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Cross-Talk between Programmed Death-1 and Suppressor of Cytokine Signaling-1 in Inhibition of IL-12 Production by Monocytes/Macrophages in Hepatitis C Virus Infection

Ying Zhang,*†,1 Cheng J. Ma,*†,1 Lei Ni,* Chun L. Zhang,* Xiao Y. Wu,* Uday Kumaraguru,‡ Chuan F. Li,§ Jonathan P. Moorman,*†,6 and Zhi Q. Yao*†,†,4

Hepatitis C virus (HCV) dysregulates innate immune responses and induces persistent viral infection. We previously demonstrated that HCV core protein impairs IL-12 expression by monocytes/macrophages (M/Mφ) through interaction with a complement receptor gC1qR. Because HCV core-mediated lymphocyte dysregulation occurs through the negative immunomodulators programmed death-1 (PD-1) and suppressor of cytokine signaling-1 (SOCS-1), the aim of this study was to examine their role in HCV core-mediated IL-12 suppression in M/Mφ. We analyzed TLR-stimulated, primary CD14+ M/Mφ from chronically HCV-infected and healthy subjects or the THP-1 cell line for PD-1, SOCS-1, and IL-12 expression following HCV core treatment. M/Mφs from HCV-infected subjects at baseline exhibited comparatively increased PD-1 expression that significantly correlated with the degree of IL-12 inhibition. M/Mφs isolated from healthy and HCV-infected individuals and treated with HCV core protein displayed increased PD-1 and SOCS-1 expression and decreased IL-12 expression, an effect that was also observed in cells treated with gC1qR’s ligand, C1q. Blocking gC1qR rescued HCV core-induced PD-1 upregulation and IL-12 suppression, whereas blocking PD-1 signaling enhanced IL-12 production and decreased the expression of SOCS-1 induced by HCV core. Conversely, silencing SOCS-1 expression using small interfering RNAs increased IL-12 expression and inhibited PD-1 upregulation. PD-1 and SOCS-1 were found to associate by coimmunoprecipitation studies, and blocking PD-1 or silencing SOCS-1 in M/Mφ led to activation of STAT-1 during TLR-stimulated IL-12 production. These data suggested that HCV core/gC1qR engagement on M/Mφ triggers the expression of PD-1 and SOCS-1, which can associate to deliver negative signaling to TLR-mediated pathways controlling expression of IL-12, a key cytokine linking innate and adaptive immunity. The Journal of Immunology, 2011, 186: 3093–3103.
molecules represent such negative mechanisms at play in host immunity (19, 20). PD-1 is an inhibitory receptor predominantly expressed on activated lymphocytes, as well as M/Ms. Upon engagement with its ligand, PDL-1/PDL-2, PD-1 induces immunoreceptor tyrosine phosphorylation and recruitment of tyrosine phosphatases, including src-homology proteins (SHP-1/SHP-2), to deliver negative signals to receptor-signaling pathways (20). SOCS-1 protein is induced upon cell activation and represents another level of inhibitory machinery by binding to cytokine receptors or receptor-associated JAK to inhibit the intracellular activation of STAT-1 (19). HCV may take advantage of host-signaling pathways by upregulating inhibitory molecules, such as PD-1/SOCS-1, as a means of dampening cellular immune responses to facilitate viral persistence. For example, PD-1 was found to be upregulated on exhausted CD4+ and CD8+ T cells during chronic HCV infection (21, 22). PD-1 and SOCS-1 were also shown to regulate murine monocyte IL-10/IL-12 production against bacterial infection (23–26). Similarly, we demonstrated that HCV core protein, by interaction with a complement receptor (gC1qR), can differentially regulate T and B lymphocyte functions through modulating PD-1/SOCS-1 signaling (27–29). Little is known about the role of these two negative modulators in human innate immune cells, such as M/Ms, in HCV infection.

We previously showed that HCV core protein inhibits IL-12 production through interaction with gC1qR on M/Ms (30) and that monocytes from HCV-infected subjects who have cleared their infection after antiviral therapy regain normal IL-12 responses and PD-1 expression (34). To determine the molecular mechanisms underlying the HCV core/gC1qR-mediated IL-12 suppression, in this study we sought to determine PD-1 and SOCS-1 expression and their role in regulating IL-12 production in human blood-derived M/Ms, as well as by inoculation of uninfected Huh-7 cells (35, 36). We found that PD-1 and SOCS-1, two negative immunomodulators upregulated by HCV core protein through its interaction with gC1qR on M/Ms, are linked, such that their cross-talk may coordinate deliver negative signaling to TLR-mediated IL-12 production through the Jak/STAT pathway.

Materials and Methods

Subjects

The study protocol was approved by the institutional review board at East Tennessee State University/James H. Quillen Veterans Affairs Medical Center, which has contributed to a database for the storage of blood samples from HCV-infected individuals for the purpose of viral immunology studies. All patients gave written consent for this study. The subjects recruited were composed of two groups of populations. The first group contained 25 chronically HCV-infected patients. HCV genotype and viral load, which were performed by Lexington Veterans Affairs Medical Center, are shown in Table I. The second group was composed of 14 healthy subjects negative for hepatitis B virus, HCV, and HIV. The majority of the study subjects are patients of the Department of Hepatology at the University of Tennessee College of Medicine, and the rest are volunteers from the local community.

