RESEARCH ARTICLE

Hepatitis C virus core protein triggers expansion and activation of CD4$^+$CD25$^+$ regulatory T cells in chronic hepatitis C patients

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CD4$^+$CD25$^+$FoxP3$^+$ regulatory T cells (Tregs) are increased in patients with chronic hepatitis C, which may contribute to the sustained suppression of hepatitis C virus (HCV)-specific T-cell responses and viral persistence in HCV-infected individuals. We postulated that HCV core protein (HCVc) directly contributes to the expansion of Tregs in HCV-infected patients, and we provide evidence to support this hypothesis in the report. Peripheral blood mononuclear cells (PBMCs) and sera were collected from 87 treatment-naïve chronic HCV-infected patients, CD4$^+$CD25$^+$ Tregs were measured by flow cytometry, and HCV RNA and HCVc levels were detected using qPCR and enzyme-linked immunosorbent assay (ELISA), respectively. CD4$^+$, CD8$^+$, CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ T cells were purified from healthy donors and cultured with recombinant HCVc and Toll-like receptor (TLR) ligands. Flow cytometry was used to analyze cell proliferation, and ELISA was performed to measure cytokine production. In the 87 chronic HCV-infected patients, HCVc showed a significant correlation with HCV RNA and CD4$^+$CD25$^+$ Tregs. Mechanistic studies showed that HCVc, together with anti-CD3 antibody, augmented CD4$^+$CD25$^+$ Treg proliferation, but inhibited CD4$^+$CD25$^-$ T-cell proliferation and IFN-γ production, in a dose-dependent and Treg-dependent manner. Moreover, unlike the TLR3 ligand (poly I:C) and the TLR4 ligand (lipopolysaccharide, LPS), the TLR2 ligand (lipoteichoic acid, LTA) and HCVc both inhibited TCR-induced CD4$^+$ T-cell proliferation and IFN-γ secretion in a Treg-dependent manner. These data indicate that HCVc, like other TLR2 ligands, triggers CD4$^+$CD25$^+$ Treg activation and expansion to inhibit host immune responses, which may play a critical role in viral persistence in HCV-infected patients.

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INTRODUCTION

Chronic viral hepatitis due to hepatitis C Virus (HCV) is accompanied by immune failure. Features of this failure include the persistence of viremia, the exhaustion of effector T cells and the increased activity of regulatory T cells (Tregs).[1–5] However, the relationships between these biomarkers of immune failure are not entirely clear. We sought to clarify these relationships through a study of T cells isolated from a cohort of 87 HCV-infected patients.

We focused on the core protein of hepatitis C virus (HCVc) because it mediates diverse immunosuppressive effects. One of these actions is mediated by binding to the Toll-like receptor 2 (TLR2) pattern recognition receptor of Kupffer cells. In this context, HCVc induces diverse pro-inflammatory and anti-inflammatory cytokines but strongly inhibits the secretion of the anti-viral cytokines IFN-α and IFN-β and inhibits the upregulation of the TNF receptor-associated apoptosis-inducing ligand on the cell surface.[6] The HCVc protein also engages TLR2 on human dendritic cells, skewing their differentiation with increased expression of both macrophage markers, and PD-L1.[7]

Tregs isolated from HCV-infected patients are immunosuppressive and can inactivate both HCV-specific and bystander CD4$^+$ and CD8$^+$ T cells.[8,9] Here, we show that the concentration

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of HCVc in the blood of HCV-infected patients directly correlates with the frequency of Tregs. Furthermore, HCVc directly caused proliferation of Tregs and caused them to secrete immunosuppressive cytokines.

While it was previously documented that HCVc inhibits T-cell proliferation,[10] here we reveal the mechanism: HCVc inhibited CD4^{+} T-cell proliferation and IFN-γ secretion in a Treg-dependent manner. Both of these effects were also seen with the TLR2 ligand lipoteichoic acid (LTA). Therefore, we conclude that these effects of HCVc on T cells are also mediated by TLR2.

These results clarify the multifaceted problem of immunosuppression and exhaustion in the context of chronic HCV infection and may also shed light on the immunology of other chronic infections, such as hepatitis B Virus infection, in which exhausted effector T cells co-exist with Tregs.[11] Therapeutic interventions to reverse exhaustion and initiate immunological self-cure in chronic hepatitis patients depend on a full understanding of these mechanisms.

