Cytokine production by innate immunity is critical for shaping the adaptive immunity through regulation of T cell differentiation. In this report, we studied T cell immunoglobulin mucin domain protein 3 (Tim-3) expression on monocytes and its regulatory effect on interleukin-12 (IL-12)/IL-23 production by CD14+ monocytes, as well as IL-17 production by CD4+ T cells in individuals with chronic hepatitis C virus (HCV) infection. We found that Tim-3 and IL-23p19 are highly expressed and that IL-12p35 is inhibited in human CD14+ monocytes, while IL-17 expression is upregulated in CD4+ T cells, in chronically HCV-infected individuals compared to healthy subjects. Interestingly, Tim-3 expression is closely associated with the differential regulation of IL-12/IL-23 expression in CD14+ monocytes and correlated to IL-17 production by CD4+ T cells. These Tim-3-associated IL-12/IL-23/IL-17 dysregulations in HCV-infected individuals are also recapitulated in vitro by incubating healthy monocytes or peripheral blood mononuclear cells with HuH-7 hepatoma cells transfected with HCV RNA. Importantly, blocking Tim-3 signaling on monocytes restores the balance of IL-12/IL-23 through the intracellular STAT3 signaling, which in turn reverses the upregulated IL-17 expression both ex vivo and in vitro. Our findings suggest that Tim-3-mediated differential regulation of IL-12/IL-23 drives TH17 cell development, a milieu favoring viral persistence and autoimmune phenomenon during HCV infection.
that a therapeutic strategy targeting this inhibitory pathway might be of clinical benefit, especially for HCV/HIV infection (26–28). While Tim-3 has been identified as an inhibitory receptor preferentially expressed on exhausted T cells (24–28), its role in monocyte/macrophage (M/MΦ) modulation remains less well understood.

We have previously demonstrated that Tim-3 is upregulated on M/MΦ and negatively regulates IL-12 expression during HCV infection (20, 21). The role of Tim-3 expression on M/MΦ in controlling the IL-12/IL-23 balance, which in turn may affect Th17 differentiation during HCV infection, remains largely unknown.

In the present study, we assessed Tim-3 expression on M/MΦ and its regulatory effect on IL-12 and IL-23 expression, as well as T cell IL-17 expression, in individuals with HCV infection. We provide pilot evidence suggesting that Tim-3-mediated differential regulation of IL-12/IL-23 promotes Th17 cell development. Since deficiency of IL-12 is crucial to impaired T cell responses against intruding pathogens and may facilitate chronic infection, whereas excess IL-23 is associated with autoimmune disorders and contributes to Th17, as well as regulatory Treg development, the present study, demonstrating Tim-3-mediated differential regulation of inflammatory cytokines, suggests the formation of a milieu that favors the viral persistence and autoimmune phenomenon observed in the setting of chronic HCV infection.

MATERIALS AND METHODS

Subjects. The study protocol was approved by an institutional review board at East Tennessee State University and James H. Quillen VA Medical Center (ETSU/VA IRB, Johnson City, TN), which has contributed to a database for the storage of blood samples from HCV-infected individuals for the purpose of viral immunology studies. The study subjects are composed of two populations: 60 HCV-infected subjects and 16 healthy subjects. Written informed consent was obtained from all participants. HCV genotype (70% type 1, 30% type 2 or 3) and viral load (ranging from 12,300 to 500,000 IU/ml) were performed by Lexington VAMC, and all subjects were virologically and serologically positive for HCV, prior to the antiviral treatment. Healthy subjects are negative for HBV, HCV, and HIV infections. The majority of the study subjects are male. The mean age of HCV-infected individuals is comparable to healthy subjects (P > 0.05).

Cell isolation and culture. Human peripheral blood mononuclear cells (PBMCs) were isolated from the peripheral blood of study subjects by Ficoll density centrifugation with Lympho-H (Atlanta Biological, Lawrenceville, GA). CD14+ monocytes and CD4+ T cells were purified from PBMCs by magnetic beads with positive selection according to the manufacturer’s instructions (purity, >95%; Miltenyi Biotec, Inc., Auburn, CA). The purified cells were cultured with RPMI 1640, containing 10% fetal bovine serum (Life Technologies, Gaithersburg, MD), 100 mg of penicillin-streptomycin (Thermo Scientific, Logan, UT)/ml, and 2 mM L-glutamine (Thermo Scientific) at 37°C with 5% CO2 atmosphere for the following experiments.