Cell isolation and culture

Human PBMCs were isolated from the peripheral blood of study subjects by Ficoll-density centrifugation with lympho-H (Atlanta Biological, Lawrenceville, GA). Human M/Ms were further isolated from PBMCs using Ficoll-Percoll gradients, per the manufacturer’s instructions (GE Healthcare, Piscataway, NJ). When indicated, M/Ms were also purified from PBMCs by anti-CD14 magnetic beads with column purification, per the manufacturer’s instructions (Miltenyi Biotec; purity of cells >95%). PBMCs and M/Ms were cryopreserved in freezing medium in liquid nitrogen (27). THP-1 cells, a human monocytic leukemia cell line, were purchased from American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 containing 10% FBS at 37°C 5% CO2 in a humidified atmosphere.

Flow cytometry

Intracellular IL-12 (p40/p70) production in activated M/Ms was determined by flow cytometric analysis using MACS Inside Stain Kit (Miltenyi Biotec, Auburn, CA), per the manufacturer’s instructions. Briefly, 1 × 10^6 purified PBMCs or THP-1 cells were stimulated with 5 μg/ml LPS or R848 in the presence or absence of HCV core protein (2 μg/ml; ViroGen, Watertown, MA) or Clq (50 and 100 μg/ml; Quidel, San Diego, CA) for 18–72 h, with 2 μg/ml secretion inhibitor brefeldin A for 6 h prior to harvesting the cells for immune staining. The stimulated cells were first stained for cell surface markers using PE-anti-human CD14 Ab and FITC–anti-PD-1 or PDL-1 Ab in FACS medium (Becton Dickinson, San Jose, CA). The primary isotype controls were used to determine the level of background staining; 20,000 events were collected after gating on M/M populations.

RT-PCR

The primary M/Ms or THP-1 cells treated by LPS/R848 with or without core, as described above, for 24–48 h were lysed, and total RNA was extracted using an RNeasy Mini Kit (Qiagen, Valencia, CA). A microgram of RNA was reverse transcribed using a GeneAmp RNA PCR kit (Applied Biosystems, Foster City, CA). SOCS-1 primers and RT-PCR conditions were described as reported previously (29). To control for genomic DNA contamination, equal amounts of cDNA from each sample were PCR amplified without reverse transcription. The resulting PCR products were separated in a 2% agarose gel (Invitrogen, Carlsbad, CA) and viewed using a multi-imager.

Western blotting and immunoprecipitation

The primary M/Ms or THP-1 cells treated by LPS/R848 with or without core, as described above, for 48–72 h, were lysed in 1× radioimmunoprecipitation assay lysis buffer (Boston BioProducts, Ashland, MA) containing protease/phosphatase inhibitors (Thermo Scientific, Rockford, IL) on ice. Cell lysates were centrifuged at 15 min at 4°C, and the protein concentrations were measured. For coimmunoprecipitation, samples were incubated with 3 μg PD-1 Abs (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C, followed by an additional 2 h of incubation with 20 μl Protein A/G PLUS-Agarose (Santa Cruz Biotechnology) at 4°C. After washing with lysis buffer, the samples were denatured with Laemmli sample buffer (Boston BioProducts) and separated by SDS-PAGE. Following transfer to Amersham Hybond-P membrane (GE Healthcare), the membrane was blocked with 2.5% BSA-PBS and probed with anti–SOCS-1 (Millipore, Temecula, CA) or β-actin (Santa Cruz Biotechnology) Ab at 4°C overnight. For detection of phosphorylated proteins, the membrane was probed with anti–phospho-STAT-1 (Tyr701), MAPK p38 (Thr 180/182), and total STAT or MAPK Ab (Cell Signaling Technology, Danvers, MA). The HRP secondary Ab incubation, signal development, and data process were carried out as described previously (28).

Huh-7 hepatocyte transfection by HCV-JFH-1 and coculture with primary M/Ms

HCV Japanese fulminant hepatitis-1 (JFH-1) (kindly provided by Dr. T. Wakita, Department of Virology II, National Institute of Infectious Diseases of Japan, through a material transfer agreement [MTA]) was transfected into Huh-7 cells. Huh-7 cells were transfected with the 48-h supernatant of JFH-1–transfected Huh-7 cells, as described previously, for 24–48 h. Huh-7 cells were transfected with the 48-h supernatant of JFH-1–transfected Huh-7 cells, as described previously. Intracellular IL-12 production was measured by cytometric analysis using MACS Inside Stain Kit (Miltenyi Biotec, Auburn, CA), per the manufacturer’s instructions. Briefly, 1 × 10^6 purified PBMCs or THP-1 cells were stimulated with 5 μg/ml LPS or R848 in the presence or absence of HCV core protein (2 μg/ml; FuroGen, Watertown, MA) or Clq (50 and 100 μg/ml; Quidel, San Diego, CA) for 18–72 h, with 2 μg/ml secretion inhibitor brefeldin A for 6 h prior to harvesting the cells for immune staining. The stimulated cells were first stained for cell surface markers using PE-anti-human CD14 Ab and FITC–anti-PD-1 or PDL-1 Ab in FACS medium (27). After fixing the cells with Intracellular Fix and Perm wash, the cells were incubated with allophycocyanin-anti-human CD14 Ab, followed by flow cytometric analysis.