MATERIALS AND METHODS

Samples
Eighty-seven treatment-naive chronic HCV-infected patients were enrolled in this study (Table 1). Venous blood was withdrawn for serum and peripheral blood mononuclear cells (PBMCs) collection. These studies were approved by the IRB of Jilin University, The First Hospital. Buffy coats from five healthy donors were provided by the Changchun Blood Center, and informed consent was provided according to the protocols of the Changchun Blood Center.

Serum viral load and HCVc level assay
HCV RNA was detected using the Cobas-TaqMan assay or Amplicor-HCV-Monitor (Roche Diagnostics, Mannheim, Germany). HCVc was quantified by automated immunoassay (i System i2000, Architect; Abbott, Wiesbaden, Germany).

HCVc, TLR agonists and reagents
Recombinant HCVc (aa 2–192 of the HCV polyprotein) was purchased from Biodesign (Saco, ME, USA); TLR agonists LTA, poly I:C and lipopolysaccharide (LPS) were purchased from Invivogen (San Diego, CA, USA).

Limulus amebocyte lysate assay for detection of LPS contamination
The QCL-1000 chromogenic LAL endpoint assay (Cam-brex, Cottonwood, AZ, USA) was used to detect endotoxin contamination of HCVc following the manufacturer's protocol as described.[6,7] Briefly, protein-free, phenol-water extracted ultrapure Escherichia coli LPS was used to create a linear standard curve from 1 to 100 pg/ml. HCVc was serially diluted to determine a concentration of agonist within the standard curve of the assay. HCVc preparations were determined to have <1 pg/ml LPS contamination.

Isolation of T lymphocyte subsets from peripheral blood
PBMCs were isolated by Ficoll/Paque density gradient centrifugation. For magnetic cell sorting, the CD8 Multisort kit (Miltenyi Biotec, Bergisch Gladbach, Germany) was used for the purification of CD8^{+} T cells without ligation of any of their surface molecules. The CD4^{+}CD25^{+} Treg Isolation kit was used for the purification of CD4^{+} T cells, CD4^{+}CD25^{−} T cells and CD4^{+}CD25^{+} Tregs. The purity of the T-cell subsets was determined by flow cytometry and was always in the 90%–95% range.

Enzyme-linked immunosorbent assay (ELISA)
Concentrations of IFN-γ, IL-10 and TGF-β in cell culture supernatants were measured by ELISA according to the manufacturer's instructions.[6,7]

T lymphocyte subsets proliferation assays
CD8^{+}, CD4^{+}, CD4^{+}CD25^{+} Treg and CD4^{+}CD25^{−} T- cell subsets were stained with 5 μM CFSE (Molecular Probes, Grand Island, NY. USA) according to the manufacturer's recommended protocol. CD3 activation was performed by using 10 μg/ml OKT3 (CD3 mAb; eBioscience, San Diego, CA. USA) bound to the plastic in the wells for subsequent cross-linking of the T-cell receptors on responding T cells. Plates could also be prepared the night before an experiment and kept in the refrigerator overnight. CFSE-labeled T lymphocyte subsets were cultured in wells pre-coated with OKT3 in the presence of HCVc and/or TLR agonists. After 5 days, when clumps were visible, cells were collected and stained with anti-CD25-APC (BD Biosciences, San Jose, CA, USA), and the proliferating T lymphocyte subsets were characterized by flow cytometry.

Flow cytometry
Staining of cells and analysis on a flow cytometer (FACScan; BD Biosciences) were done as described.[6,7] CD4^{+}CD25^{+} Tregs were identified using the following anti-human mAb: FITC Mouse Anti-Human CD4 (Clone: RPA-T4), APC Mouse Anti-Human CD25 (Clone: M-A251) and PE Mouse Anti-Human FoxP3 (Clone: 259D/C7). Intracellular FoxP3 staining

Table 1 Characteristics of study population

	HCVc serum level (fmol/l)		
	<1000	1000–5000	>5000
Age, years (mean±s.e.m.)	48.9±15.1	45.5±10.9	48.2±16.44
Sex Female/male	20/24	11/13	8/11
ALT, IU/ml (mean±s.e.m.)	71.21±9.36	68.24±20.73	60.34±13.94
HCV-DNA (mean±s.e.m.)	4.86×10⁵ ±1.98×10⁵	3.05×10⁶ ±6.01×10⁵	1.11×10⁷ ±2.53×10⁶
HCV genotype (2a/1b/n.a)	29/11/4	13/10/1	14/5/0

Abbreviations: n.a.: not applicable; SEM: standard error of mean.

and cell surface CD4 and CD25 staining were performed according to the manufacturers recommended protocol. All the antibodies were obtained from BD Biosciences. The data acquired were analyzed with FlowJo (Treestar software, Ashland, OR, USA).

