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T-bet-mediated Tim-3 expression dampens monocyte function during chronic hepatitis C virus infection

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Summary

Hepatitis C virus (HCV) induces a high rate of chronic infection via dysregulation of host immunity. We have previously shown that T-cell immunoglobulin and mucin domain protein-3 (Tim-3) is up-regulated on monocyte/macrophages (M/Mφ) during chronic HCV infection; little is known, however, about the transcription factor that controls its expression in these cells. In this study, we investigated the role of transcription factor, T-box expressed in T cells (T-bet), in Tim-3 expression in M/Mφ in the setting of HCV infection. We demonstrate that T-bet is constitutively expressed in resting CD14$^+$ M/Mφ in the peripheral blood. M/Mφ from chronically HCV-infected individuals exhibit a significant increase in T-bet expression that positively correlates with an increased level of Tim-3 expression. Up-regulation of T-bet is also observed in CD14$^+$ M/Mφ incubated with HCV$^+$ Huh7.5 cells, as well as in primary M/Mφ or monocytic THP-1 cells exposed to HCV core protein *in vitro*, which is reversible by blocking HCV core/gC1qR interactions. Moreover, the HCV core-induced up-regulation of T-bet and Tim-3 expression in M/Mφ can be abrogated by incubating the cells with SP600125 – an inhibitor for the c-Jun N-terminal kinase (JNK) signalling pathway. Importantly, silencing T-bet gene expression decreases Tim-3 expression and enhances interleukin-12 secretion as well as signal transducer and activator of transcription 1 phosphorylation. These data suggest that T-bet, induced by the HCV core/gC1qR interaction, enhances Tim-3 expression via the JNK pathway, leading to dampened M/Mφ function during HCV infection. These findings reveal a novel mechanism for Tim-3 regulation via T-bet during HCV infection, providing new targets to combat this global epidemic viral disease.

Keywords: hepatitis C virus; c-Jun N-terminal kinase pathway; monocyte/macrophages; T-bet; Tim-3.

Introduction

Hepatitis C virus (HCV), infecting nearly 3% of the world population, has imposed a tremendous burden on global health.[1] Following acute infection, approximately 80% of individuals progress to chronic hepatitis, putting these individuals at risk for developing serious sequelae including liver cirrhosis and/or hepatocellular carcinoma.[2] Notably, robust immune responses are essential for the clearance of acute HCV infection, whereas dysfunction of

Abbreviations: Blimp-1, PR domain zinc-finger protein 1; ERK1/2, extracellular signal-regulated kinase 1/2; HCV, hepatitis C virus; HIV, human immunodeficiency virus; IL-12, interleukin-12; JNK, c-jun N-terminal kinase; LCMV, lymphocytic choriomeningitis virus; M/Mφ, monocyte/macrophages; NFAT2, nuclear factor of activated T cell; p38 MAPK, mitogen-activated protein kinase; PBMC, peripheral blood mononuclear cell; pSTAT-1, phosphorylated signal transducers and activators of transcription 1; SVR, sustained virological response; T-bet, T-box expressed in T cells; Tim-3, T-cell immunoglobulin-and mucin-domain-containing molecule-3; TLR, Toll-like receptor; TNF-α, tumor necrosis factor-α; β-gal, β-galactosidase

innate to adaptive immunity is observed in individuals with chronic HCV infection.

As the first line of protection against pathogenic infections, innate immune cells, such as monocyte/macrophages (M/Mφ), are engaged in phagocytosis, antigen presentation and cytokine production; secretion of tumour necrosis factor-α and interleukin-18 (IL-18) can activate natural killer cells, and ultimately induce an interferon-γ-mediated response. However, individuals infected with HCV exhibit reduced M/Mφ function.[3] As reported in our previous studies, inhibitory receptors such as programmed cell death protein 1 (PD-1) and T-cell immunoglobulin and mucin domain protein-3 (Tim-3) are induced by HCV and can inhibit IL-12 production by M/Mφ.[4–6] The precise mechanism underlying the M/Mφ dysregulation remains unclear, in particular regarding the transcription factors involved in the regulation of the inhibitory receptors of these cells. Given that several transcriptional factors, including PR domain zinc-finger protein 1 (Blimp-1), T-box expressed in T cells (T-bet) and nuclear factor of activated T cell (NFAT2), have been implicated in controlling exhausted T cells during chronic infection,[7] here we investigated their potential roles in monocyte dysregulation in the setting of HCV infection.

T-bet was initially found in T helper type 1 (Th1) cells and later in other cell types, including monocytes and dendritic cells,[8] and could activate both Th1 and Th2 lineage-specific genes.[9] T-bet participates in the differentiation, expansion and cytotoxicity of T-cell[10,11] and antibody production in B cells.[12] It functions by binding to large domains enriched with enhancers[13] and regulating interferon-γ expression in both natural killer and T cells.[14] In HIV elite controllers, T-bet expression is up-regulated in virus-specific $CD8^+$ T cells,[15] and its deficiency is linked to $CD8^+$ T-cell dysfunction in lymphocytic choriomeningitis virus (LCMV) infection.[16] A similar phenomenon is also observed in hepatitis B virus and HCV infection, indicating a critical role of T-bet in a successful CD8 T-cell response against chronic viral infection.[17,18] Although the molecular mechanisms of T-bet are well-studied in T-cell immunity, little is known about such mechanisms in innate immune cells, especially in M/Mφ. As we and others have previously demonstrated, Tim-3 expression on $CD14^+$ M/Mφ is up-regulated by HCV,[4] and T-bet binds directly to the Tim-3 promoter in $CD4^+$ Th1 cells.[19] We therefore postulated that Tim-3 might be a direct transcriptional target of T-bet in M/Mφ. Whether T-bet is an important modulator in M/Mφ exhaustion induced by HCV remains unknown.

In this study, we investigated the regulatory effects of T-bet on Tim-3 expression in M/Mφ during HCV infection. Our results suggest a crucial role for transcription factor T-bet in controlling Tim-3-mediated inhibition of M/Mφ function during chronic HCV infection.

Materials and methods

Subjects

Twenty-five patients with chronic HCV infection and 25 healthy participants were enrolled in this study. The characteristics of the participants including age, gender and HCV viral load are shown in Table 1. All the patients recruited were from Tangdu Hospital (Shaanxi, China), before antiviral therapy. Healthy participants who shared features comparable to the patients with chronic HCV infection were chosen as controls. All participants were negative for HIV, hepatitis B virus infection and other liver diseases. The study was approved by Institutional Review Boards of the Fourth Military Medical University. All participants enrolled have signed a consent form.