Human M/Ms were further isolated from PBMCs using anti-CD14 magnetic beads with column purification, per the manufacturer’s instructions (Miltenyi Biotec, Auburn, CA), per the manufacturer’s instructions. Briefly, 1 × 10^6 purified PBMCs or THP-1 cells were stimulated with 5 μg/ml LPS or R848 in the presence or absence of HCV core protein (2 μg/ml; FuroGen, Watertown, MA) or Clq (50 and 100 μg/ml; Quidel, San Diego, CA) for 18–72 h, with 2 μg/ml secretion inhibitor brefeldin A for 6 h prior to harvesting the cells for immune staining. The stimulated cells were first stained for cell surface markers using PE-anti-human CD14 Ab and FITC–anti-PD-1 or PDL-1 Ab in FACS medium (27). After fixing the cells with Intracellular Fix and Perm wash, the cells were incubated with allophycocyanin-anti-human CD14 Ab, followed by flow cytometric analysis.
same way as the negative control. For the coculture experiments, highly purified M/M₄₈s with anti-CD14 magnetic beads were incubated with hepatocytes at a ratio of 10:1 at 48 h after HCV/mock transfection and were stimulated with or without LPS/R848 for another 6–48 h. The expressions of PD-1 and IL-12 in M/M₄₈s were examined by flow cytometry.

**HCV core/gC1qR or PD-1/PDL-1 blockade**

Primary M/M₄₈s from healthy subjects or HCV patients or monocytic THP-1 cells were incubated with 10 μl anti-gC1qR (1:10 dilution, blocking Ab was generously provided by Dr. Young S. Hahn, University of Virginia), 10 μg/ml anti-PD1 or anti-PDL-1, or control IgG Abs (eBioscience, San Diego, CA) overnight, followed by stimulation with LPS and R848 in the presence or absence of HCV core protein for 18–72 h. IL-12/PD-1/STAT-1 expression in the treated cells was described as previously published (33, 37).

**SOCS-1 small interfering RNA silencing**

A total of 3 × 10⁶ THP-1 cells was incubated with 60 pmol SOCS-1 small interfering RNA (siRNA) duplex or control siRNA in 200 μl siRNA Transfection Medium in a six-well plate, per the manufacturer’s instructions (Santa Cruz Biotechnology). Following 6 h of incubation at 37°C, normal growth medium containing 2× concentrations of normal serum and antibiotics was added. The transfected cells were stimulated with 5 μg/ml LPS and R848 in the presence of 2 μg/ml HCV core for 48–72 h, followed by detection of PD-1/PDCL/STAT1/IL-12, as described above.

**Statistical analysis**

Study results were summarized, and results are expressed as mean ± SD. A comparison between two groups was performed using multiple-comparison testing least significant difference or the Tukey test, depending on the ANOVA F-test, by SPSS 18 software. Paired t tests were used to compare data with anti–PDL-1 versus control IgG treatment. A Pearson correlation program was used to determine the correlation between PD-1 and IL-12 expression on M/M₄₈s from the study subjects; p values <0.05 and 0.01 were considered significant or very significant, respectively.

**Results**

**HCV core protein suppresses IL-12 production by M/M₄₈s from healthy and HCV-infected individuals**

We previously demonstrated that HCV core protein inhibits M/M₄₈s IL-12 production through interaction with a complement receptor (gC1qR) in vitro (33). To consolidate the data and explore the mechanisms of IL-12 inhibition during HCV infection, PBMCs isolated from HCV-infected patients and healthy subjects (Table I) were stimulated with TLR ligand LPS/R848 in the presence or absence of HCV core protein, followed by flow cytometric analysis for intracellular IL-12 production in CD14⁺ M/M₄₈s. Fig. 1 (upper panel) shows the gating strategy for M/M₄₈s subsets and a representative dot plot of IL-12 inhibition in CD14⁺ M/M₄₈s by HCV core protein; the bottom panel shows the summary data of the percentage of IL-12⁺ cells in CD14⁺ M/M₄₈s treated with or without HCV core protein. Intracellular IL-12 secretion by CD14⁺ M/M₄₈s in response to TLR stimulation without core treatment was significantly suppressed in patients with chronic HCV infection compared with healthy subjects. Notably, HCV core treatment significantly suppressed IL-12 production by CD14⁺ M/M₄₈s from healthy subjects and HCV patients. Thus, cells isolated from HCV patients and treated with LPS/R848 and core protein also produced less IL-12 compared with those from healthy subjects with the same treatment ex vivo. These results suggested that IL-12 secretion by M/M₄₈s is impaired in patients with chronic HCV infection, and this suppression can be further enhanced by ex vivo exposure of the cells to HCV core protein.

**PD-1 expression on M/M₄₈s of healthy and HCV-infected individuals is upregulated by HCV core protein**

Several investigators, including us, showed that PD-1 is upregulated on CD4⁺ and CD8⁺ T cells, leading to Th1/Tc1 dysfunction during HCV infection (21, 22, 27). To determine whether the PD-1 pathway is involved in the regulation of IL-12 production in M/M₄₈s during HCV infection, we used flow cytometry to examine PD-1 expression on the surface of M/M₄₈s stimulated with LPS/ R848 in the presence or absence of HCV core protein. Fig. 2 (upper panel) shows the gating strategy for M/M₄₈s subsets and a representative dot plot of PD-1 upregulation on CD14⁺ M/M₄₈s by HCV core protein; the lower panel shows the summary data of the percentage of PD-1⁺ cells on CD14⁺ M/M₄₈s treated with or without HCV core protein. In contrast to the suppression of IL-12 production during HCV infection, PD-1 expression on M/M₄₈s was significantly greater than that of healthy subjects. Notably, HCV

![Image](http://www.jimmunol.org/)  
**FIGURE 1.** HCV core protein suppresses IL-12 production by M/M₄₈s from healthy and HCV-infected individuals. PBMCs isolated from healthy subjects (n = 14) and chronically HCV-infected patients (n = 25) were stimulated with TLR ligand LPS/R848 in the presence or absence of HCV core protein, followed by flow cytometric analysis for the expression of CD14 and IL-12. *Core,* Gating strategy for M/M₄₈ subsets and a representative dot plot of IL-12 inhibition in CD14⁺ M/M₄₈s by HCV core protein. **Core,** Summary data of the percentage of IL-12⁺ cells in CD14⁺ M/M₄₈s treated with or without HCV core protein. Each symbol represents an individual subject, and the horizontal lines represent the mean of IL-12⁺CD14⁺ M/M₄₈s. *p < 0.05, **p < 0.01.

