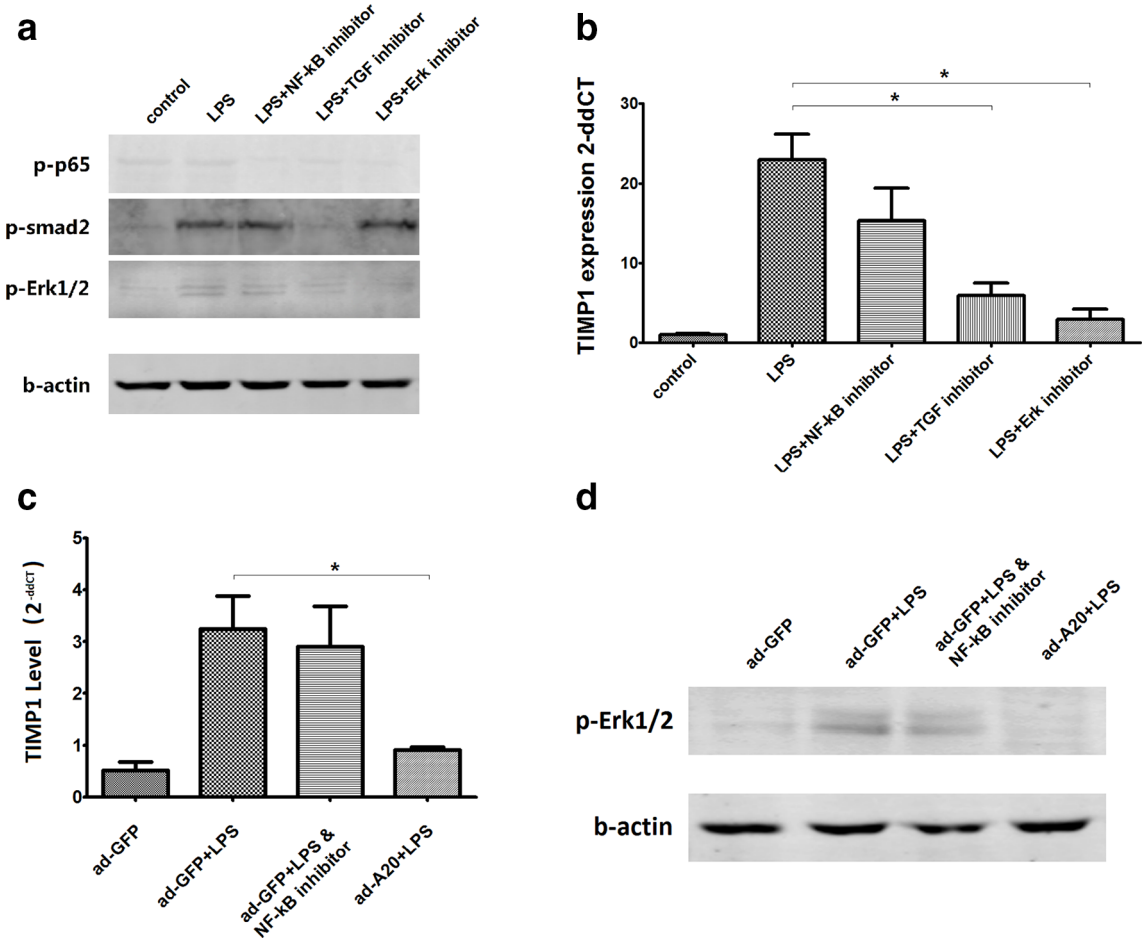

Fig. 4. TIMP1 was negatively regulated by A20 through the TGF-β/Erk pathway. Macrophages were preincubated with HCVcc supernatant and then stimulated with LPS. Each stimulated cell was co-incubated with TGF-β inhibitor, Erk inhibitor, or transfected with A20 expressing vector during LPS stimulation. (a) p-p65, p-smad2 and p-Erk were examined regarding the NF-κB, TGF-β and Erk pathways. (b) TIMP1 expression was tested in different signal inhibition groups. (c) A20 overexpression significantly suppressed TIMP1 expression during LPS stimulation. (d) Erk phosphorylation was suppressed by A20 overexpression but not NF-κB inhibition, suggesting that A20 suppression of Erk signal is independent of A20 negative regulation of NF-κB. *, P<0.05.

signal transduction [7]. Furthermore, TIMP1 expression was inhibited by NS3 antibody incubation (Fig. 5b), implying that NS3 may participate in TIMP1 upregulation during the cross-tolerance. This was interpreted to mean that NS3 protein could induce TGF-β signal activation by direct binding of the receptor [8]. Therefore, TGF-β activity was examined. As expected, TGF-β/Erk signalling activity was suppressed by NS3 neutralization (Fig. 5c), indicating that persistent NS3 stimulation from HCVcc supernatant may contribute to TGF-β activation and TIMP1 upregulation.

HCV antigen/LPS-induced cross-tolerance promotes collagen deposition

A co-culture system including isolated macrophage and HCV-infected Huh7.5 cells was established. MMP1 and TIMP1 expression was examined when co-incubated with Huh7.5 or HCV-transfected Huh7.5 cells after LPS stimulation. With HCV-infected Huh7.5 cell incubation, additional LPS-induced MMP1 expression in macrophage significantly decreased compared to the HCV uninfected group. However, the expression of TIMP1 only differed slightly between the two groups, and exhibited a tolerance/resistance phenotype (Fig. 5d). Therefore, during HCV infection, LPS stimulation may induce a transient imbalance between MMP1 and TIMP1.