Flow cytometry. To determine which Toll-like receptor (TLR) is critical to regulate IL-12/IL-23 production and Th17 development during HCV infection, we determined intracellular IL-12 and IL-23 expression by CD14+ monocytes and IL-17 expression by CD4+ T cells stimulated with specific TLR ligands. Specifically, PBMCs isolated from HCV patients were stimulated (6 and 18 h) with 2 μg of peptideycgon/l (E. coli strain O111:B4 PGN; InvivoGen, San Diego, CA) for TLR2, 2 μg of poly(I:C) (Amersham Pharmacia, Minneapolis, MN)/ml for TLR3, 1 μg of lipopolysaccharide (LPS; BD Pharmingen)/ml for TLR4, 20 ng of flagellin (rec-FLA-ST; InvivoGen)/ml for TLR5, 2.5 μg of R848 (InvivoGen)/ml for TLR7/8, or 20 μg of ODN2395 (InvivoGen)/ml for TLR9. PBMCs were also stimulated with 100 ng of phorbol 12-myristate 13-acetate (PMA)/ml and 1 μg of ionomycin mitogens (InvivoGen)/ml, followed by flow cytometry analysis. IL-12/IL-23 expression was detected in CD14+ monocytes with PBMCs stimulated with TLR4/7/8 and PMA/ionomycin (levels high at 6 h and low at 18 h), and IL-23 was also detected by TLR2 stimulation, whereas Th17 cells were only detectable with PBMCs stimulated with PMA/ionomycin at 6 h. Annexin V/PI apoptosis staining of the purified CD14+ monocytes and CD4+ T cells stimulated with LPS/R848 or PMA/ionomycin for 6 h exhibits slightly increased annexin v (Av) expression, but no significant dead cells within 6 h stimulation. Therefore, in the following experiments, PBMCs or purified CD14+ monocytes were stimulated by 1 μg of TLR4 ligand LPS/ml and 2.5 μg of TLR 7/8 ligand R848/ml for 6 h. Brefeldin A (BioLegend, San Diego, CA) was added 5 h prior to harvesting the cells, inhibiting cytokine secretion. PBMCs or CD4+ T cells were stimulated by 100 ng of PMA/ml and 1 μg of ionomycin/ml for 6 h, with brefeldin A added 5 h prior to harvest the cells. The use of specific antibody direct conjugates for cell surface staining was carried out using Tim-3-APC (R&D, Minneapolis, MN), CD4-APC or CD14-FITC (Miltenyi Biotec), followed by intracellular staining for IL-12p35-APC (R&D), IL-23p19-PE (eBioscience), IL-17A-PE (eBioscience), or pSTAT3-perCP (BD Pharmingen). The intracellular cytokine staining was carried out using Inside Stain kit (Miltenyi Biotec) according to the manufacturer’s instructions. Isotype-matched control antibodies (eBioscience) and fluorescence minus one (FMO) controls were used to determine background levels of staining and adjust multicolor compensation as gating strategy. The cells were analyzed on a FACS Calibur flow cytometer (BD, Franklin Lakes, NJ) and FlowJo software.

Healthy CD14+ monocytes or PBMCs cocultured with HCV+/− Huh-7 hepatocytes. Transfection of Huh-7 hepatocytes (kindly provided by T. J. Liang, Liver Section, National Institutes of Health [NIH]/National Institute of Diabetes and Digestive and Kidney Diseases [NIDDK]) with HCV JFH-1 strain (kindly provided by T. Wakita) was carried out as described previously (20, 21). For coculture experiments, HCV+/− Huh-7 hepatocytes were starved for 18 h and then activated with rhIFN-Y (0.1 μg/ml; R&D Systems) for 48 h. Activated hepatocytes were removed from plates by 0.05% trypsin-EDTA and then plated at 5 × 10^5 cells/well in a 12-well plate. Purified healthy CD14+ monocytes, CD4+ T cells, or peripheral blood mononuclear cells (PBMCs) were added to the adherent hepatocytes in RPMI media, cocultured for another 72 h in the presence or absence of specific inhibitor or blockers, stimulated with LPS/R848 or PMA/ionomycin for 6 h, and then analyzed by flow cytometry or Western blotting.

Western blot analyses. Purified CD14+ monocytes from patients or healthy monocytes cocultured with HCV+/− hepatocytes were treated as described above, and the expressions of phosphorylated and total STAT3 were measured by Western blotting. The cells were lysed in 1× radioimmunoprecipitation assay lysis buffer (Boston BioProducts, Inc., Ashland, MA) supplied with protease inhibitors/phosphorylase inhibitors (Thermo Scientific, Rockford, IL) and EDTA on ice. Cell lysates were centrifuged for 15 min at 4°C, and the protein concentrations were measured. Protein samples were thereafter combined with 4× Laemmli sample buffer (Boston BioProducts, Ashland, MA), denatured, and separated by SDS-PAGE. After transfer to an Amersham Hybond-P membrane (GE Healthcare, Piscataway, NJ), the membrane was blocked and probed with phospho-STAT3 (Tyr705) or total STAT3 antibody (Cell Signaling Technology, Inc., Danvers, MA) at 4°C overnight. Finally, the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody (Millipore, Temecula, CA) and developed by using Amersham ECL Plus Western blot detection reagents (GE Healthcare Biosciences, Pittsburgh, PA) on Kodak X-Omat-LS X-ray film (Sigma-Aldrich, St. Louis, MO).