Isolation of $CD14^+$ M/Mφ and cell culture

Human peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation using Ficoll–Hypaque (Sigma-Aldrich, St Louis, MO), and then were frozen in fetal bovine serum (FBS; Gibco, Grand Island, NY) containing 10% DMSO. $CD14^+$ M/Mφ were purified from PBMCs using a human CD14 microbeads kit following the manufacturer's instruction (Miltenyi Biotech, Auburn, CA). The M/Mφ purity was $> 90\%$. M/Mφ or monocytic THP-1 cells were cultured with 10% FBS RPMI-1640 containing 2 μg/ml HCV core protein (American Research Products Inc, Grandville, MI, USA) or β-galactosidase (Sigma-Aldrich) for 24 hr, followed by the assays as described below.

Co-culture of M/Mφ with HCV-infected Huh7.5 cells

Plasmid HCV JFH-1 was isolated from *Escherichia coli* by a plasmid miniprep kit (Tiangen Biotech (Beijing) Co., Ltd., Beijing, China), and linearized with *Xba*I (Takara, Otsu, Shiga, Japan). RNA from purified DNA was

Table 1. Characteristics of participants in this study

Group	*n*	Age, years Mean (range)	Gender (male/female)	HCV-RNA (10^6 IU/ml) Mean (range)
Patients with Chronic HCV infection	25	46 (24–62)	9/16	13·8 (0·0381–74·6)
Healthy participants	25	37 (25–53)	7/18	N/A

N/A, not applicable.

obtained using a transcript Aid T7 high-yield transcription kit (Thermo Scientific, Rockford, IL). Huh7.5 cells were transfected with RNA or control in a 24-well plate using DMRIE-C reagent (Invitrogen, San Diego, CA) following the manufacturer's instruction. Cells were collected and stained by anti-HCV core antibody (Abcam, Cambridge, MA), followed by a conjugated secondary antibody, and a fluorescence microscope was applied to observe the intensity. HCV RNA copies were quantified by quantitative PCR from supernatants of the cultures (Qiagen, Hilden, Germany). For co-culture, purified M/Mφ were added to the transfected Huh7.5 cells and collected at different time-points to detect the T-bet expression.

Blockade of gC1qR and signal pathways

Cells from healthy participants (1×10^6 PBMCs) were cultured in RPMI-1640 (ATCC, Manassas, VA) with 10% FBS. Then, 2 μg/ml HCV core protein was incubated with PBMCs in the presence of 2 μg/ml anti-gC1qR (Abcam) or mouse IgG for 24 hr. To identify the signal pathway involved in this process, 20 μM PD98059, 50 μM SP600125, 10 μM SB203580 (Cell Signaling Technology, Beverly, MA) or 1 : 1000 DMSO was added to PBMCs in the presence of 2 μg/ml HCV core protein for 24 hr. The cells were stained with phycoerythrin-conjugated anti-CD14 antibody (BioLegend, San Diego, CA), permeabilized with the Foxp3/Transcription Factor Staining buffer set (eBioscience, San Diego, CA), and incubated with phycoerythrin-Cy7-conjugated anti-T-bet antibody (eBioscience). The isotype controls were used to determine the level of background staining. Fixed samples were detected on a FACS Aria II (BD Biosciences, San Jose, CA) and data were analysed by Flowjo7.6 software (Tree Star, Inc. Ashland, OR, USA).

Silencing T-bet with small interfering RNA

To transiently knock-down T-bet expression, we synthesized small interfering RNA (siRNA) against T-bet 5′-UAGGAGAGGAGAGUAGUGAUCUCCC-3′ [20] and negative control siRNA from GenePharma Co., Ltd. (Shanghai, China). After 1×10^6 THP-1 cells were transfected with 100 pmol siRNA, the efficiency was assessed by flow cytometry and real-time PCR. After 6 hr incubation at 37° in 5% CO_2, the cells were centrifuged and cultured in 2 × concentration of normal medium with 2 μg/ml HCV core, 5 μg/ml lipopolysaccharide (LPS) and 5 μg/ml R848 for another 72 hr, followed by adding Brefeldin A (BioLegend) 6 hr before harvest. Cell surface staining was carried out by using allophycocyanin-conjugated anti-Tim-3 (R&D, Minneapolis, MN), followed by intracellular staining with phycoerythrin-conjugated anti-IL-12 (BD Biosciences) and phycoerythrin-Cy7-conjugated anti-T-bet. The supernatants were collected to measure the level of IL-12p70 by ELISA (eBioscience).

Western blot

THP-1 cells were lysed by RIPA buffer (150 mM NaCl, 50 mM Tris–HCl, 0·5% deoxycholic acid, 0·1% SDS, 1% Triton X-100) containing PhosSTOP (Roche Applied Science, Mannheim, Germany) on ice. The protein concentration was measured, and equal loads of proteins were separated by 10% SDS–PAGE, then transferred onto PVDF membranes (PVDF, Millipore Corp., Bedford, MA, USA), blocked by 3% bovine serum albumin in TBST for 1 hr, incubated with phosphorylated signal transducers and activators of transcription 1 (STAT-1) antibody or STAT-1 antibody (Cell Signaling Technology) at 4° overnight. Horseradish peroxidase-conjugated antibodies (Proteintech Group, Wuhan, China) were added and the protein bands were captured and quantitatively analysed by ChemiDoc XRS (BioRad, Hercules, CA, USA).

Statistical analysis

All the data were analysed using GraphPad Prism (version 5; GraphPad Software Inc., San Diego, CA). Student's *t*-test was used to determine the statistical significance of the differences between groups, and $P < 0{\cdot}05$ was considered to be statistically significant.

Results

T-bet expression is up-regulated and positively correlates with Tim-3 expression in M/Mφ from chronic HCV patients

We have previously shown that Tim-3 is up-regulated and involved in inhibiting M/Mφ functions during HCV infection. The mechanisms involved in regulating its expression and function, however, remain undefined. Recently, it has been reported that T-bet is a transcription factor that controls Tim-3 expression in T cells.[19] To determine whether T-bet also controls Tim-3 expression in M/Mφ, we measured T-bet and Tim-3 expressions in PBMCs isolated from 25 chronically HCV-infected patients and 25 healthy participants by flow cytometry. Representative histogram and summary data of T-bet expression are shown in Fig. 1(a). T-bet is constitutively expressed in resting $CD14^+$ M/Mφ in the peripheral blood. Chronically HCV-infected individuals exhibit a marked and significantly higher level of T-bet expression in M/Mφ, with almost twofold increases compared with healthy participants.