Since the MMP1/TIMP1 ratio could influence ECM formation, the collagen I deposition effect was tested by ELISA and Western blotting. Accordingly, while pro-collagen I secretion showed slight change, more collagen I was detected in cell supernatant when HCVcc antigens exist (Fig. 5e). In summary, this result indicated that HCV antigens and LPS-induced endotoxin tolerance might lead to transient MMP/TIMP imbalance and may facilitate the process of liver fibrosis.

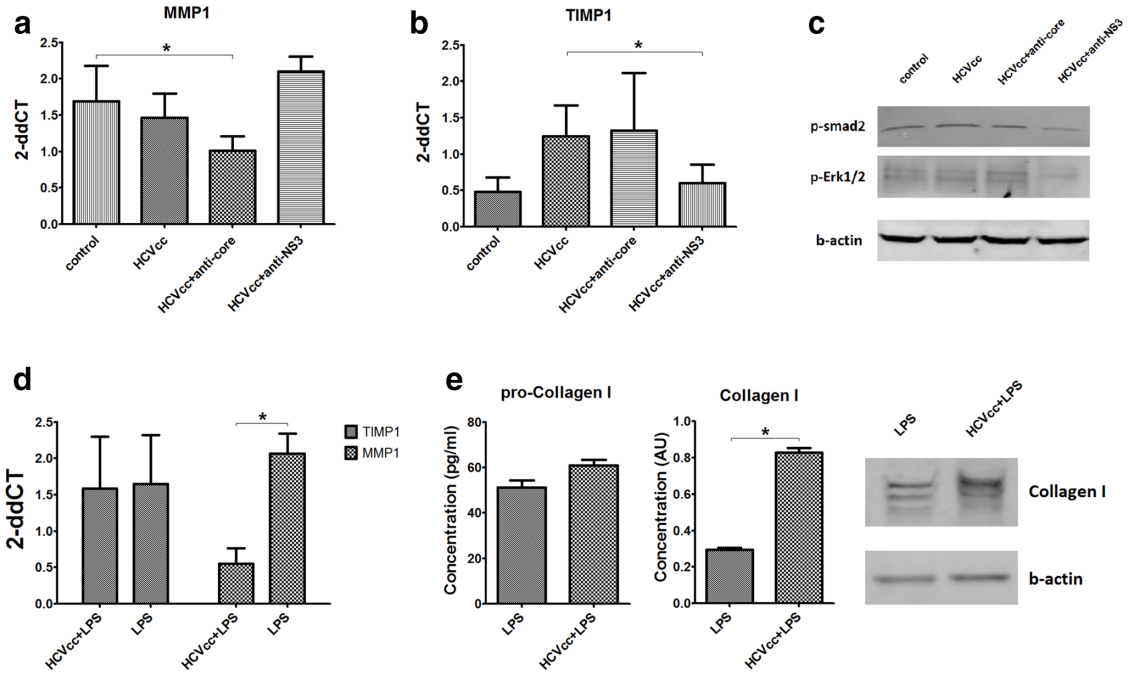

Fig. 5. TGF-β/Erk upregulation associated with NS3 induces MMP/TIMP imbalance and collagen I deposition. Macrophages were prestimulated with or without HCVcc supernatant. Prestimulated cells were co-incubated with HCV core antibody or NS3 antibody for antigen neutralization. In addition, MMP1 (a) and TIMP1 (b) expression and TGF-β/Erk signal activation (c) were examined. Macrophage and Huh7.5 cells with or without HCV infection were co-cultured. (d) MMP1 and TIMP1 expression was examined in HCV-infected and uninfected groups. (e) Collagen I deposition effect was tested by ELISA and Western blotting. *, P<0.05.

DISCUSSION

LPS is a gut microbial antigen that has important effects on the macrophage and immune responses [3, 13]. Our results showed that the serum LPS levels were elevated in HCV and liver fibrosis patients, and were significantly different from those in healthy individuals. It was also proved that LPS levels fluctuated during HCV infection, although the underlying mechanism has not yet been elucidated. Normally, upregulated LPS can increase both MMP1 and TIMP1 expression [3, 14]. In our experiment, it appears that transient LPS upregulation induces MMP1/TIMP1 imbalance in macrophage in the presence of HCV antigens. Because MMP1/TIMP1 balance has a profound effect on extracellular matrix remodelling, it is implied that LPS transient upregulation during HCV infection may be an important factor responsible for liver fibrosis progress.

In the current study, the activity ratio of NF-κB to TGF-β likely determines the balance between MMP and TIMP expression. In HCV infection, NF-κB was activated by HCVcc, core and NS3 [7]. Endotoxin tolerance was established by negative regulators, such as A20, IRAK-M and SHIP1. However, with the effects of additional LPS stimulation, increased IRAK-M and SHIP1 further suppressed NF-κB activation, and caused A20 downregulation and released the inhibition of TGF-β/Erk signalling through ubiquitinoylation of TRAF6. Meanwhile, the effect of NS3 on the activation of TGF-β signalling was enhanced during NF-κB suppression. Therefore, MMP, as an NF-κB promoting gene, was restricted to a certain level. However, TIMP1 was transiently upregulated due to TGF-β/Erk activation. Since the MMP1/TIMP1 ratio could influence ECM formation, collagen I deposition was tested. Accordingly, more collagen I was detected during HCVcc antigen incubation. In summary, LPS fluctuation during HCV infection may lead to transient MMP/TIMP imbalance in macrophages and facilitate the process of liver fibrosis.

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Author contributions
C. F.: data collection, acquisition, analysis and interpretation, statistical analysis, and manuscript writing; X. X. Z.: conducted part of the experiments; P. Z. and J. Z.: collection of patient material; Y. Z. and X. W.: revision of the manuscript; Z. J.: selection of samples and critical revision; Y. W.: experiment design and critical revision.

Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
The protocol was approved by the Research and Ethical Committee of Tangdu Hospital of the Fourth Military Medical University.

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