Tim-3 blocker or STAT3 inhibitor. Purified CD14+ cells or PBMCs were incubated with LEAF anti-human Tim-3 antibody (10 μg/ml; BioLegend) or control IgG for 72 h, followed by stimulation with LPS/R848 for 6 h or PMA/ionomycin for 6 h, and then subjected to flow cytometric analysis of IL-12p35, IL-23p19, IL-17A, or pSTAT3 expression as described above. CD14+ cells were also cocultured with HCV-expressing hepatocytes in complete RPMI medium, with or without 100 μM STAT3-specific inhibitor S3I-201 (NSC 74859; Sigma-Aldrich, St. Louis, MO).
RESULTS

Tim-3 overexpressed on CD14⁺ monocyte is associated with downregulation of IL-12p35 expression and upregulation of IL-23p19 expression in patients with chronic HCV infection. As an initial approach to investigate the role of Tim-3 in M/Mφ regulation, Tim-3 expression, along with intracellular IL-12p35 and IL-23p19 production, in resting and TLR-stimulated monocytes derived from chronically HCV-infected patients and healthy subjects was examined by flow cytometry. The representative dot plots and summary data of Tim-3, IL-12p35, and IL-23p19 expression by CD14⁺ monocytes of healthy subjects (HS; n = 16) and HCV-infected individuals (HCV; n = 40) are shown in Fig. 1. Notably, chronically HCV-infected individuals exhibit significantly elevated Tim-3 expression, not only in the frequency of Tim-3 expression on CD14⁺ cells but also in the level of Tim-3 mean fluorescence intensity (MFI) per CD14⁺ cell, compared to those in HS (Fig. 1A). As shown previously (20, 21), with stimulation of purified CD14⁺ monocytes by LPS/R848 for 6 h, Tim-3 expression rapidly declined, accompanied by an increase of IL-12 and IL-23 expression by activated monocytes. Notably, IL-12p35 is downregulated, whereas IL-23p19 is upregulated, in response to LPS/R848 stimulation of CD14⁺ monocytes in individuals with chronic HCV infection compared to HS (Fig. 1B). The increase in IL-23p19-positive cells observed in HCV infection over HS holds true upon analysis of the MFI of IL-23 expression levels in CD14⁺ monocytes, whereas no significant difference in IL-12p35 MFI level is detected between HCV and HS (data not shown). With Pearson correlation analysis, Tim-3 expression was found to be inversely associated with monocyte IL-12p35 inhibition (Fig. 1C) and positively correlated with monocyte IL-23p19 expression (Fig. 1D), suggesting that Tim-3 may function as a marker or regulator for controlling IL-12/IL-23 expression.

Tim-3-associated differential regulation of IL-12/IL-23 expression by monocytes is correlated with an increase of IL-17-producing T H17 cells in HCV infection. IL-12 and IL-23 share a chain, but their production in response to pathogens is differentially regulated, and their functions are distinct and often antithetical (6–9). IL-12 is involved in the induction or amplification of the T H1 response, whereas IL-23 has been associated with the generation of the T H17 response (5–10). To determine whether the observed differential regulation of IL-12/IL-23 may lead to the generation of IL-17-producing T helper cells (T H17) during HCV infection, PBMCs isolated from chronically HCV-infected individuals and HS were stimulated with PMA/ionomycin, and the prevalence of T H17 CD4⁺ T cells was assessed by flow cytometry. As shown in Fig. 2A, individuals with chronic HCV infection exhibit significantly higher frequencies of IL-17⁺ cells in CD4⁺ T cell populations than HS. In addition, the IL-17 expression level (MFI) per CD4⁺ T cells is also higher in HCV-infected patients versus HS. Importantly, the increased frequency of IL-17⁺ CD4⁺ cells positively correlated with the ratio of IL-23/IL-12 productions (Fig. 2B), as well as Tim-3 expression by CD4⁺ monocytes (Fig. 2C), suggesting that Tim-3-associated differential regulation of IL-12/IL-23 might contribute to the development of T H17 cells during HCV infection.