**Table I.** Demographic features of the study subjects

<table>
<thead>
<tr>
<th>Group</th>
<th>Subjects (n)</th>
<th>Mean Age (y; range)</th>
<th>Males (%)</th>
<th>Genotype 1 (%)</th>
<th>HCV-RNA (IU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCV-infected patients</td>
<td>25</td>
<td>53 (31–69)</td>
<td>96</td>
<td>72</td>
<td>23,000–50,000,000</td>
</tr>
<tr>
<td>Healthy subjects</td>
<td>14</td>
<td>45 (27–58)</td>
<td>85</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

NA, not applicable.

Group Subjects: M/M₄₈s from healthy and HCV-infected individuals. PBMCs isolated from healthy subjects or HCV patients or monocytic THP-1 cells were incubated with 10 μl anti-gC1qR (1:10 dilution, blocking Ab was generously provided by Dr. Young S. Hahn, University of Virginia), 10 μg/ml anti-PD1 or anti-PDL-1, or control IgG Abs (eBioscience, San Diego, CA) overnight, followed by stimulation with LPS and R848 in the presence or absence of HCV core protein for 18–72 h. IL-12/PD-1/STAT-1 expression in the treated cells was described as previously published (33, 37).

**Statistical analysis**

Study results were summarized, and results are expressed as mean ± SD. A comparison between two groups was performed using multiple-comparison testing least significant difference or the Tukey test, depending on the ANOVA F-test, by SPSS 18 software. Paired t tests were used to compare data with anti–PDL-1 versus control IgG treatment. A Pearson correlation program was used to determine the correlation between PD-1 and IL-12 expression on M/M₄₈s from the study subjects; p values <0.05 and 0.01 were considered significant or very significant, respectively.

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CD14 expression. HCV core protein, followed by flow cytometric analysis for PD-1 and were stimulated with TLR ligand LPS/R848 in the presence or absence of M/M demonstrated by the negative line trend, in reverse association between PD-1/PDL-1 expression and IL-12 production, as demonstrated by the negative line trend, in the same group of cells from chronically HCV-infected individuals. In contrast, the expression of PDL-1, although also upregulated on M/M from HCV-infected patients compared with those from healthy subjects, did not significantly correlate with IL-12 production with or without HCV core treatment ex vivo.

HCV core protein induces SOCS-1 and PD-1 expression in primary M/M\textsubscript{qs} and monocytic THP-1 cells

In addition to PD-1 dysregulation, we previously demonstrated that HCV core protein differentially regulated T and B lymphocyte functions by modulation of SOCS-1 (28–31). To determine whether SOCS-1 is also involved in the negative regulation of M/M\textsubscript{qs} IL-12 production during HCV infection, primary M/M\textsubscript{qs} were purified from healthy subjects and HCV-infected patients and stimulated with LPS/R848 for 24–48 h, followed by detection of SOCS-1 gene expression by RT-PCR and Western blotting. As shown in the representative experiment in Fig. 4A, SOCS-1 mRNA (left panel) and protein levels (right panel) were greater in M/M\textsubscript{qs} from chronically HCV-infected individuals compared with healthy subjects, whereas the amount of loading controls (β2M and β-actin) were comparable. These data were re-

PD-1 expression is inversely associated with IL-12 production in M/M\textsubscript{qs} of HCV-infected patients

To determine whether the upregulated PD-1 expression on the surface of M/M\textsubscript{qs} correlated with intracellular IL-12 production during HCV infection with or without HCV core treatment, PBMCs isolated from HCV-infected patients and stimulated ex vivo with LPS/R848 in the presence or absence of core protein were assayed for expression of PD-1, PDL-1, and IL-12 and compared by Pearson correlation analysis using SPSS 18 software. As shown in Fig. 3A and 3B, the upregulation of PD-1 on M/M\textsubscript{qs}, in response to ex vivo TLR stimulation with or without core treatment ($r^2 = -0.464$, $p = 0.039$; and $r^2 = -0.448$, $p = 0.048$, respectively), was inversely associated with IL-12 production, primarily by PD-1$^+$ CD14$^+$ M/M\textsubscript{qs} (representative dot plot experiment in inset), in the same group of cells from chronically HCV-infected individuals. In contrast, the expression of PDL-1, although also upregulated on M/M\textsubscript{qs} from HCV-infected patients compared with those from healthy subjects, did not significantly correlate with IL-12 production with or without HCV core treatment ex vivo.