Statistical analysis

All data were analyzed and found to be significant using the D'Agostino and Pearson omnibus normality test. Mean values were compared using either a paired t-test (two groups) or ANOVA ($>$two groups), followed by a Bonferroni correction for multiple comparisons test. P values $<$0.05 were considered to be significant. All statistical tests were performed by Prism software (GraphPad, San Diego, CA, USA).

RESULTS

HCVc correlation with HCV RNA and CD4$^+$CD25$^+$ Tregs

A cohort of 87 treatment-naive chronic HCV-infected patients was enrolled in this study and divided into three groups based on serum HCVc levels (44 with HCVc levels $<$1000 fmol/l, 24 with HCVc levels between1000 and 5000 fmol/l and 19 with HCVc levels $>$5000 fmol/l) (Table 1). Serum HCV
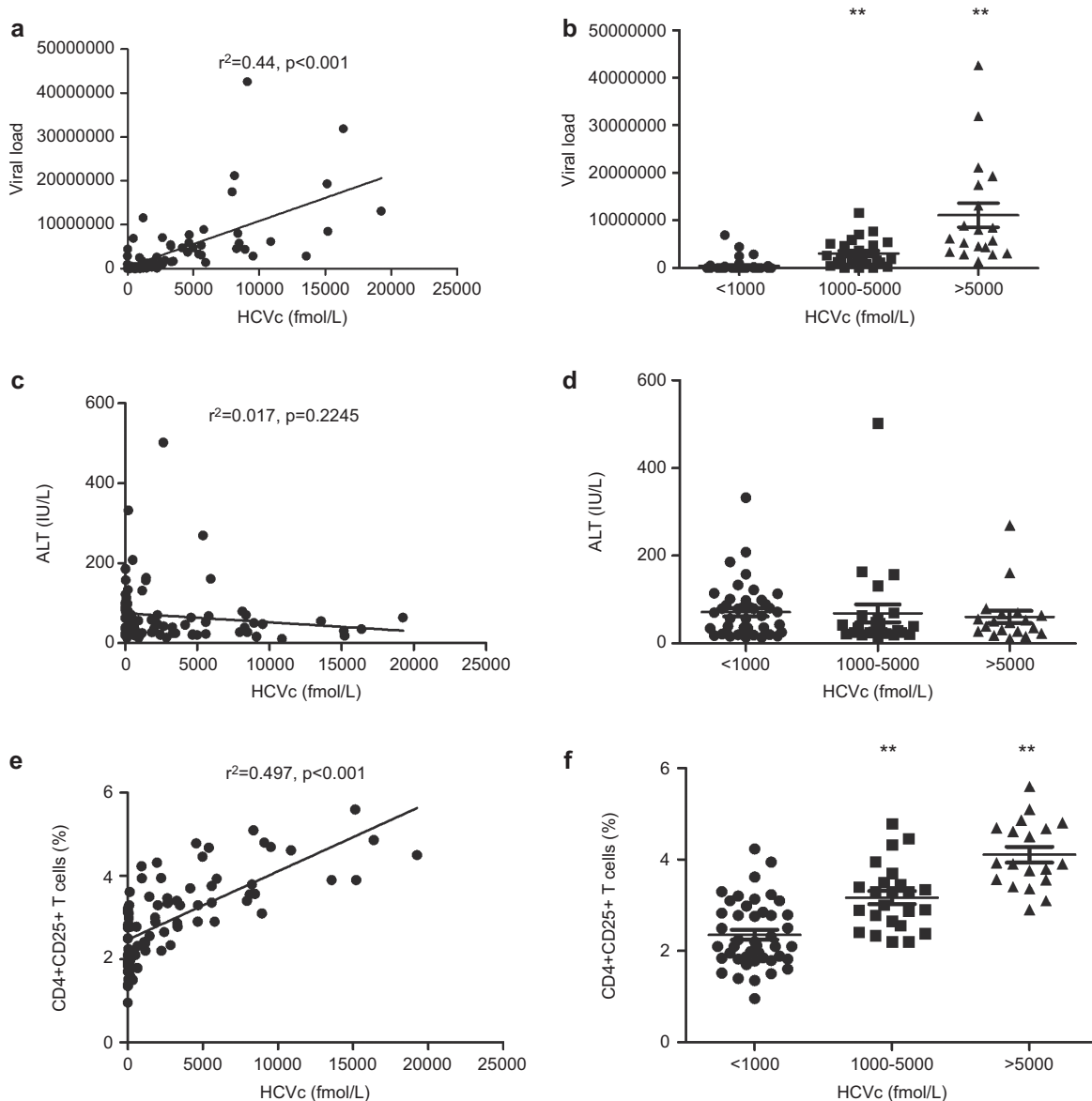