Additionally, we found that Tim-3 is highly expressed in $T\text{-}bet^+$ cells in chronically HCV-infected patients and healthy participants. As shown in Fig. 1(b), both Tim-3 and T-bet expressions in M/Mφ were significantly increased in patients with chronic HCV infection, and up-regulation of T-bet was primarily found in those $Tim\text{-}3^+$ cells. To

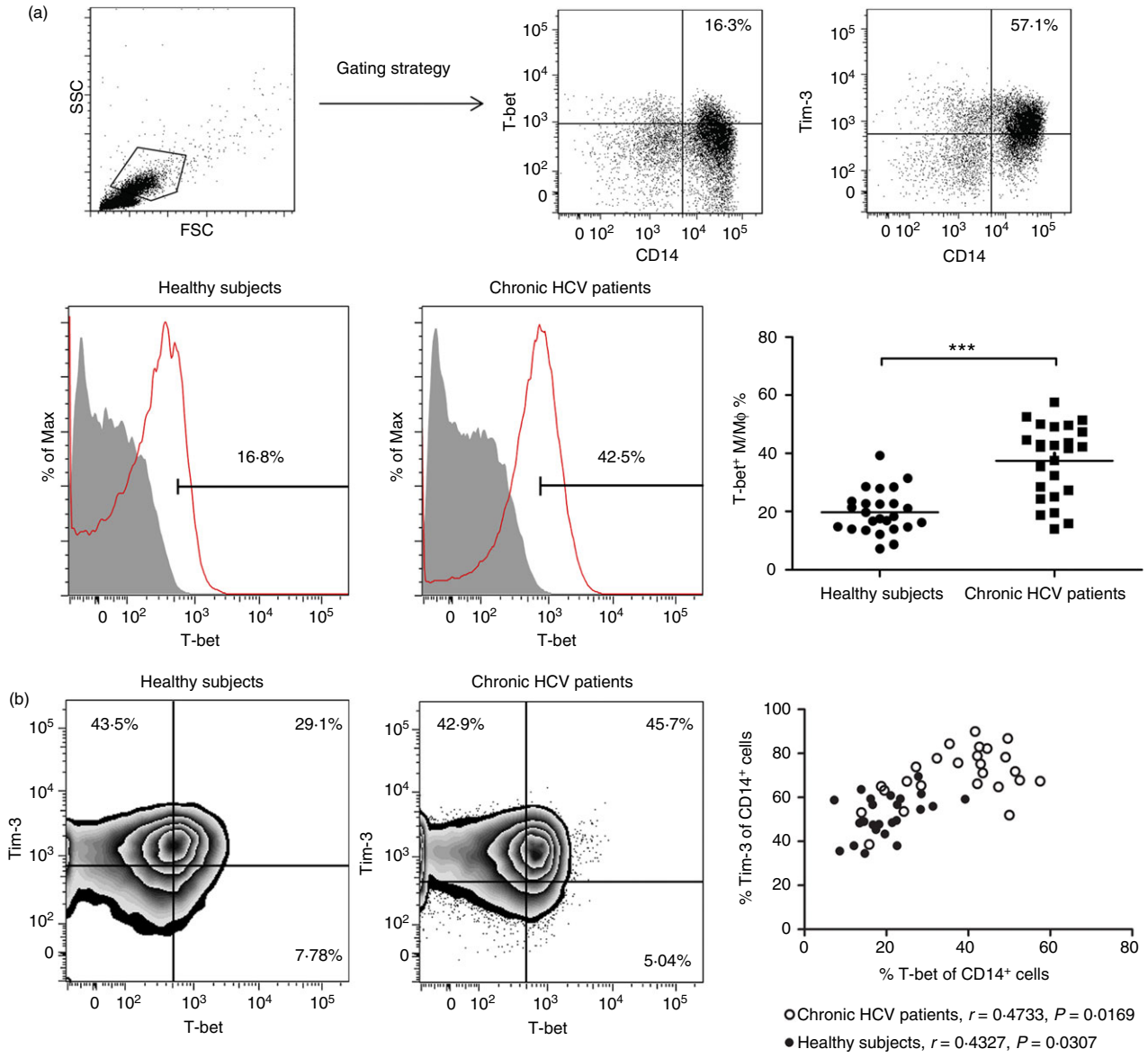

Figure 1. T-bet is up-regulated and positively associated with Tim-3 expression in monocyte/macrophages (M/Mφ) from patients with chronic hepatitis C virus (HCV) infection. (a) Cells were gated and then analysed for the percentage of T-bet and Tim-3. Peripheral blood mononuclear cells (PBMCs) were stained with anti-CD14 and anti-T-bet antibodies, followed by flow cytometric analysis. The representative histograms for the expression of T-bet in CD14$^+$ M/Mφ from HCV patients and healthy participants are displayed, isotype controls (filled grey area) are used to identify the level of background staining. Summary data of each group (n = 25) are shown, and the horizontal bar represents the median value. ***$P < 0{\cdot}001$. (b) Representative dot plots of T-bet$^+$ and Tim-3$^+$ CD14$^+$ M/Mφ, and their correlation are shown. The open circles represent data from chronically HCV-infected patients, and the filled circles represent data from healthy participants.

determine whether the up-regulated T-bet expression in M/Mφ correlated with the Tim-3 expression, PBMCs isolated from HCV-infected patients and healthy participants were stimulated with LPS/R848, then assessed for the expression of T-bet and Tim-3, and analysed by Pearson correlation using SPSS 18 (SPSS Inc., Chicago, IL, USA). Indeed, the up-regulated T-bet in CD14$^+$ M/Mφ was positively associated with Tim-3 expression (chronically HCV infected: $r^2 = 0{\cdot}4733$, $P = 0{\cdot}0169$; healthy participants: $r^2 = 0{\cdot}4327$, $P = 0{\cdot}0307$, respectively). Taken together, these results indicate that T-bet expression is significantly up-regulated, and closely correlates to the elevated level of Tim-3 expression, in M/Mφ from HCV-infected patients.