HCV core protein upregulates PD-1 expression on M/M\textsubscript{qs} from healthy and HCV-infected individuals. PBMCs isolated from healthy subjects (○, $n = 14$) and chronically HCV-infected patients (▲, $n = 25$) were stimulated with TLR ligand LPS/R848 in the presence or absence of HCV core protein, followed by flow cytometric analysis for PD-1 and CD14 expression. Upper panel, Gating strategy for M/M\textsubscript{qs} subsets and a representative dot plot of PD-1 upregulation on CD14$^+$ M/M\textsubscript{qs} by HCV core protein. Lower panel, Summary data of the percentage of PD-1$^+$ CD14$^+$ M/M\textsubscript{qs} treated with or without HCV core protein. Each symbol represents an individual subject, and the horizontal lines represent the mean of PD-1$^+$CD14$^+$ M/M\textsubscript{qs}. *$p < 0.05$, **$p < 0.01$.

core significantly increased PD-1 expression on M/M\textsubscript{qs} of healthy subjects, as well as HCV-infected individuals. Thus, cells isolated from HCV patients and exposed ex vivo to core protein expressed relatively more PD-1 compared with those cells from healthy subjects. These results suggested that M/M\textsubscript{qs} from HCV-infected patients express higher levels of PD-1 than do those from healthy subjects and that HCV core significantly increases PD-1 expression on M/M\textsubscript{qs} from healthy and HCV-infected individuals.

FIGURE 2. HCV core protein upregulates PD-1 expression on M/M\textsubscript{qs} from healthy and HCV-infected individuals. PBMCs isolated from healthy subjects and that HCV core significantly increases PD-1 expression on M/M\textsubscript{qs}.

FIGURE 3. PD-1, but not PDL-1, expression is inversely associated with IL-12 production by M/M\textsubscript{qs}, with or without HCV core treatment. Each symbol represents an individual expression level of PD-1 (A, B), PDL-1 (C, D), and IL-12 by CD14$^+$ M/M\textsubscript{qs} treated (B, D) or not (A, C) with HCV core protein. The inverse association between PD-1/PDL-1 expression and IL-12 production, as demonstrated by the negative line trend, in M/M\textsubscript{qs} from chronically HCV-infected patients was evaluated using Pearson correlation analysis. Pearson correlation and two-tailed significance are shown in the upper right corner of each analysis. *$p < 0.05$. Representative dot plots of PD-1 and IL-12 expression by CD14$^+$ M/M\textsubscript{qs} (insets) illustrate that IL-12 is primarily expressed by PD-1$^+$ CD14$^+$ M/M\textsubscript{qs}.

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produced in repeated experiments using M/M$_{\alpha}$ highly purified from six healthy donors and six chronically HCV-infected individuals (Fig. 4A, lower panel). To examine HCV core’s effect on the induction of SOCS-1 gene expression, purified healthy M/M$_{\alpha}$ or human monocytic THP-1 cells (which were used in subsequent gene-silencing experiments) were incubated with LPS/R848 in the presence or absence of HCV core protein for 48 h, followed by RT-PCR or Western blot. As shown in Fig. 4B, SOCS-1 expression in primary M/M$_{\alpha}$ (left panel) and THP-1 cell line (right panel) was significantly augmented by exposure to HCV core protein, whereas the expression of internal controls was not. PD-1 upregulation and IL-12 downregulation were also observed in THP-1 cells stimulated with LPS/R848 and HCV core protein (Fig. 4C). Additionally, we transfected Huh-7 hepatocytes with the JFH-1 strain (through an MTA) and detected HCV core expression in the HCV-transfected cells by immunofluorescent staining (Fig. 4D, upper panel), as well as in the supernatant of the culture by RT-PCR (middle panel), but not in the mock-transfected Huh-7 cells. Importantly, we demonstrated, by FACS analysis, that PD-1 was upregulated, whereas IL-12 was downregulated, in the highly purified M/M$_{\alpha}$ cocultured with HCV$^+$ Huh-7 cells versus HCV$^-$ Huh-7 cells (green line) by FACS analysis, whereas IL-12 (lower right panel) was inhibited in purified M/M$_{\alpha}$ cocultured with HCV$^+$ Huh-7 cells (red line) versus HCV$^-$ Huh-7 cells (green line).

HCV core mimics C1q to regulate M/M$_{\alpha}$ functions through interaction with gC1qR

We previously demonstrated that HCV core protein differentially regulates T and B lymphocytes and inhibits monocyte IL-12 production through interaction with a complement receptor (gC1qR) expressed on these immune cells (27–34, 37–40). gC1qR is an immunoreceptor initially identified by its ability to bind the globular head of C1q, the first component of the C1 complex in the complement system, which plays a crucial role in innate immunity against microbial Ags circulating in the blood of the
infected host (40). Engagement of C1q with gC1qR leads to multiple cellular activities, including immunosuppression (40). To determine whether HCV core protein mimics C1q function on innate immune cells through interaction with gC1qR, we stimulated THP-1 cells and primary M/M_Fs with LPS/R848 in the presence of gC1qR’s natural ligand, C1q (50 and 100 μg/ml), for 18–72 h, followed by examination of the expression of PD-1 and IL-12 by flow cytometry. As shown in Fig. 5A, PD-1 was upregulated, whereas IL-12 was inhibited, in THP-1 cells. Similar results were observed in primary M/M_Fs; PD-1 was dose-dependently upregulated by C1q, whereas IL-12 was inhibited (Fig. 5B). These data were reproducible in three repeated experiments (Fig. 5B, lower panel), suggesting that HCV core can mimic the function of C1q, at least in part, in inhibiting M/M_F IL-12 production by regulation of PD-1 expression.