Figure 1 Correlation of HCVc levels with that of HCV RNA and CD4$^+$CD25$^+$ Tregs. Data are shown for 87 treatment-naive chronic HCV-infected patients. Serum HCV RNA and HCVc levels were detected using qPCR and ELISA, respectively. The frequency of CD4$^+$CD25$^+$ Tregs from PBMCs was measured by flow cytometry. A positive correlation was shown between the serum level of HCVc and HCV RNA (r^2=0.44, P<0.001) (**a**). The 87 chronic HCV-infected patients were divided into three groups according to serum HCVc levels (19 with HCVc level $>$5000 fmol/l, 24 with HCVc level 1000–5000 fmol/l and 44 with HCVc level $<$1000 fmol/l). The correlation between serum HCVc levels and the serum level of HCV RNA (**b**), ALT (**c**) or the frequency of CD4$^+$CD25$^+$ Tregs from PBMCs (**d**), was also analyzed. HCV, hepatitis C virus; HCVc, HCV core protein; PBMC, peripheral blood mononuclear cell; Treg, regulatory T cell.

RNA and HCVc levels were detected using qPCR and ELISA, respectively.

Figure 1a and b show that HCVc levels positively correlate with HCV RNA levels ($r^2 = 0.44$, $P < 0.001$), but not with ALT levels ($r^2 = 0.017$, $P = 0.2245$) (Figure 1c and d), indicating that HCVc concentration represents a stable and reliable marker of HCV viral replication. $CD4^+CD25^+$ Tregs from PBMCs of chronic HCV-infected patients were measured by flow cytometry. Intracellular FoxP3 staining showed that 87% of the $CD4^+CD25^+$ Tregs, 11% of the $CD4^+CD25^{dim}$ cells and 0.81% of the $CD4^+CD25^-$ cells are $FoxP3^+$ (Supplementary Figure 1). The frequency of $CD4^+CD25^+$ Tregs was positively correlated with serum HCVc levels ($r^2 = 0.497$, $P < 0.001$) (Figure 1e). Further analysis shows that the frequency of $CD4^+CD25^+$ Tregs was significantly higher among the 19 chronic HCV-infected patients with HCVc levels >5000 fmol/l (4.107 ± 0.1693, $N = 19$) than in the 24 with HCVc levels between 1000 and 5000 fmol/l (3.167 ± 0.1452, $N = 24$) ($P < 0.001$) and the 44 with HCVc levels <1000 fmol/l (2.352 ± 0.1093, $N = 44$) ($P < 0.001$) (Figure 1f and Supplementary Figure 2). These results suggest that the HCV-encoded core protein circulating in the blood may induce the expansion of natural $CD4^+CD25^+$ Tregs or stimulate their generation from $CD4^+$ T cells.

HCVc induces proliferation of $CD4^+CD25^+$ Tregs

In order to analyze the effects of HCVc on the proliferation of $CD4^+CD25^+$ Tregs, freshly isolated $CD4^+$, $CD4^+CD25^+$ Tregs and $CD4^+CD25^-$ T cells from healthy HCV-negative blood donors were activated by anti-CD3 mAb for 5 days in the presence of increasing concentrations of HCVc protein. Our results show that HCVc induced $CD4^+CD25^+$ Treg proliferation in a dose-dependent manner but inhibited total $CD4^+$ T-cell proliferation (Figure 2b). However, depletion of $CD4^+CD25^+$ Tregs from the total $CD4^+$ T-cell population abrogated the inhibitory effect of HCVc on anti-CD3-induced $CD4^+$ T-cell proliferation (Figure 2a and b) supplementary Figure 3. Additionally, HCVc alone did not induce T-cell proliferation (data not shown). These data suggest that the HCVc protein may stimulate Tregs to actively suppress other $CD4^+$ T cells.