T-bet is up-regulated in M/Mφ co-cultured with HCV$^+$ hepatocytes

To better understand the mechanisms for T-bet up-regulation by HCV, we employed a newly established HCV culture system *in vitro* to simulate the infected liver

in vivo. To this end, purified JFH-1 plasmids were transfected into Huh7.5 hepatocytes. 48 hr after transfection, HCV core protein was detected by immunofluorescent staining; and HCV RNA was measured in the supernatant of the medium ($1{\cdot}43 \times 10^8$ to $1{\cdot}2 \times 10^9$ IU/ml) by RT-PCR (Fig. 2a). Purified $CD14^+$ M/Mφ were then added into the transfected Huh7.5 cells for 24 hr. As shown in Fig. 2(b), T-bet was significantly up-regulated in M/Mφ incubated with HCV^+ Huh7.5 cells compared with the negative controls. Interestingly, T-bet expression gradually increased following the incubation time, reaching its peak at 44 hr (Fig. 2c). These data suggest that HCV induces

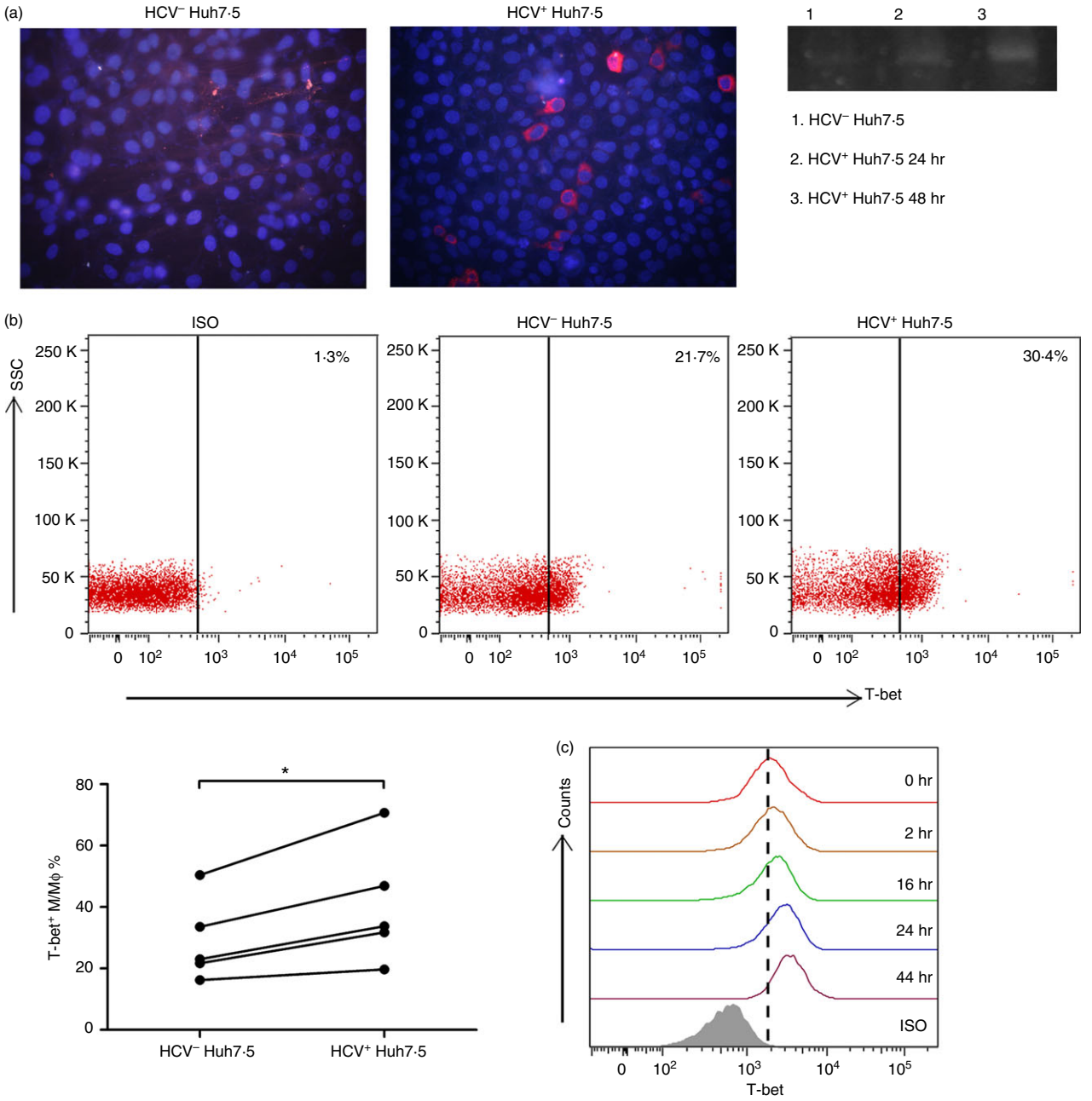

Figure 2. Hepatitis C virus (HCV) induces T-bet expression in $CD14^+$ monocyte/macrophages (M/Mφ) in a time-dependent manner. (a) Huh7.5 cells were transfected with JFH-1 or a control (left panel) plasmid, and HCV core protein (red) was detected by immunofluorescence staining 48 hr after transfection. DAPI was used to visualize cell nuclei. HCV RNA from the culture supernatant was measured by RT-PCR at 24 and 48 hr after transfection (right panel). (b) $CD14^+$ M/Mφ were isolated from healthy participants and incubated with HCV^+ Huh7.5 at different time-points, the expression of T-bet was assessed by flow cytometry. The representative plot of 24 hr after treatment and summary data from five individuals are shown. $^*P < 0{\cdot}05$. (c) Histograms of various time-points indicate the dynamic expression of T-bet. The dashed line is isotype control.

T-bet expression in $CD14^+$ M/Mφ in a time-dependent manner.

HCV core protein induces T-bet expression through interaction with gC1qR

As the first protein to be synthesized upon HCV infection, HCV core protein has been previously shown to be a strong stimulator of inhibitory receptors, such as PD-1 and Tim-3.[4] We postulated that HCV core may potentially up-regulate T-bet expression in $CD14^+$ M/Mφ. To test this hypothesis, purified M/Mφ were treated with HCV core protein. T-bet was induced to a greater extent after 24 hr incubation from baseline in comparison with the control protein β-galactosidase (Fig. 3a). Similar results were also found using PBMCs or THP-1 cells treated with HCV core or control protein (Fig. 3b), indicating that HCV core could up-regulate T-bet expression in multiple contexts.

