Up-regulation of FOXP3 and induction of suppressive function in CD4⁺ Jurkat T-cells expressing hepatitis C virus core protein

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ABSTRACT

HCV (hepatitis C virus) infection is a serious health care problem that affects more than 170 million people worldwide. Viral clearance depends on the development of a successful cellular immune response against the virus. Interestingly, such a response is altered in chronically infected patients, leading to chronic hepatitis that can result in liver fibrosis, cirrhosis and hepatocellular carcinoma. Among the mechanisms that have been described as being responsible for the immune suppression caused by the virus, Treg-cells (regulatory T-cells) are emerging as an essential component. In the present work we aim to study the effect of HCV-core protein in the development of T-cells with regulatory-like function. Using a third-generation lentiviral system to express HCV-core in CD4⁺ Jurkat T-cells, we describe that HCV-core-expressing Jurkat cells show an up-regulation of FOXP3 (forkhead box P3) and CTLA-4 (cytotoxic T-lymphocyte antigen-4). Moreover, we show that HCV-core-transduced Jurkat cells are able to suppress CD4⁺ and CD8⁺ T-cell responses to anti-CD3 plus anti-CD28 stimulation.

INTRODUCTION

HCV (hepatitis C virus) infection is a serious health care problem affecting more than 170 million people worldwide. HCV infection leads to chronicity in more than 80% of the patients, resulting in liver fibrosis, cirrhosis and hepatocellular carcinoma [1]. HCV is an enveloped, positive single-stranded RNA virus that belongs to the Flaviviridae family. Its genome is translated into a single polyprotein which gives rise to ten different proteins. Core, spanning nucleotides 1–573, is the first to be translated and codes for the viral nucleocapsid [2]. Although the primary HCV target is the hepatocyte, there is increasing evidence showing that the virus is also able to infect tissues such as heart [3] as well as cells from the immune system such as Jurkat and Molt-4 CD4⁺ T-cell lines [4], as well as PBMCs (peripheral blood mononuclear cells) in vitro [5–8] and lymph nodes in vivo [9,10]. We have also shown recently that HCV RNA is detected in peripheral blood CD4⁺ T-cells from chronically infected patients [11]. Adaptive immune responses are pivotal for the outcome of HCV infection, with T-cells playing an essential role in viral clearance [12–15]. Viral clearance has been associated with a strong and multispecific T-cell response in acute infections [16–18], whereas chronically infected patients show a weak, short-lived, dysfunctional or even absent HCV-specific T-cell response [19–23].

Key words: CD4, hepatitis C virus, forkhead box P3 (FOXP3), immune evasion, immune tolerance, regulatory T-cell.

Abbreviations: APC, allopachocyanin; CsA, cyclosporine A; Cₜ, comparative threshold; CTLA-4, cytotoxic T-lymphocyte antigen-4; DMEM, Dulbecco’s modified Eagle’s medium; DTT, dithiothreitol; FBS, fetal bovine serum; FOXP3, forkhead box P3; GFP, green fluorescent protein; GITR, glucocorticoid-induced tumour necrosis factor receptor; HCV, hepatitis C virus; HEK, human embryonic kidney; HRP, horseradish peroxidase; IL-2, interleukin-2; MFI, mean fluorescence intensity; NEAA, non-essential amino acid; NFAT, nuclear factor of activated T-cells; PBMC, peripheral blood mononuclear cell; PE, phycoerythrin; Treg, regulatory T-cell; nTreg, natural Treg.

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Regarding virus mediated T-cell dysfunction; we and others have reported the effect of HCV-core expression in CD4+ T-cells, showing that this protein induces a state of unresponsiveness similar to clonal anergy [11,24].

There are several additional mechanisms that have been suggested to contribute to CD4+ T-cell unresponsiveness during chronic HCV infection, among which suppression of T-cell function by CD4+CD25 Treg− cells (regulatory T-cells) is emerging as one of the most important [25–32].