HCVc induced production of IL-10 and TGF-β in $CD4^+CD25^+$ T cells and inhibited IFN-γ production in $CD4^+$ T cells through $CD4^+CD25^+$ Tregs

To test whether HCVc affected Treg function and to assess its effects on IFN-γ secretion from other T cells, freshly isolated HCV-negative, human $CD4^+$, $CD4^+CD25^+$ Tregs and $CD4^+CD25^-$ T cells were activated by anti-CD3 mAb in the
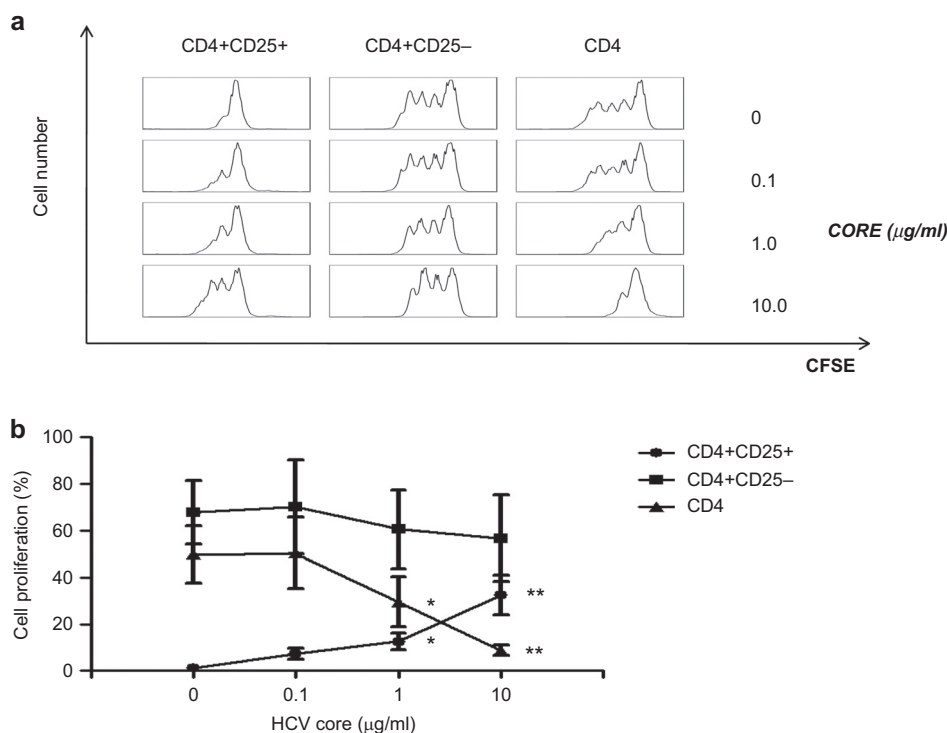

Figure 2 HCV core protein induces $CD4^+CD25^+$ T-cell proliferation. Primary $CD4^+$ T cells were isolated from peripheral blood of healthy donors by using a CD4 isolation kit first, and further purification of $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells was performed by using a Treg isolation kit according to the manufacturer's instructions. The purified total $CD4^+$, $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were labeled with CFSE and then cultured for 5 days in plates pre-coated with OKT3 (CD3 mAb) and various concentrations of HCVc. Flow cytometry was used to analyze cell proliferation. Representative experiment results from one donor out of five are shown (**a**) and the percentage of cell proliferation as mean±s.d. from five separate experiments are also shown (**b**) ($n = 5$, *$P < 0.05$, **$P < 0.01$). HCV, hepatitis C virus; HCVc, HCV core protein; Treg, regulatory T cell.

absence or presence of HCVc for 2 days. We found that HCVc inhibited TCR-induced IFN-γ secretion of CD4$^+$ T cells in a dose-dependent manner, and depletion of CD4$^+$CD25$^+$ Tregs from the CD4$^+$ T cells abrogated the inhibitory effect of HCVc on IFN-γ production. HCVc did not induce IFN-γ production in CD4$^+$CD25$^+$ Tregs (Figure 3a). More importantly, we also found that HCVc induced IL-10 and TGF-β secretion by CD4$^+$CD25$^+$ Tregs, but showed no effect on cytokine production by CD4$^+$CD25$^-$ T cells (Figure 3b and c).