As reported in previous studies,[21–23] the complement receptor gC1qR is widely expressed on immune cells and is a receptor for HCV core protein. HCV core protein regulates T and B lymphocytes and inhibits monocyte IL-12 production through interaction with gC1qR expressed on these cells.[4] Here we investigated whether gC1qR was employed by HCV core to regulate T-bet expression in

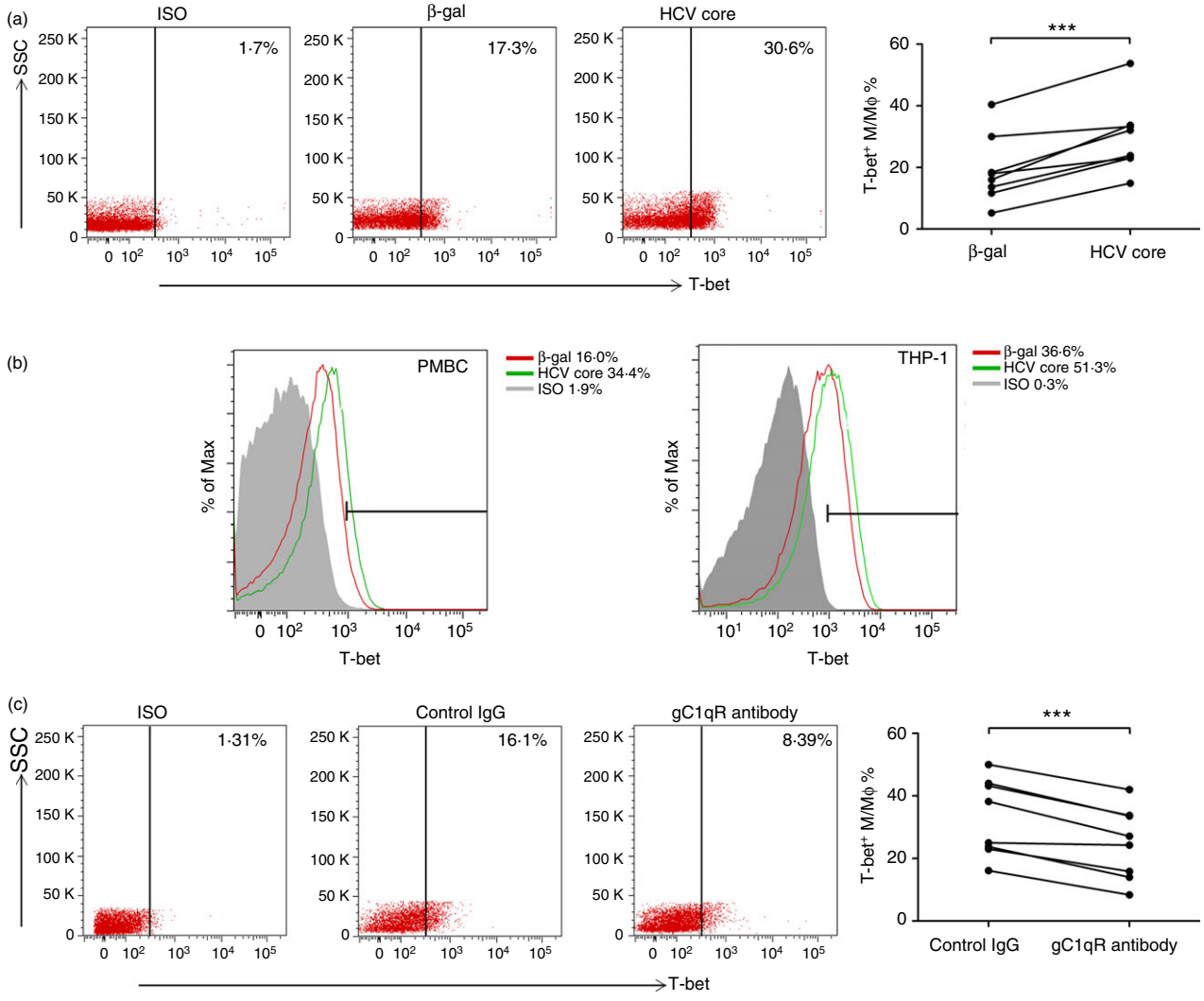

Figure 3. Hepatitis C virus (HCV) core up-regulates T-bet through interaction with gC1qR in monocytes. (a) Monocyte/macrophages (M/Mφ) from healthy participants were treated with HCV core protein or β-galactosidase (β-gal; 2 μg/ml) for 24 hr, and T-bet expression was assessed by flow cytometry. Representative flow cytometric dot plots measuring T-bet expression are shown (left panel). Right panel shows the summary data from different healthy participants tested comparing the effect of HCV core to β-gal control ***$P < 0{\cdot}001$. (b) Histogram of T-bet expression in $CD14^+$ cells from peripheral blood mononuclear cells (PBMC) (left panel) and in THP-1 cells (right panel) after HCV core and β-gal treatment. (c) PBMCs from healthy participants were treated with HCV core protein in the presence of gC1qR antibody or a control IgG for 24 hr. T-bet expression was detected by flow cytometry. ***$P < 0{\cdot}001$.

monocytes. To this end, PBMCs from healthy controls were treated with HCV core protein in the presence of gC1qR antibody, or control IgG. As shown in Fig. 3(c), HCV core-induced T-bet expression was significantly inhibited in $CD14^+$ M/Mφ when gC1qR was blocked, suggesting that gC1qR is required for up-regulating T-bet expression by HCV core protein.

c-Jun N-terminal kinase signalling participates in up-regulating T-bet and Tim-3 expression induced by HCV core

Although HCV core protein has been shown to regulate various pathways, including extracellular signal-regulated kinase 1/2 (ERK), c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (p38 MAPK),[24–26] it remains unclear whether these signalling pathways are involved in T-bet up-regulation by HCV core protein. To investigate the role of these signalling pathways in HCV core-induced T-bet expression, we employed specific inhibitors for these signalling pathways in our experimental system. To this end, T-bet expression was measured by flow cytometry after healthy PBMCs were treated with MAPK kinase (MEK) inhibitor PD98059, JNK inhibitor SP600125, p38 MAPK inhibitor SB203580, or 1/1000 DMSO, following 24 hr exposure to HCV core protein. As shown in Fig. 4(a), there was no significant difference in T-bet expression among the treatments when compared with the DMSO control, except in those cells treated with the JNK inhibitor SP600125, which exhibited an obvious decrease in T-bet expression. Moreover, Tim-3 expression was also significantly suppressed with this treatment (Fig. 4b), especially in $T\text{-bet}^+$ M/Mφ (Fig. 4c), suggesting that when the JNK pathway is suppressed, HCV core protein cannot promote Tim-3 and T-bet expression. We therefore demonstrate, for the first time, an important role for the JNK pathway in regulating T-bet and Tim-3 expression by HCV core protein.