It is widely accepted that CD4+CD25high Treg− cells can be divided into nTreg− cells (natural Treg− cells) and induced (or adaptive) Treg− cells [33]. nTreg− cells develop in the thymus as a defined population [34] and constitutively express CTLA-4 (cytotoxic T-lymphocyte antigen-4) [35], GITR (glucocorticoid-induced tumour necrosis factor receptor) [36] and FOXP3 (forkhead box P3), with the latter being the marker that better identifies Treg− cells [37–39]. Adaptive Treg− cells from which several subpopulations have been identified [40], develop in the periphery from naive CD4+CD25− T-cells under certain environmental conditions [41]. There are various reports in the literature showing that there is a higher frequency of T-cells with regulatory function in peripheral blood of chronically infected patients compared with healthy or recovered controls [26]. Treg− cells from HCV-infected individuals have been shown to inhibit HCV-specific and non-specific CD4+ and CD8+ T-cell responses [30,31,42]. However, it is not clear whether these Treg− cells are originated in the thymus and/or arise from CD4+ T-cells in the periphery upon HCV antigen contact, or upon internalization of viral particles or proteins. In this work we aim to study the effect of HCV-core protein in the development and function of cells with regulatory function. Using a third-generation lentiviral packaging system to express HCV-core in CD4+T-cells, showing that this protein induces a state of unresponsiveness similar to clonal anergy [11,24].

** MATERIALS AND METHODS **

**Cell culture**

Jurkat cells (A.T.C.C.) were maintained in RPMI 1640 medium containing 2 mM l-glutamine, 10 mM Heps, 10% (v/v) heat-inactivated FBS (fetal bovine serum), 1% (v/v) NEAA (non-essential amino acid), 1% (v/v) sodium pyruvate, 50 μM 2-mercaptoethanol, 100 units/ml penicillin, 100 μg/ml streptomycin, at 37°C, 5% CO2. HEK (human embryonic kidney)-293-FT cells (Invitrogen) were maintained in DMEM (Dulbecco’s modified Eagle’s medium) supplemented with 10% (v/v) FBS, 10 mM Heps, 100 units/ml penicillin, 100 μg/ml streptomycin at 37°C, 10% CO2.

**PBMC and CD4+/CD8+ T-cell purification**

Human peripheral blood samples were obtained from healthy donors with informed consent of the subjects and following ethical approval. PBMC were purified by density gradient centrifugation using Ficoll-Paque (Amersham Pharmacia Biotech). CD4+/CD8+ T-cells were enriched by positive immunomagnetic selection (CD8/CD4 Cell Adembeads™; Ademtech) resulting in over 95% purity as assessed by FACS staining (CyanADP-MLE™; DakoCytomation). CD4+ T-cells were resuspended at 106 cell/ml in DMEM containing 10% (v/v) FBS, 1% (v/v) NEAA, 1% (v/v) sodium pyruvate, 2 mM l-glutamine, 10 mM Heps, 50 μM 2-mercaptoethanol, 100 μg/ml streptomycin, 100 units/ml penicillin. IL-2 (interleukin-2; 40 units/ml) was added to the cultures every other day for a total of 6 days before performing the experiments.

**Lentiviral plasmid construction and packaging system**

HCV-core gene, comprising the first 191 amino acids of the HCV polyprotein, was amplified by PCR from a vector containing the complete HCV genome from the H77 strain (serotype 1a, provided by Charles M. Rice, Laboratory of Virology and Infectious Disease, The Rockefeller University, New York, U.S.A.), using the following primers: sense 5’-GGATTCC-ATATGACCAATCTTGGTAGGCTGAAGGATGTAGGCGTGG-3’ and antisense 5’-CGCGGGATCCACCTTGGTAGGCTGAAGCGGCGG-3’. Amplicons were subcloned in frame with the GFP (green fluorescent protein) gene into an NdeI/BamHI site of the third-generation lentiviral transfer plasmid pHRSINcPPTSEWGF.