HCVc and LTA inhibit the proliferation of CD4$^+$ T cells in a Treg-dependent fashion

HCVc is a ligand for TLR2, which induces the production of inflammatory cytokines by activating the MyD88-dependent TLR signaling pathway.[12] Therefore, we further examined whether HCVc triggers the expansion and activation of CD4$^+$CD25$^+$ regulatory T cells *via* TLR2 signaling. CD4$^+$ T cells and CD8$^+$ T cells were purified from the peripheral blood of healthy donors by CD4 and CD8 microbeads and then activated by anti-CD3 mAb and ligands of TLR2, TLR3 and TLR4 (LTA, poly I:C and LPS, respectively) in the absence or presence of HCVc. The results showed that the TLR2 ligand LTA, like HCVc, inhibited the proliferation of CD4$^+$ T cells, but poly I:C and LPS did not affect CD4$^+$ T-cell proliferation (Figure 4a and c). In addition, neither HCVc nor any of the ligands of TLR2, TLR3 and TLR4 affected the proliferation of purified CD8$^+$ T cells (Figure 4b and d).

LTA and HCVc inhibit IFN-γ production in TCR-activated CD4$^+$ T cells

To explore whether LTA also inhibits IFN-γ secretion in CD4$^+$ T cells through CD4$^+$CD25$^+$ Tregs, total CD4$^+$ T cells and CD4$^+$CD25$^-$ T cells were purified from healthy HCV-negative donors and activated by anti-CD3 mAb and TLR2, TLR3 and TLR4 ligands for 2 days in the presence of HCVc as described above. We found that neither TLR2, TLR3 and TLR4 ligands nor HCVc alone induced CD4$^+$ T-cell IFN-γ production, whereas anti-CD3 mAb induced IFN-γ secretion from both CD4$^+$ and CD4$^+$CD25$^-$ T cells (Figure 5). However, LTA and HCVc inhibited anti-CD3 mAb-induced IFN-γ secretion from CD4$^+$ T cells, but not from CD4$^+$CD25$^-$ T cells. Poly I:C and LPS did not affect IFN-γ secretion in TCR-activated CD4$^+$ T cells. Therefore, HCVc inhibited CD4$^+$ T-cell activation in a Treg-dependent fashion.

DISCUSSION

CD4$^+$CD25$^+$FoxP3$^+$ Tregs are increased in patients with chronic hepatitis C, and this may mediate the sustained suppression of HCV-specific T-cell responses and the viral persistence in HCV-infected individuals.[13–15] The mechanism of Treg proliferation and its activity in HCV infection are not completely understood. In the current study, we confirm that HCVc showed a significant, positive correlation with HCV RNA and the frequency of CD4$^+$CD25$^+$ Tregs in 87 chronic HCV-infected patients. Furthermore, we evaluated the role of HCVc in Treg proliferation and suppressive functions and
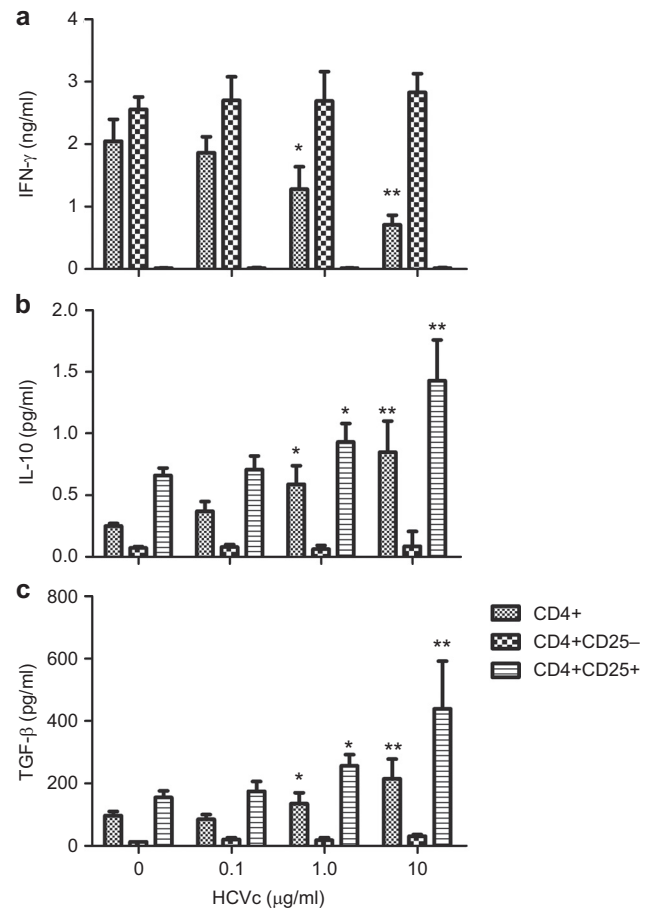
Figure 3 HCVc induces IL-10 and TGF-β production in CD4$^+$CD25$^+$ T cells and inhibits IFN-γ production in CD4$^+$ T cells through CD4$^+$CD25$^+$ T cells. Total CD4$^+$, CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ T cells were cultured for 2 days in wells pre-coated with OKT3 (CD3 mAb) and various concentrations of HCVc. The supernatants were then harvested and assayed by ELISA for IFN-γ (**a**), IL-10 (**b**) and TGF-β (**c**) levels ($n=5$, *$P<0.05$, **$P<0.01$). HCVc, HCV core protein.