Tim-3 blockade on monocytes upregulates IL-12 expression, downregulates IL-23 expression, and inhibits IL-17 expression in HCV-infected individuals. The observed Tim-3 expression and differential regulation of IL-12, IL-23, and IL-17 production might be a concurrent but unrelated phenomenon during HCV infection. To determine the role of Tim-3 on IL-12, IL-23, and IL-17 expression, we isolated CD14⁺ monocytes, CD4⁺ T cells, or PBMCs from 16 chronically HCV-infected individuals and 16 HS, incubated ex vivo with anti-Tim-3 (αTim-3) or a control antibody (IgG) for 72 h and then stimulated with LPS/R848 or PMA/ionomycin for 6 h, followed by detecting IL-12/IL-23 and IL-17 expression by flow cytometry. As shown in Fig. 3A, representative dot plots and summary data of IL-12p35 versus IL-23p19 expression in CD14⁺ monocytes, blocking Tim-3 signaling significantly improves IL-12 expression yet inhibits IL-23 expression by monocytes in HCV-infected individuals, but not in HS. We also examined Tim-3 expression and its blockade on IL-17 production in purified CD4⁺ T cells. As shown in Fig. 3B, IL-17 is primarily produced by Tim-3⁻ T H17 cells, and blocking Tim-3 on CD4⁺ T cells enhances IL-17 production, suggesting a directly inhibitory role of Tim-3 expression on IL-17 expression in T H17 cells. Given its negative signaling on IL-17-producing CD4⁺ T cells, Tim-3 blockade on purified T cells would result in a decrease in Tim-3⁻ but increase in Tim-3⁺ cells, likely through regulating cell apoptosis and proliferation (as we have reported in T cell regulation [36]), thus leading to enhanced IL-17 secretion.

We then sought to determine whether the Tim-3 signaling on monocytes that alters the IL-12/IL-23 balance may indirectly affect IL-17 expression by T H17 cells. To this end, we first depleted CD14⁺ monocytes from PBMCs and then carried out a Tim-3 blocking experiment in monocyte-free PBMCs, followed by the detection of T H17 cells. Interestingly, we observed enhanced IL-17-producing CD4⁺ T cells by Tim-3 blockade (Fig. 3C), similar to the results seen in purified CD4⁺ T cells upon Tim-3 blockade, suggesting that Tim-3 signaling on cell populations free of monocytes (including NK cells, B cells, and T cells) has an overall inhibitory effect on IL-17 production. To further address the role of Tim-3 on monocyte IL-12/IL-23 expression, which in turn, may affect T H17 cell differentiation, we next carried out the Tim-3 blocking experiment in purified CD14⁺ monocytes for 72 h and then changed the culture medium containing blocking antibody and secreted cytokines (such as IL-12/IL-23) and added homogeneous CD4⁺ T cells, followed by PMA/ionomycin stimulation for 6 h. We observed little IL-17-producing T H17 cells in this experimental setting (data not shown), supporting the notion that IL-23 might be necessary for T H17 cell development or survival.

We thus inhibited Tim-3 signaling in whole PBMCs from HCV-infected individuals (n = 16) or HS (n = 16) to determine the overall effect of blockade on IL-17 production by T H17 cells. As shown in Fig. 3D, representative dot plots and summary data of IL-17A expression in CD4⁺ T cells, blocking Tim-3 signaling in PBMCs ex vivo for 72 h, followed by PMA/ionomycin stimulation for 6 h significantly reduces the number of T H17 cells upregulated
during chronic HCV infection, but not in HS. These results, in conjunction with the above data, further reinforce the role of Tim-3-mediated differential regulation of IL-12/IL-23 expression by monocytes affecting Th17 cell development in HCV infection, and it is feasible that Tim-3 signaling on monocytes overrides its effect on other types of cells in PBMCs, resulting in an overall stimulatory effect on IL-17 production or TH17 cell development, perhaps by an indirect effect through stimulating IL-23 production.