T-bet knockdown decreases Tim-3 expression and improves IL-12 production and STAT-1 phosphorylation in THP-1 cells

To further explore the role of T-bet in Tim-3 expression, THP-1 cells were transfected with siRNA specific for T-bet or a negative control for 6 hr, and then treated with HCV core protein and LPS/R848 for another 72 hr. The efficacy of T-bet knockdown is shown in Fig. 5(a). The expression of T-bet was greatly silenced at both the RNA and protein levels. We measured Tim-3 expression and IL-12 production in THP-1 cells after T-bet silencing and LPS/R848/core treatment for 72 hr. As shown in

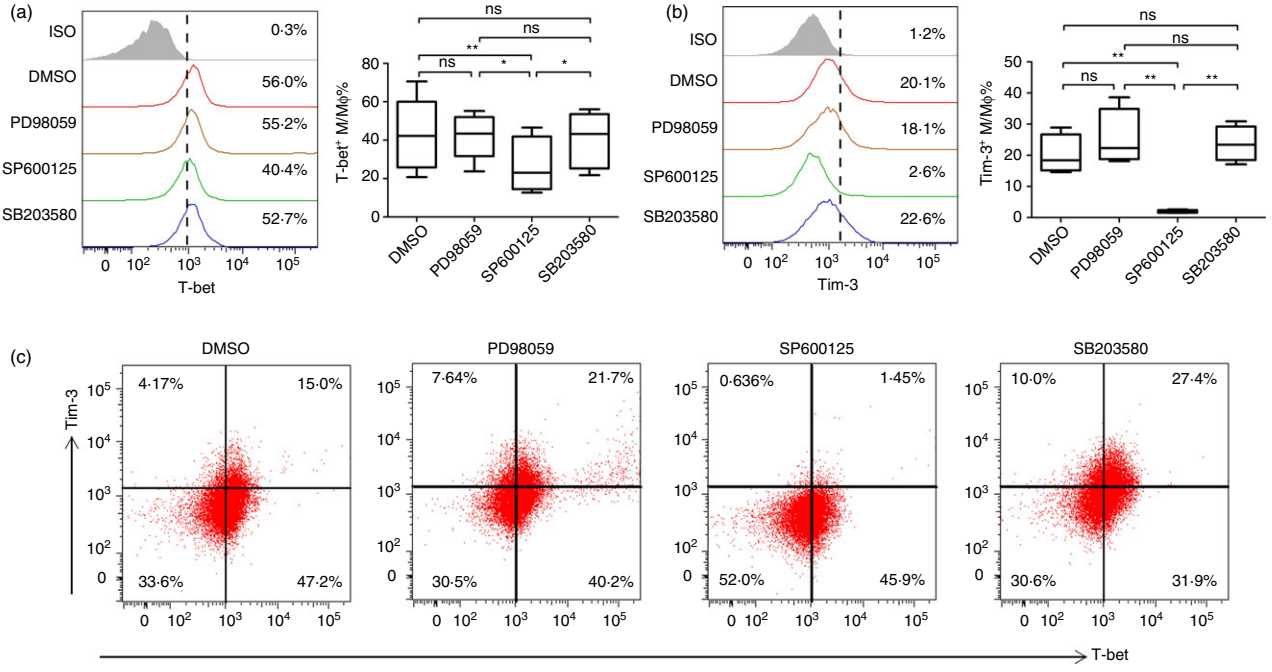

Figure 4. T-bet expression induced by hepatitis C virus (HCV) core requires activation of the c-Jun N-terminal kinase (JNK) pathway. (a–b) Peripheral blood mononuclear cells (PBMCs) from healthy participants were treated with HCV core in the presence of mitogen-activate protein kinase (MAPK) kinase 1/2 (MEK1/2) inhibitor PD98059, JNK inhibitor SP600125, p38 MAPK inhibitor SB203580 or DMSO (1 : 1000), respectively, for 24 hr. T-bet (a) and Tim-3 (b) expressions and summary data in monocyte/macrophages (M/Mφ) are shown. $*P < 0{\cdot}05$, $**P < 0{\cdot}05$, NS, not significant. (c) Representative dot plots for T-bet and Tim-3 expressions were detected in $CD14^+$ M/Mφ treated with HCV core in the presence of specific inhibitors for different signal pathways.