found that HCVc induced CD4$^+$CD25$^+$ Treg proliferation and IL-10 and TGF-β secretion in a dose-dependent manner. Moreover, HCVc inhibited CD4$^+$ T-cell proliferation and IFN-γ production in a Treg-dependent fashion. In addition, a TLR2 agonist, LTA, inhibited CD4$^+$ T-cell proliferation and IFN-γ production to the same extent as HCVc did, but poly I:C (TLR3 agonist) and LPS (TLR4 agonist) did not. These results suggest that HCVc, as a ligand for TLR2, may play a role in activating Treg proliferation and inducing their suppressive function. This mechanism may contribute to the sustained suppression of HCV-specific T-cell responses and the consequently persistent HCV infection.

HCV has developed different strategies to evade the host's innate and adaptive immune responses. HCV has been shown to interfere with innate antiviral defenses through multiple mechanisms. HCVc protein induces the expression of SOCS-3, which suppresses JAK-STAT signaling. Also, the NS3/4A protease antagonizes IRF-3 activation and interferon-β
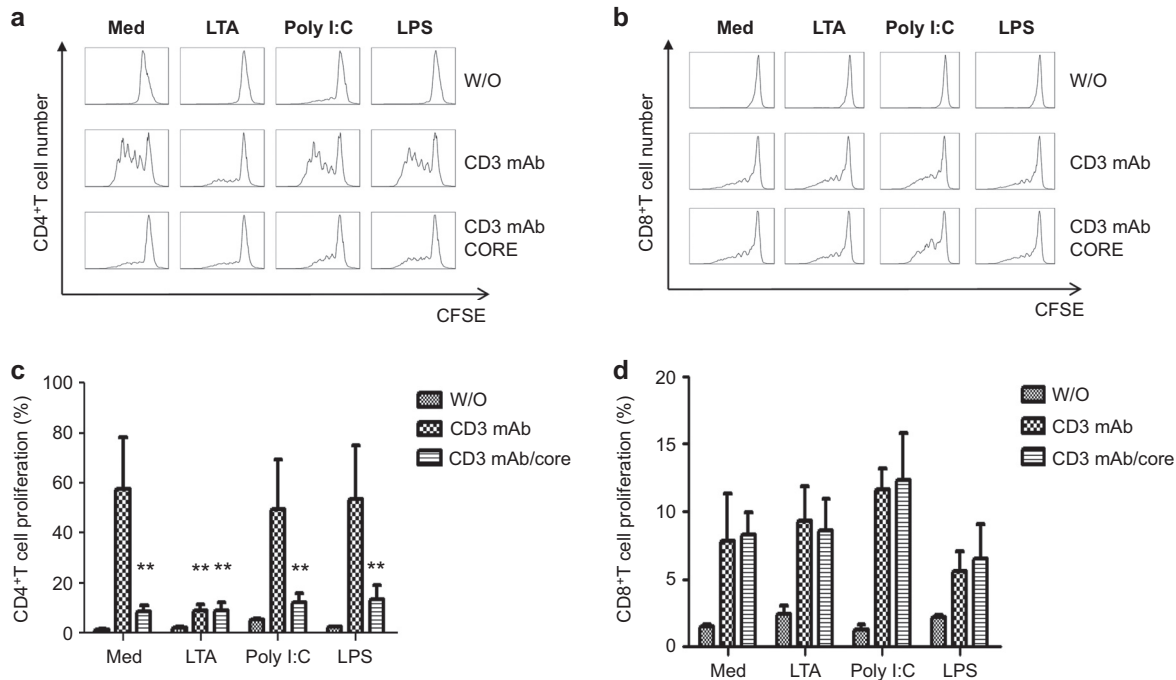
Figure 4 Both HCVc and LTA inhibit the proliferation of CD4$^+$ T cells but not CD8$^+$ T cells. Primary CD4$^+$ T cells and CD8$^+$ T cells were purified, and CFSE-labeled CD4$^+$ and CD8$^+$ T cells were cultured for 5 days in wells pre-coated with OKT3 (CD3 mAb) and HCVc in the presence of different TLR ligands. Flow cytometry was used to analyze cell proliferation. Representative experiment results from one donor out of five are reported in (**a**) and (**b**), and the percentage of proliferating CD4$^+$ and CD8$^+$ T cells and mean±s.d. from five separate experiments are shown as (**c**) and (**d**) (n=5, *P<0.05, **P<0.01). HCVc, HCV core protein; LTA, lipoteichoic acid; TLR, Toll-like receptor.