IL-12p35, IL-23p19, and IL-17A expression is differentially regulated in healthy CD14+ monocytes or PBMCs cocultured with Huh-7 hepatoma cells transfected with HCV RNA. The Tim-3 expression observed above might be a result, rather than a cause, of differential regulation of IL-12/IL-23 expression and/or TH17 cell development during HCV infection. In addition, the driving force for Tim-3 upregulation during HCV infection remains to be determined. To further elucidate the role of HCV in the regulation of Tim-3 expression and cytokine production by...
inflammatory cells, we used a newly established cell culture system by transfecting Hub-7 hepatocytes with the HCV-JFH-1 strain in vitro to mimic the in vivo setting of early HCV infection (20, 21). To this end, healthy CD14+ monocytes or PBMCs were purified from HS and cocultured with HCV−/− Hub-7 hepatocytes for 48 h, followed by stimulation with αTim-3 or IgG control for another 72 h, followed by LPS/R848 or PMA/ionomycin stimulation for 6 h. Tim-3 and IL-12/IL-23/IL-17 expression were detected by flow cytometric analysis. As shown in Fig. 4 in a histogram, dot plots, and bar figures derived from multiple repeated experiments (n = 6), Tim-3 expression is upregulated on purified monocytes cocultured with Huh-7 hepatocytes expressing HCV compared to HCV− hepatocytes (Fig. 4A). These data are consistent with the results we have shown previously (20, 21). Notably, IL-12p35 and IL-23p19 expression is differently regulated by Huh-7 hepatoma cells transfected with HCV RNA, i.e., HCV− Huh-7 significantly inhibits IL-12p35 expression and upregulates IL-23p19 expression by TLR-activated CD14+ monocytes compared to those cocultured with HCV− Huh-7 hepatocytes (Fig. 4B). Importantly, blocking Tim-3 signaling significantly corrects the imbalance of monocyte IL-12/IL-23 expression induced by Huh-7 hepatoma cells expressing HCV (Fig. 4B). These data indicate that HCV inhibits IL-12 expression and stimulates IL-23 expression by TLR-activated monocytes through Tim-3 signaling.

To determine whether HCV may drive T\textsubscript{H}17 cell differentiation and Tim-3 may play a role in this process, we cocultured healthy PBMCs with HCV−/− Huh-7 cells for 48 h, in the presence of αTim-3 or IgG for another 72 h, and stimulated with PMA/ionomycin for 6 h, followed by detecting IL-17 expression by CD4+ T cells. Similar to what we observed in chronically HCV-infected individuals who have an increased number of T\textsubscript{H}17 cells over HS, T\textsubscript{H}17 cells are detected higher in PBMCs cocultured with HCV+ hepatocytes versus HCV− hepatocytes, and blocking Tim-3 signaling while coculturing these cells inhibits the HCV-induced IL-17 expression by T\textsubscript{H}17 cells (Fig. 4C). It should be noted that T\textsubscript{H}17 cells generated in this in vitro model, which mimics the early phase of HCV infection, recapitulated the phenomena observed in the setting of chronic HCV infection in vivo (Fig. 2A and Fig. 3D). This suggests that HCV triggers T\textsubscript{H}17 cell development, at least in part through Tim-3-mediated differential regulation of IL-12/IL-23 expression by monocytes.

HCV upregulates Tim-3 expression and induces IL-23 production in CD14+ monocytes through the STAT3 pathway. Since HCV induces Tim-3 and IL-23 expression, whereas blockade of Tim-3 signaling inhibits HCV-mediated IL-23 expression in TLR-stimulated CD14+ monocytes, we next sought to explore the intracellular signaling pathway involved in this process. It has been reported that phosphorylation of signal transducer and activator of transcription 3 (STAT3) play a pivotal role in IL-12/IL-23 expression in macrophages infiltrated in the tumor microenvironment (29). We thus hypothesized that HCV-induced Tim-3 might modulate TLR-stimulated monocyte IL-23 expression through the Jak/STAT pathway. To test this hypothesis, we first determined the relationship of Tim-3 and STAT3 expression in monocytes cocultured with HCV+/− Huh-7 cells (48 h) in the presence or absence of TLR stimulation (6 h). As shown in Fig. 5A, phosphorylation of STAT3 is barely detectable in non-TLR-stimulated monocytes, regardless of coculturing of monocytes with HCV+ or HCV− hepatocytes. Nevertheless, Tim-3 expression is significantly upregulated in nonstimulated monocytes cocultured with HCV+ Huh7 compared to HCV− Huh7. After TLR stimulation, Tim-3 expression is significantly reduced, and STAT3 phosphorylation is upregulated, in monocytes cocultured with both HCV+ and HCV− hepatocytes. However, STAT3 phosphorylation is sig-