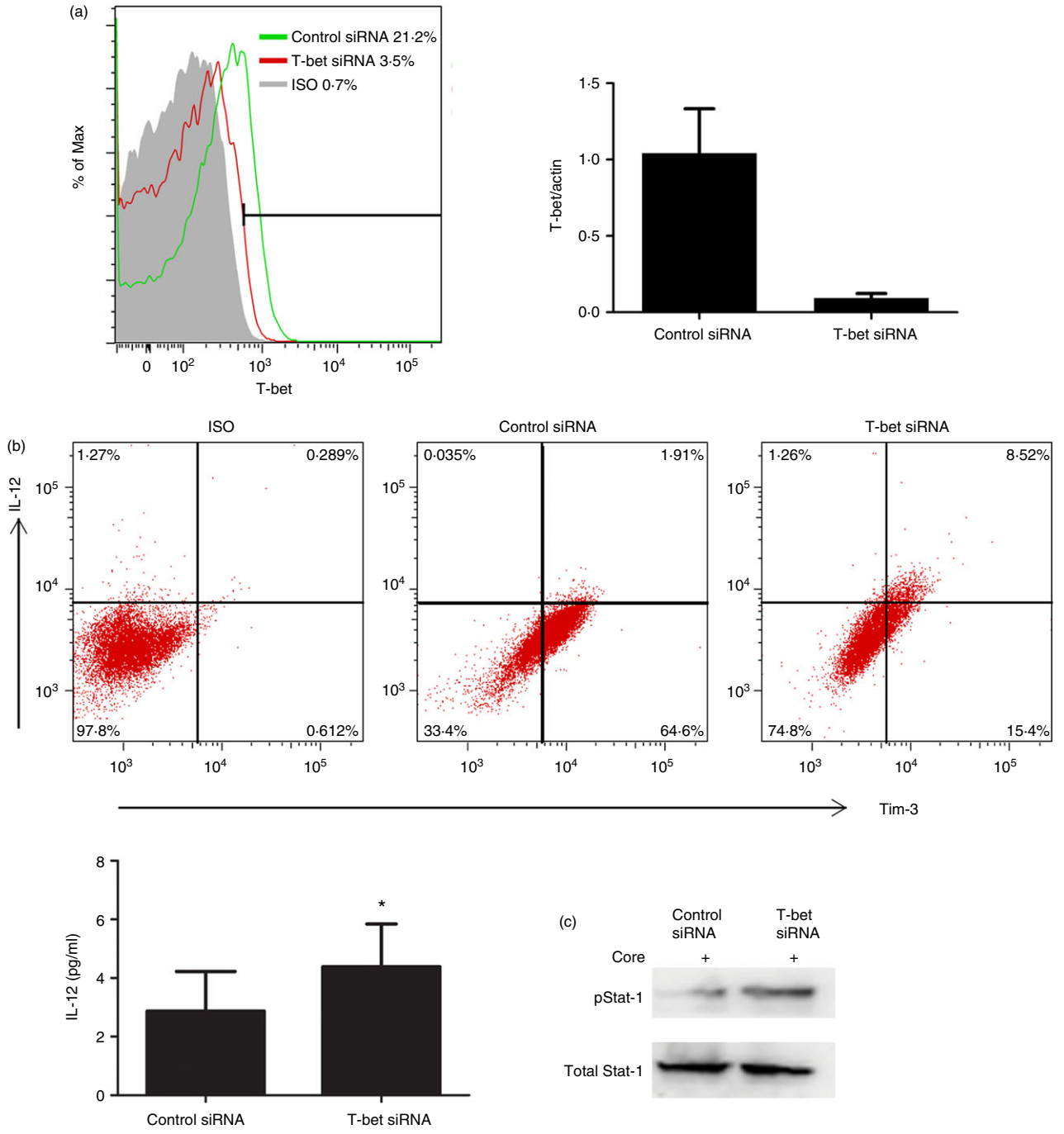

Figure 5. T-bet knockdown regulates hepatitis C virus (HCV) core-induced Tim-3 and interleukin-12 (IL-12) expression in THP-1 cells. (a) THP-1 cells were transfected with T-bet small interfering RNA (siRNA) or a control siRNA for 6 hr, and stimulated with lipopolysaccharide (LPS)/R848 and HCV core for another 72 hr. T-bet expression was measured by both flow cytometry (left panel) and RT-PCR (right panel). (b) Representative dot plots showing Tim-3 and IL-12 expressions in THP-1 cells transfected with T-bet siRNA and control siRNA, respectively. (c) IL-12 levels (left panel) were measured by ELISA from the culture supernatant, and the phosphorylation of signal transducer and activator of transcription 1 (STAT-1) (right panel) was assessed by Western blot.

Fig. 5(b), compared with the control siRNA, Tim-3 expression is significantly suppressed in THP-1 cells with T-bet knockdown. Functionally, transfection of T-bet siRNA significantly abrogated HCV core-mediated IL-12 suppression when compared with cells transfected with control siRNA. In addition, silencing T-bet expression also rescued HCV core-induced STAT-1 dephosphorylation (Fig. 5c). Collectively, these data suggest that T-bet

is essential in HCV-induced M/Mφ dysfunction, as silencing T-bet gene expression significantly decreases Tim-3 expression and enhances IL-12 secretion as well as STAT-1 phosphorylation.

Discussion

Hepatitis C virus is characterized by its ability to establish persistent infection in the majority of infected individuals. The high rate of escape from host immunity relies on several strategies, including viral mutation and immune exhaustion. Monocytes are the first line of antiviral response and play an important role in presenting antigens and cooperating with effector T cells to clear acute viral infection. However, monocytes are profoundly dysfunctional during chronic viral infection, a dysfunction induced by the elevated expression of negative regulators such as PD-1, Tim-3 and SOCS-1. This results in diminished production of IL-12, a key cytokine in the effective transition of innate to adaptive immune responses.[4] Our previous study has demonstrated the role of Tim-3 in monocyte dysfunction, but the transcription factor that controls Tim-3 expression and the mediators participating in the signalling transmission remained poorly defined.

In this study, we explored the regulatory role of T-bet on Tim-3 expression in $CD14^+$ monocytes during chronic HCV infection. We found that T-bet was significantly up-regulated and positively correlated with the level of Tim-3 expression in monocytes from HCV-infected individuals. Up-regulation of T-bet was also observed in $CD14^+$ M/Mφ incubated with HCV^+ Huh7.5 cells as well as in primary M/Mφ or monocytic THP-1 cells exposed to HCV core protein *in vitro*, and this was reversible by blocking HCV core/gC1qR interactions. Moreover, the HCV core-induced up-regulation of T-bet and Tim-3 expression in M/Mφ can be abrogated by incubating cells with SP600125 – an inhibitor of the JNK signalling pathway. Importantly, silencing T-bet gene expression decreases Tim-3 expression and enhances IL-12 secretion as well as STAT-1 phosphorylation.

T-bet was initially found in Th1 cells;[19] but was recently found to be widely expressed in $CD8^+$ T cells, B cells, natural killer cells, dendritic cells and monocytes[12,16,27–29] and participates in secretion of interferons by dendritic cells,[30] promoting development of Th1 cells,[31] and preventing Th17 cell differentiation.[32] However, its function in regulating innate immunity, especially monocyte function, is still unclear. Here, we found that T-bet expression in $CD14^+$ M/Mφ of patients with chronic HCV infection is significantly up-regulated compared with healthy participants. The up-regulation of T-bet positively correlated with Tim-3 expression in the study participants. In addition, by employing the HCV cell culture system, we demonstrated that T-bet expression in M/Mφ was induced by HCV^+ Huh7.5 in a time-dependent pattern. The similar phenomenon was observed in $CD4^+$ T cells, dendritic cells from mouse[19] and natural killer cells,[33] establishing a universal relationship between the transcriptional factor T-bet and the inhibitory receptor Tim-3. In addition, T-bet is expressed selectively in Tim-3^+ $CD8^+$ T cells in HIV-infected individuals.[34] However, T-bet mRNA (but not protein level) is negatively associated with the number of Tim-3^+ T cells in hepatitis B virus infection.[35] Whether different cell types or viral infections contribute to this disparity should be further explored.