expression by blocking RIG-I and TLR3 signaling.[16,17] In addition, HCV proteins have been shown to upregulate TLR2 and TLR4 expression on Raji cells and peripheral blood mononuclear cells, thereby modulating the cell's pro-inflammatory response.[18,19] Moreover, several studies by our group and others

Figure 5 HCVc and LTA inhibit IFN-γ production by activated CD4$^+$ T cells in a Treg-dependent fashion. Purified CD4$^+$ and CD8$^+$ T cells were cultured with pre-coated OKT3 (CD3 mAb) and HCVc and TLR ligands for 2 days. The supernatants were then harvested and assayed by ELISA for IFN-γ levels (n=5, *P<0.05, **P<0.01). HCVc, HCV core protein; LTA, lipoteichoic acid; Treg, regulatory T cell; TLR, Toll-like receptor.

have provided evidence that the HCV core can activate TLR2 on human monocytes, macrophages and Kupffer cells, which induces production of inflammatory cytokines by activating the MyD88-dependent TLR signaling pathway.[6,12,19] An effect of HCV core expression on CD4$^+$ T cells has been reported, showing that this protein induces a state of unresponsiveness similar to clonal anergy or T-cell exhaustion.[20–22] HCVc can bind the human complement receptor C1qR, thus inhibiting T-cell responses.[23,24] More recently, using a lentiviral vector to express HCVc in CD4$^+$ Jurkat T cells, Dominguez-Villar et al.[25] report that FoxP3 and CTLA-4 (cytotoxic T-lymphocyte antigen-4) were upregulated in HCVc-expressing Jurkat cells and that HCVc-transfected Jurkat cells were able to suppress CD4$^+$ and CD8$^+$ T-cell responses to anti-CD3 plus anti-CD28 stimulation.[25] In agreement with these findings, our results showed that HCVc inhibited CD4$^+$ T-cell proliferation and IFN-γ production in the presence of anti-CD3 mAb, and further mechanistic studies showed that HCVc triggers CD4$^+$CD25$^+$ Treg expansion and activation to mediate the inhibition.

Our results shed new light on the expansion of Treg cells by HCVc and provide evidence that this HCV protein is responsible for enhanced Treg activity in the periphery during chronic HCV infections. Interestingly, the canonical TLR2 ligand lipoteichoic acid had the same effect on Tregs as HCVc, suggesting that other pathogens that engage TLR2 may also induce the expansion and activation of CD4$^+$CD25$^+$ Tregs.
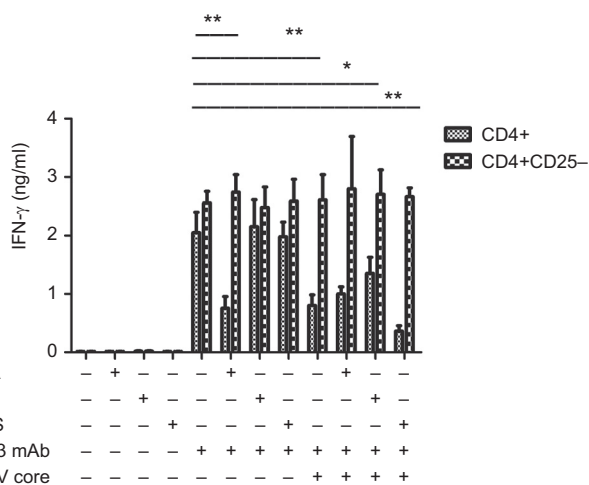
CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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