FIG 2 T\textsubscript{H}17 cells upregulation in HCV-infected individuals is associated with Tim-3 expression and differential regulation of IL-23/IL-12 production by monocytes. PBMCs from HCV-infected individuals and HS were stimulated with PMA/ionomycin for 6 h, stained with CD4 and IL-17A, followed by flow cytometric analysis. (A) Representative dot plots of IL-17A expression in CD4+ T cells. Summary data of percentages of the positive cell frequency and the mean MFI values + the SD of for IL-17A expression in CD4+ T cells (*, P < 0.01 (as determined by Student t test). (B) IL-17 expression by CD4+ cells positively correlates to Tim-3 expression on CD14+ cells (**, P < 0.05. (C) IL-17 expression by CD4+ cells positively correlates with the ratio of IL-23/IL-12 produced by CD14+ cells (*, P < 0.05).
nificantly increased in monocytes cocultured with HCV-expressing Huh-7 cells. We also measured the phosphorylation level of STAT3 protein in monocytes cocultured with HCV-expressing Huh-7 cells by Western blotting. As shown in Fig. 5B, the phosphorylation of STAT3 in monocytes is increased by HCV-expressing Huh-7 cells, whereas the levels of total STAT3 protein remained static between HCV-H11001 and HCV-H11002 cultures. The densitometry ratio of phosphorylated STAT3 versus total STAT3 proteins from four independent experiments is summarized on the right. Notably, although IL-23p19 is also upregulated in monocytes cocultured with HCV-H11001 Huh-7 versus HCV-H11002 Huh-7 (Fig. 4B), its expression is remarkably diminished in the presence of a STAT3-specific inhibitor (S3I-201, rather than a dimethyl sulfoxide control) when the cells are cocultured (48 h) after TLR stimulation (6 h) (Fig. 5C), supporting the notion that IL-23 expression is STAT3 dependent.

We further characterized the role of Tim-3 on IL-23 production through regulating TLR-mediated STAT3 activation in monocytes isolated from HCV-infected individuals. To this end, purified CD14+ monocytes from chronically HCV-infected patient were incubated with Tim-3 or control IgG antibody for 72 h and then stimulated with LPS/R848 for 6 h, followed by flow cytometric analysis of IL-12p35 and IL-23p19 expression. Representative dot plots and summary data measuring IL-12p35 and IL-23p19 production in CD14+ monocytes in HCV versus HS with the blockade of Tim-3 or IgG antibody are shown. Each symbol represents an individual subject, connected by a line meaning the same cells treated with IgG or aTim-3, the horizontal bars represent median values. *, P < 0.05; NS, no significance by paired t test. (B) Representative experiment of purified CD4+ T cells from an HCV-infected individual that were incubated with aTim-3 and IgG for 72 h, stimulated with PMA/ionomycin for 6 h, followed by flow cytometric analysis of IL-17 versus Tim-3 expression in CD4+ T cells. (C) Representative experiment of CD14+ monocyte-depleted PBMCs from an HCV-infected individual that were incubated with aTim-3 and IgG for 72 h and stimulated with PMA/ionomycin for 6 h, followed by flow cytometric analysis of IL-17 versus Tim-3 expression in CD4+ T cells. (D) PBMCs from HCV-infected individuals and HS were incubated with aTim-3 and control IgG antibody for 72 h and then stimulated with PMA/ionomycin for 6 h, followed by flow cytometric analysis of IL-17A expression in CD4+ T cells. Representative dot plots and summary data measuring IL-17 production in CD4+ T cells in HCV versus HS with the blockade of Tim-3 or IgG antibody are shown. Each symbol represents an individual subject, connected by a line meaning the same cells treated with IgG or aTim-3, the horizontal bars represent median values. *, P < 0.05; NS, no significance by paired t test.
with TLR stimulation and further decreased by Tim-3 blockade. IL-23 is barely detectable in unstimulated monocytes; its expression is significantly increased by TLR stimulation. Importantly, blocking Tim-3 signaling inhibits IL-23 expression, suggesting that Tim-3 stimulates TLR signaling to positively upregulate IL-23 expression in monocytes. Similarly, phosphorylation of STAT3 is only observed in TLR-activated monocytes, and Tim-3 blockade significantly inhibits STAT3 phosphorylation. To summarize these findings, the percentages of cell frequency of phosphorylated STAT3 in unstimulated, or TLR/IgG-stimulated, and TLR/Tim-3-stimulated CD14⁺ monocytes from six subjects are shown as a bar figure in the right panel.

In addition, lysates from cells treated the same as described above were also immunoblotted to detect phosphorylated STAT3 protein, and total STAT-3 levels were measured to serve as loading controls. As shown in Fig. 6B, pSTAT3 protein is significantly upregulated in TLR-activated monocytes from HCV-infected patients, whereas Tim-3 blocking significantly downregulates pSTAT3 expression in monocytes compared to cells treated with control IgG. The statistical analysis of densitometry data from multiple experiments (n = 4) is significant and is summarized in the bar figures shown in the right panel. Taken together, these results suggest that HCV-induced Tim-3 expression positively regulates IL-23 production, likely through synergistic stimulation of TLR-mediated STAT3 phosphorylation in monocytes in the setting of viral infection. This is an extension of our recent finding that HCV-induced Tim-3 expression negatively regulates IL-12 production, though inhibi-
TLR-mediated STAT1 phosphorylation in monocytes during HCV infection (20).