To determine whether gC1qR is involved in core’s ability to affect M/M_Fs, we incubated THP-1 cells with HCV core in the presence of anti-gC1qR or control Ab, followed by FACS analysis of core binding on the cell surface. As shown in Fig. 5C, the percentage of HCV core+ cells or the total/geometric mean fluorescence intensity of the detected signal is decreased by blockade of core/gC1qR engagement with anti-gC1qR but not by the control Ab. To determine whether HCV core protein alters gC1qR signaling to disrupt host PD-1 expression and IL-12 inhibition, M/M_Fs or THP-1 cells were preincubated with anti-gC1qR or a control IgG Ab overnight and then stimulated with LPS/R848 and HCV core protein for 18–72 h, followed by flow cytometry analysis. As shown in the representative graph overlay in Fig. 5D, cells preincubated with anti-gC1qR Ab, followed by LPS/R848/core stimulation, exhibited reduced PD-1 expression and increased IL-12 production compared with the cells treated with IgG control Ab and otherwise identical stimulations. These data were reproducible in at least five independent experiments in THP-1 cells, as well as in primary M/M_Fs (Fig. 5E), suggesting that HCV core-induced PD-1 and IL-12 dysregulation is gC1qR dependent. Collectively, these results indicated that PD-1 and SOCS-1 are overexpressed in M/M_Fs of chronically HCV-infected individuals and that HCV core protein can mimic the function of C1q, inducing PD-1 expression and IL-12 inhibition through interaction with gC1qR on M/M_Fs.

**FIGURE 5.** PD-1 and IL-12 expressions in THP-1 cells and M/M_Fs are differentially regulated by C1q, and HCV core-mediated PD-1/IL-12 dysregulation is abrogated by blocking gC1qR. A, Dose-dependent regulation of PD-1 and IL-12 expression in THP-1 cells by complement C1q. THP-1 cells were stimulated with LPS/R848 in the presence of C1q (0, 50, or 100 μg/ml) for 48–72 h, followed by flow cytometric analysis of PD-1 (left panel) and IL-12 (right panel). Representative overlay of graph and summary data from three independent experiments are shown. B, PD-1 and IL-12 expressions in primary M/M_Fs treated by complement C1q. PBMCs were stimulated with LPS/R848 in the presence of C1q (0, 50, or 100 μg/ml) for 18 h, followed by flow cytometric analysis of PD-1 and IL-12 expressions. Representative overlay of graph of PD-1 or IL-12 expression and summary data from three healthy subjects are shown. C, Blocking gC1qR prevents core from cell surface binding. THP-1 cells were incubated with HCV core protein in the presence of anti-gC1qR or control IgG for 2 h, followed by flow cytometric analysis of core binding on the cell surface, as described in Materials and Methods. One representative experiment of core-binding graph overlay (left) and summary data from three independent experiments (right) are shown. D and E, Blocking HCV core/gC1qR interaction corrects IL-12 and PD-1 dysregulation. THP-1 cells or PBMCs isolated from healthy subjects were preincubated with anti-gC1qR or control IgG overnight and then stimulated with LPS/R848/core for 18–72 h, followed by flow cytometric analysis of IL-12 and PD-1 expression. Representative overlay of graphs of PD-1 or IL-12 expression and summary data from five repeated experiments are shown.
Blocking the PD-1 pathway regulates SOCS-1 expression and rescues HCV core-induced IL-12 suppression in M/MFs

Because PD-1 and SOCS-1 are upregulated during HCV infection in vivo and by HCV core treatment ex vivo in M/MFs, which displayed impaired IL-12 production, we next sought to define the relationship between PD-1 and SOCS-1 upregulation as a potential mechanism underlying HCV core-mediated IL-12 inhibition. As an initial approach, we investigated whether blockade of the PD-1 pathway affected HCV core-induced SOCS-1 expression in M/MFs. Thus, THP-1 cells and M/MFs isolated from healthy or HCV-infected patients were preincubated with anti–PD-1, anti–PDL-1, or control IgG Abs overnight and then stimulated with LPS/R848 and HCV core protein for 48 h, followed by Western blotting to detect SOCS-1 protein expression. Incubation of THP-1 cells (Fig. 6A) or healthy M/MFs (Fig. 6B) with anti–PD-1 or anti–PDL-1 Ab, followed by TLR stimulation and core treatment, resulted in suppression of SOCS-1 compared with the control IgG treatment. Anti–PDL-1 Ab resulted in a greater blocking effect than did anti–PD-1 Ab; thus, it was used in M/MFs purified from multiple healthy subjects. As shown in Fig. 6C and 6D, HCV core-induced upregulation of SOCS-1 expression and IL-12 inhibition were abrogated by anti–PDL-1 treatment. Upregulated SOCS-1 in M/MFs during chronic HCV infection was also reduced by ex vivo incubation of the cells with anti–PDL-1, followed by TLR stimulation without core treatment (Fig. 6E); the data were reproducible in repeated experiments using M/MFs purified from three chronically HCV-infected patients. These data were in agreement with our observation that antiviral therapy and PDL-1 blockade resulted in a significant improvement in IL-12 production by restoring PD-1 and SOCS-1 expression in M/MFs from chronically HCV-infected patients (34), as well as reports of Th1/Tc1 functional recovery in chronically HCV-infected patients with