Our data illustrate that HCV core protein and its receptor, gC1qR, are pivotal elements in promoting T-bet expression in M/Mφ through the JNK pathway. It has been reported that HCV core protein can up-regulate inhibitory receptors and cell transformation in several cell types.[36,37] It can be secreted into peripheral blood in

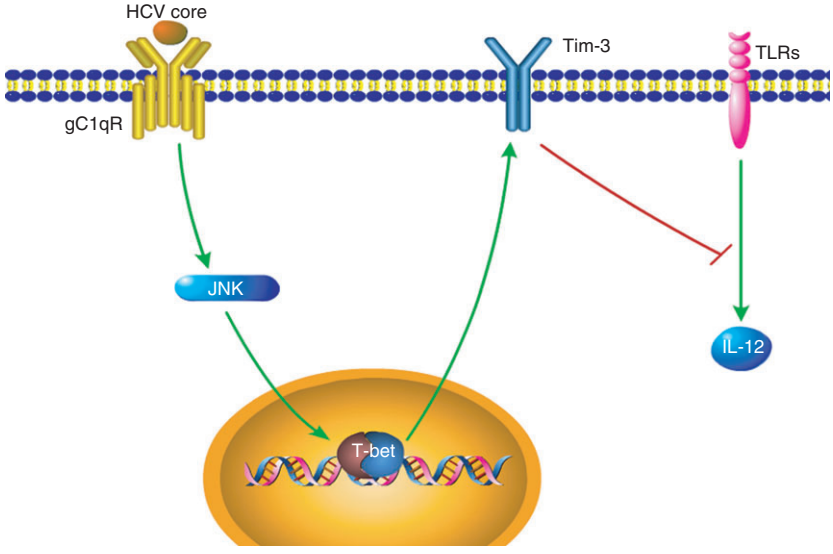

Figure 6. Schematic model for hepatitis C virus (HCV) core/gC1qR interaction-induced T-bet/Tim-3 expression via the c-Jun N-terminal kinase (JNK) pathway. Our results indicate that HCV core/gC1qR interaction induces T-bet and Tim-3 expression in monocyte/macrophages (M/Mφ). Blocking the JNK pathway may abrogate the HCV core/gC1qR-induced up-regulation of T-bet and Tim-3 expressions in these cells. T-bet silencing significantly reduces the HCV core-induced Tim-3 expression, and increases interleukin-12 (IL-12) expression, suggesting that T-bet is an essential transcriptional factor controlling Tim-3-mediated inhibition of M/Mφ functions.

HCV-infected patients,[38] and so could be a strong inducer for circulating monocytes. For the first time, we confirmed that HCV core protein is a stimulator of T-bet, not only in $CD14^+$ M/Mφ, but also in monocytic THP-1 cells. In addition, HCV core protein can regulate several signalling pathways that include ERK, JNK and p38 MAPK.[39,40] Notably, via the JNK and MEK1/2 pathways, HIV infectivity is enhanced by adding HCV core protein.[26] Here we reveal that the JNK pathway, rather than the p38 MAPK or ERK1/2 pathways, is involved in T-bet and Tim-3 up-regulation by HCV core protein. Interestingly, Tim-3 expression is all but totally inhibited when T-bet is suppressed by a JNK inhibitor. There is still the possibility that other molecules may also participate in Tim-3 regulation beyond T-bet. For example, we have recently reported that miR-155 is required for Tim-3 expression in natural killer cells during HCV infection;[33] whether miR-155 is involved in regulating T-bet/Tim-3 expression in M/Mφ is under investigation in our laboratory.

By knocking down T-bet in THP-1 cells, we found that HCV core-induced Tim-3 expression is significantly suppressed. Meanwhile, HCV core-mediated inhibition of IL-12 production and STAT-1 phosphorylation are rescued, indicating a negative regulatory role for T-bet in monocytic function. In conjunction with our previous studies, we believe that T-bet is an upstream regulator of Tim-3 that impairs IL-12 production during HCV infection. T-bet could directly bind to the promoter of Tim-3 to activate its expression.[19] It is reported that T-bet is highly expressed in CMV-specific T cells that sustain an exhausted phenotype.[11,6]

Although we conclude that T-bet may be a critical factor contributing to innate immune dysregulation in persistent infections, the situation may change depending on the context. For example, T-bet positively regulates $CD4^+$ T-cell function, but contributes to maintaining $CD8^+$ T-cell exhaustion during LCMV infection.[16,41] During acute LCMV infection, increased T-bet facilitates terminal differentiation of $CD8^+$ T cells, partly determines spontaneous resolution, and decreases when persistent infection establishes in response to constant antigenic stimulation.[16] However, when infected with HIV, enhanced T-bet may facilitate the expression of perforin and granzyme B in HIV-specific $CD8^+$ T cells.[15]

In conclusion, this study describes a crucial role for the transcription factor T-bet in controlling Tim-3-mediated inhibition of M/Mφ function during chronic HCV infection. HCV appears to hijack inherently inhibitory receptors by enhancing T-bet and Tim-3 expressions to facilitate persistent infection. We propose a schematic model, shown in Fig. 6, in which T-bet is induced by HCV core/gC1qR interaction via the JNK pathway and enhances Tim-3 expression, leading to dampened M/Mφ function during chronic HCV infection. Indeed, T-bet-dependent Tim-3 up-regulation is found in both innate and adaptive immune cells and plays a crucial role in dampening host immune responses. Although the precise mechanisms for T-bet-mediated Tim-3 suppression have yet to be fully elucidated, the identification of the relationship between T-bet and Tim-3 expression and its function in innate immune regulation in a human disease model reveals novel immunotherapeutic targets for HCV and other chronic viral infections.

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Disclosures

The authors declared that no competing interests exist.

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