DISCUSSION

Chronic HCV infection is a worldwide infectious disease that can lead to chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. Although the pathogenesis of HCV infection remains only partly elucidated, it has become evident that the dysregulation of innate to adaptive immune responses plays a major role in viral persistence and disease progression. In this study, we demonstrate that (i) Tim-3 is upregulated on monocytes in HCV-infected individuals or by incubation with HCV-expressing hepatocytes, (ii) that this HCV-induced Tim-3 expression can inhibit IL-12 and stimulate IL-23 production through regulating Jak/STAT signaling pathways in monocytes in response to TLR stimulation, and (iii) that HCV-induced, Tim-3-mediated differential regulation of IL-12/IL-23 might play a pivotal role in T\textsubscript{H}17 cell development during viral infection. To our knowledge, this is the first report to demonstrate that HCV-induced Tim-3 expression differentially regulates IL-12/IL-23 production through modulating TLR signaling pathways in monocytes, leading to T\textsubscript{H}17 cell development during HCV infection.

Pathogen-associated molecule patterns are recognized by TLRs expressed on APCs to secret IL-12 and IL-23. IL-12 is important in stimulating T\textsubscript{H}1 responses that are essential for host defense and viral clearance, whereas IL-23 is a proinflammatory cytokine that plays an important role in inflammatory diseases, infection, and tumor environments (29). IL-23 signals through an IL-23 receptor complex consisting of the IL-12 receptor \textbeta\textsubscript{1} chain and a specific IL-23 receptor subunit. Despite their similarities in ligand structure and receptor complexes, it has become evident that IL-12 and IL-23 have common but divergent activities (30). Recent studies demonstrated that mice deficient in IL-12p40 (thus lacking both IL-12 and IL-23) or IL-23p19 (lacking IL-23 but not...
IL-12) were resistant to experimental autoimmune encephalomyelitis (EAE) and collagen-induced arthritis (CIA), whereas IL-12p35-deficient mice (lacking IL-12 but not IL-23) were more susceptible to disease, indicating that IL-23, but not IL-12, is critical in the development of autoimmune inflammation (31, 32). Further studies revealed that IL-23-deficient mice had normal TH1 responses but did not produce IL-17A, whereas IL-12p35-deficient mice exhibit an increased number of IL-17-producing CD4^+ T cells in inflamed tissues (31,32). Functionally, TH17 cells contribute to host defense as a new effector TH cell subset with a role in protection against pathogens through activities on immune and non-immune epithelial cells. Their activities, however, are also pivotal in the development of autoimmune diseases under pathological conditions. For example, mice deficient in IL-17 were found to be resistant to EAE or CIA, compared to wild-type (WT) mice, IL-17A-deficient mice had significantly suppressed EAE as indicated by delayed disease onset, reduced maximum severity scores, attenuated histological changes, and early recovery from the disease, whereas overexpression of IL-17 exacerbated these autoimmune diseases (33, 34). Therefore, IL-12 and IL-23 both appear to have distinct roles in vivo in promoting antimicrobial immune responses and diseases. IL-12 suppresses IL-23 and IL-17 production and vice versa, IL-23 inhibits IL-12 and IFN-γ production, indicating cross-regulation between the IL-23/Th17 and IL-12/Th1 pathways (35,36). It appears that IL-12 and IL-23/IL-17 may be paradoxically regulated, but the precise mechanism for their differential expression remains unclear.

We have recently shown that HCV-induced Tim-3 expression inhibits IL-12 production through the delivery of negative signaling to TLR-mediated STAT1 activation in monocytes (20). Here, we further demonstrated that HCV-induced Tim-3 expression upregulates IL-23 expression by stimulating TLR-mediated STAT3 phosphorylation. Based on these data and our previous studies, we propose a model in which HCV-induced Tim-3 controls the balance of IL-12/IL-23 through differential regulation of STAT1/STAT3, promoting either differentiation or expansion of
T\textsubscript{H}17 cells during viral infection (Fig. 7). This novel model is plausible and provides an insight into understanding the pathogenesis of HCV persistence.

Based on this model, interactions between HCV-infected hepatocytes and infiltrating immune cells might determine viral clearance or persistence and disease progression. We have recently demonstrated that HCV not only upregulates Tim-3 expression on activated T cells, but also a Tim-3 ligand, galectin-9 (Gal-9), on the surfaces of infected hepatocytes (37). The HCV-mediated Tim-3/Gal-9 interactions drive naïve CD4\textsuperscript{+} T cells differentiation into CD4\textsuperscript{+}CD25\textsuperscript{−}Foxp3\textsuperscript{+} T regulatory cells (Tregs) but inhibit CD4\textsuperscript{+}CD25\textsuperscript{−}Foxp3\textsuperscript{−} T effector cells (Teffs), indicating an immunomodulatory role of HCV-infected hepatocytes (37, 38). We show here that HCV also promotes TLR-mediated STAT3 signaling within the virally infected microenvironment and induces a heterodimeric cytokine, IL-23, while inhibiting its counterpart cytokine, IL-12, thereby shifting the balance of antiviral immunity toward a condition favoring viral persistence and autoimmune disorders in HCV infection.