**FIGURE 6.** PD-1 signaling negatively regulates SOCS-1 expression in M/MFs treated with or without HCV core protein. A and B, Blocking PD-1 signaling inhibits SOCS-1 upregulation in M/MFs. THP-1 cells (A) or healthy M/MFs (B) were preincubated with anti–PD-1 or anti–PDL-1 or a control IgG Ab overnight and then stimulated with LPS/R848 and HCV core protein for 48 h, followed by Western blot detection of SOCS-1 expression. β-actin served as a loading control. C and D, Blocking PD-1 signaling in HCV core-treated healthy M/MFs inhibited SOCS-1 expression and improved IL-12 production. Upper panels, Representative experiment using M/MFs isolated from a healthy subject pretreated with anti–PDL-1 or a control IgG overnight and then stimulated with LPS/R848/core for 48 h, followed by detection of SOCS-1/β-actin expression by Western blot. Lower panel, Summary densitometry data of SOCS-1 downregulation by PDL-1 blockade, normalized to β-actin control, in M/MFs from three healthy subjects and summary data of IL-12 expression with various treatment in six healthy subjects. **p < 0.01. E, Blocking PD-1 signaling inhibits SOCS-1 expression in M/MFs from HCV-infected patients. Upper panel, Representative experiment using M/MFs isolated from an HCV-infected individual and pretreated with anti–PDL-1 or a control IgG overnight and then stimulated with LPS/R848/core for 48 h, followed by detection of SOCS-1/β-actin expression by Western blot. Lower panel, Summary densitometry data of SOCS-1 downregulation by PDL-1 blockade, normalized to β-actin control, in M/MFs from three HCV patients.
Affecting STAT-1 activation (28, 29, 34). To determine whether experiments.

IgG control. These data were reproducible in five repeated ex-

periments.

imunoprecipitated with anti–PD-1 or control IgG Ab, followed

treated with LPS/R848/core protein. To this end, cell lysates were

to STAT-1, we used flow cytometry to examine the amount of

phosphorylated STAT-5 in M/Mas pretreated with PDL-1 or con-
trol IgG overnight, followed by stimulation with LPS/R848/core for

18 h. As shown in Fig. 8D, blockade of PD-1 signaling im-

proved HCV core-induced inhibition of STAT-1 and STAT-5. We

previously demonstrated that HCV core protein, through in-
teraction with gC1qR, induced IL-8 expression in pulmonary

fibroblasts through activation of p38 MAPK (41). Interestingly,

STAT activation, but not MAPK p38 phosphorylation (data not

shown), was affected by blocking PD-1 signaling in core-treated

M/Mas, suggesting that HCV core regulates cell function through
distinct intracellular-signaling pathways that may be cell-type de-
pendent (Fig. 9).

Discussion

In this study, we analyzed the roles and relationships between PD-1 and SOCS-1, two negative immunomodulators induced by HCV core protein, in inhibiting TLR-mediated IL-12 production in M/Mas from healthy subjects and HCV-infected individuals. We found that PD-1 expression at baseline was increased and corre-
lated with the degree of IL-12 inhibition in M/Mas isolated from
chronically HCV-infected patients versus healthy subjects. Addi-
tionally, PD-1 expression was augmented and IL-12 expression was inhibited in M/Mas treated with HCV core protein in healthy
subjects, and they were further augmented and inhibited, re-
spectively, in HCV-infected patients, a phenomenon that could be
recapitulated by treatment with C1q and corrected by blocking
gC1qR, a receptor for HCV core protein. PD-1 upregulation and
IL-12 inhibition were also observed in highly purified M/Mas
cocultured with Huh-7 hepatocytes expressing live HCV. Import-
tantly, blocking the PD-1 pathway enhanced IL-12 production and
reduced the expression of SOCS-1 induced by HCV core pro-
tein; conversely, silencing SOCS-1 gene expression promoted
IL-12 secretion and inhibited PD-1, suggesting that these two
inhibitory molecules are mechanistically linked, which was con-
firmed by their physical association with coimmunoprecipitation.
Additionally, activation of the intracellular signaling pathways
STAT-1 and STAT-5, but not p38 MAPK, was involved in the
improvement of TLR-mediated IL-12 production in M/Mas upon
PD-1 blockade or SOCS-1 silencing. These data indicated that
HCV core/gC1qR engagement on M/Mas triggered the expression of
PD-1 and SOCS-1, which can associate and/or cross-talk with
each other to coordinately inhibit TLR-mediated IL-12 production
through the Jak/STAT pathway.

Antiviral CD4+ T cell responses are crucial for the resolution of viral infections by providing help for CD8+ T cell priming and B cell Ab generation. However, HCV-specific T and B cells are not detected until 1–3 mo postinfection, suggesting that there is a de-
lay in the generation of adaptive immunity during HCV infection
compared with other viruses. These data support the concept that the early stages of innate-immune responses against HCV are
impaired. Indeed, compelling studies showed that M/Mas or DCs
isolated from patients chronically infected with HCV display a
defective maturation and function, as well as a reduced capacity
to produce IL-12 during HCV infection (7–14). IL-12 is a 70–75-kDa heterodimeric cytokine composed of a 40-kDa H chain and a 35-kDa L chain. It is produced mainly by activated M/MFs, as well as myeloid DCs (mDCs), primarily in response to stimulation by bacterial or viral Ags via TLRs expressed on the cell surface. Microbial gene products also stimulate upregulation of costimulatory or inhibitory receptors on M/MFs or mDCs and modulate the secretion of IL-12. M/MF- or mDC-produced IL-12 induces various effects on NK and T cells, playing an essential role in the transition between innate- and adaptive-immune responses; this is particularly crucial for CD4+ T cell differentiation and the production of Th1 cytokines, such as IFN-γ (36, 42). Inhibition of IL-12 leading to impaired Th1 polarization and CTL responses represents a common mechanism for host immune evasion by several viral infections, including hepatitis C (7–18). However, the underlying mechanism(s) for IL-12 inhibition during viral infections remains largely elusive.