The adaptive immune system is crucial for the elimination of viral infections, but dysregulation of adaptive immune responses can also lead to the development of inflammatory and autoimmune diseases. The TH1-TH2 paradigm of CD4\textsuperscript{+} T cell lineage was described by Mosmann et al. based upon distinct cytokine profiles and characteristic functions (39). T\textsubscript{H}1 cells develop in the presence of IL-12, produce primarily IL-2/IFN-γ, and are involved in cell-mediated immunity. T\textsubscript{H}2 cells differentiate in the presence of...
IL-4, produce IL-4/IL-5/IL-13, and are critical for humoral immunity. T_{H}17 cells are newer members of T_{H} cell family and are distinct from T_{H}1 and T_{H}2, being characterized by their ability to produce specific cytokines such as IL-17A, IL-17F, IL-22, and CCL20. The conditions for the differentiation of T_{H}17 cells remains unclear, but the ratio of IL-12/IL-23 produced by monocytes may play a pivotal role in several disease models (40). IL-23 was essential for development of pathogenic T_{H}17 cells in autoimmune inflammation as indicated by undetectable T_{H}17 cells in IL-23p19 deficient mice (41, 42). Moreover, IL-23 and T_{H}17 cells were found to be critical for the induction, but not the effector phase, of EAE, an animal model of human multiple sclerosis (42). In addition, IL-23 and T_{H}17 cells were found to be linked to the development of multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus, chronic immune thrombocytopenia, psoriasis, asthma, atopic dermatitis, and chronic hepatitis B in humans (14–19, 30–32). In accordance with these findings, cytokines such as IL-17, IL-17F, IL-22, and IL-6 produced by Th17 cells are found to be elevated in several human inflammatory diseases, and both IL-23 and the Th17 pathway correlate with disease severity and immunopathology in diverse infections (33, 34).

In the present study, we provide pilot evidence indicating that T_{H}17 cells accumulate in the peripheral blood of HCV-infected individuals. Increased T_{H}17 cells are also observed in healthy PBMCs cocultured with hepatocytes expressing HCV. In conjunction with monocyte depletion or Tim-3 blockade, which reverses T_{H}17 cell development both in vitro and in vivo, these data suggest that HCV-induced, Tim-3-mediated differential regulation of IL-12/IL-23 expression by APCs may be critical to T_{H}17 cell development. It would follow that the STAT3/IL-23/IL-17 pathway, rather than or, more likely, in addition to the STAT1/IL-12/IFN-γ axis, may be crucial to viral persistence and the autoimmune phenomena observed during HCV infection.

The mechanisms, by which HCV-mediated Tim-3 expression regulates innate to adaptive immune responses leading to viral persistence and autoimmune disorders during HCV infection, are likely multiple. We and others have shown that HCV-induced negative signaling molecules, including program death-1 (PD-1), suppressor of cytokine signaling-1 (SOCS-1), and Tim-3, play pivotal role in inhibiting several intracellular signaling pathways, including MAPK, Jak/STAT, and Akt/PI3K, and lead to the inhibition of monocyte IL-12 production, the suppression of virus-specific T cell responses, the promotion of B cell activation and proliferation, and the induction of CD4^-CD25^-Foxp3^- regulatory T cells during HCV infection (20, 21, 37, 38, 43). Notably, it has been reported that HCV-specific T_{H}17 cells were suppressed by production of anti-inflammatory cytokines IL-10 and transforming growth factor β (TGF-β) (44). Since Foxp3^- Tregs suppress inflammatory effector T cells (Teffs) through the secretion of IL-10 and TGF-β, Th17 cells would also be suppressed by anti-inflammatory cytokines IL-10 and TGF-β. Importantly, Tim-3 controls the ratio of Tregs/Teffs by regulating cell proliferation and apoptosis and fine-tunes the balance of T-cell-mediated antiviral immunity and self-injury pathology in the setting of chronic HCV infection (38). Nevertheless, the present study, demonstrating HCV-induced Tim-3 expression differentially regulates IL-12/IL-23 expression by monocytes that leads to T_{H}17 cell development, adds new insight to the larger picture underlying HCV pathogenesis, and underscores the potential importance of immunotherapy in conjunction with antiviral treatment in the management of HCV infection.

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