Although ample evidence supports the roles of PD-1/SOCS-1 in the negative regulation of adaptive-immune responses, less is known about their expression and function in innate cells and immunity. Recently, it was demonstrated that, in addition to T and B lymphocytes, PD-1 can be induced on murine macrophages or DCs by bacterial infection or TLR engagement, and SOCS-1 negatively regulates IL-12 production in a murine model (23–26). We previously showed that HCV core protein regulates T and B lymphocyte activation and M/MF IL-12 production through interaction with a complement receptor (gC1qR) (27–33). In this translational study, we further demonstrated that PD-1 and SOCS-1 are significantly upregulated in peripheral M/MFs by HCV core protein, and this upregulation is inversely associated with IL-12 production by PD-1+CD14+ cells in chronically HCV-infected patients compared with healthy subjects. Importantly, blockade of PD-1 signaling rescued HCV core-induced STAT-1 inhibition. M/MFs isolated from healthy subjects were treated with or without anti–PDL-1 or a control IgG Ab overnight and then stimulated with LPS/R848 in the presence or absence of HCV core for 48 h, followed by Western blot detection of phospho–STAT-1. Total STAT-1 served as loading control. PD-1 signaling improved STAT-1 and STAT-5 activation in M/MFs from HCV-infected patients. PBMCs isolated from chronically HCV-infected patients were preincubated with anti–PDL-1 or control IgG overnight, followed by stimulation with LPS/R848 for 18 h, and phosphorylated STAT proteins were examined by flow cytometric analysis. Representative dot plots of STAT-1 and STAT-5 and summary data from six HCV subjects are shown. *p < 0.05, **p < 0.01.

**FIGURE 8.** Phosphorylation of STAT protein was improved in SOCS-1–silenced THP-1 cells and PD-1–blocked human M/MFs. A, Coimmunoprecipitation of PD-1 and SOCS-1 in THP-1 cells stimulated with LPS/R848/core. The cell lysates were immunoprecipitated with anti–PD-1 or control IgG Ab overnight, followed by Western blot probed with anti–SOCS-1 Ab. Data were reproducible in five repeated experiments. B, Silencing SOCS-1 gene expression improved STAT-1 activation. THP-1 cells transfected with SOCS-1 siRNA or control siRNA, followed by stimulation with LPS/R848/core, were subjected to Western blot detection of phospho–STAT-1. Summary densitometry data of phospho–STAT-1, normalized to total STAT-1 protein, in SOCS-1 siRNA-transfected cells versus control siRNA-transfected cells. C, Blocking PD-1 signaling rescued HCV core-induced STAT-1 inhibition. M/MFs isolated from healthy subjects were treated with or without anti–PDL-1 or a control IgG Ab overnight and then stimulated with LPS/R848 in the presence or absence of HCV core for 48 h, followed by Western blot detection of phospho–STAT-1. Total STAT-1 served as loading control. D, Blocking PD-1 signaling improved STAT-1 (left panels) and STAT-5 (right panels) activation in M/MFs from HCV-infected patients. PBMCs isolated from chronically HCV-infected patients were preincubated with anti–PDL-1 or control IgG overnight, followed by stimulation with LPS/R848 for 18 h, and phosphorylated STAT proteins were examined by flow cytometric analysis. Representative dot plots of STAT-1 and STAT-5 and summary data from six HCV subjects are shown. *p < 0.05, **p < 0.01.
control of TLR-mediated STAT-1 activation and potentially promote viral clearance.

well as the relatively small number of subjects recruited in this study, such that it is more difficult to detect a statistically significant difference with TLR stimulation and/or HCV core treatment. However, interaction with PDL-1 is required for PD-1–mediated suppression of IL-12 production. Although PDL-1 was shown to be upregulated in various tissues and cells in response to viral infection, it may not be the rate-limiting molecule (like PD-1 receptor) in the dysregulation of M/Mϕ cytokine responses. One should bear in mind that in addition to the PD-1/PDL-1 engagement that may occur between M/Mϕ, it is feasible that the ample PDL-1 available on other cells/tissues, including hepatocytes and endothelial cells, interacts with upregulated PD-1 on M/Mϕ to signal IL-12 inhibition during chronic HCV infection. The molecular mechanism underlying PD-1/SOCS-1–mediated IL-12 inhibition by HCV core protein has yet to be clarified. It was proposed that PD-1 may suppress T cell responses via the immunoreceptor tyrosine inhibitory motif and/or the immunoreceptor tyrosine switch motif in its cytoplasmic tail, delivering a negative signal to the JAK/STAT pathway (43). In B cells, cross-linking of PD-1 along with the BCR leads to tyrosine phosphorylation of the PD-1 cytoplasmic domain, recruitment of SHP-2 phosphatase, and reduced phosphorylation of BCR proximal kinases (44). SOCS-1 was shown to bind to cytokine receptors or receptor-associated JAKs to inhibit the activation of STAT and kinases (44). SOCS-1 was shown to bind to cytokine receptors or

FIGURE 9. A model for HCV core/gC1qR-induced PD-1 and SOCS-1 upregulation in modulating IL-12 and T cell responses during HCV infection. Our data support the proposed notion that HCV core/gC1qR interaction upregulates PD-1 and SOCS-1 negative signaling molecules, which associate and/or cross-talk with each other and coordinate inhibitory TLR-related STAT-signaling pathways that mediate IL-12 production, leading to Th1/Tc1 dysregulation and HCV persistence. Blocking these inhibitory pathways may rejuvenate M/Mϕ activation and potentially promote viral clearance.

References

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Disclosures
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