**Production of lentiviral supernatant and transduction of Jurkat cells**

HEK-293-FT cells were used as packaging cell line to produce lentiviral supernatant by co-transfecting the corresponding transfer vector (pHRSINcPPT-SEWcore-GFP or pHRSINcPPTSEWGF) with plasmids encoding for HIV-1 GAG-POL proteins (pCMVΔR8.91) and a plasmid coding for the VSVG (vesicular stomatitis virus G) protein pMD2.G. Transfection was performed using Lipofectamine™ 2000 (Invitrogen), according to manufacturer’s guidelines. Lentiviral supernatants were collected at 48 and 72 h post-infection. Transfection efficiency was evaluated by FACS analysis.

**Lentiviral transduction**

Jurkat cells were plated at a density of 5 x 10⁵ cells/ml, and cells were cultured for 48 h upon addition of lentiviral supernatants. Infection efficiency was analysed by FACS.
(CyanADP-MLE; DakoCytomation), being > 95% in all experiments.

**Nuclear protein extracts**

Nuclear extracts were purified from 4 × 10^7 cells. Cells were collected and resuspended in a buffer containing 10 mM Tris/HCl, pH 7.5, 10 mM NaCl, 3 mM MgCl₂, 1 mM DTT (dithiothreitol) and protease inhibitors, and incubated on ice for 10 min. Nonidet P40 was added at a final concentration of 0.05% and cells were centrifuged for 5 min at 1800 g. Nuclei were resuspended in a buffer containing 20 mM Hepes with Immobilon™ Western Chemiluminescent HRP antibody (Bio-Rad Laboratories). Bands were detected conjugated secondary goat anti-mouse polyclonal AbCam), followed by an HRP (horseradish peroxidase)-anti-human FOXP3 antibody (Clone mAbcam 22510; collected.

**SDS/PAGE and Western blot analysis**

To analyse FOXP3 expression, nuclear extracts were resolved by SDS/PAGE (10% gel) and transferred on to a PVDF membrane (Immobilon-PTM; Millipore). FOXP3 was detected with 1 μg/ml monoclonal mouse anti-human FOXP3 antibody (Clone mAbcam 22510; AbCam), followed by an HRP (horseradish peroxidase)-conjugated secondary goat anti-mouse polyclonal antibody (Bio-Rad Laboratories). Bands were detected with Immobilon™ Western Chemiluminescent HRP Substrate (Millipore), and images were obtained by means of a VersaDoc Imaging System (Bio-Rad Laboratories). Relative band intensities were analysed using the Quantity One™ software (Bio-Rad Laboratories). To control for equivalent loading, membranes were stripped in a buffer containing 2% (w/v) SDS, 62.5 mM Tris/HCl, pH 6.8, and 0.8% 2-mercaptoethanol, pre-heated at 50 °C for 45 min with continuous shaking, washed with PBS and subsequently stained in a buffer containing Coomassie solution [0.1% Coomassie Brilliant Blue, 50% (v/v) methanol and 7% (v/v) acetic acid] for 2 min at room temperature (22 °C).

**RNA purification and cDNA synthesis**

Total RNA was extracted from HCv-core- or GFP-expressing Jurkat cells using TRI reagent (Sigma), following the manufacturer’s recommendations. Total RNA was precipitated with isopropyl alcohol and washed twice with 70% (v/v) ethanol. RNA concentration and quality was assessed by spectrophotometry (Smart Spec; Bio-Rad Laboratories) and agarose-formaldehyde gel electrophoresis respectively. cDNA was synthesized from total RNA using iScript cDNA synthesis kit (Bio-Rad Laboratories) following manufacturer’s recommendations.

**Real-time PCR**

FOXP3 mRNA expression was quantified by real time PCR using SYBR Green I supermix (Bio-Rad Laboratories) in an iCycler (Bio-Rad Laboratories). L32 was used as an internal control for data normalization. Primer sequences used are as follows: FOXP3 forward: GAAAGCGACATTCCCA-GAGTTT; reverse: ATGCCCCAGGGATGAG; L32 forward: CCGAGTTCTCGTTCCACA; reverse: GCACATCGACGACTTCCA. Purity of the amplified sequences was checked by melting curve analysis and agarose gel electrophoresis. For quantification, a threshold was set in the linear region of the amplification curve, and the number of cycles needed to reach it was calculated for each sample [Ct (comparative threshold)]. Real-time PCRs were run in parallel with cDNA from untransduced Jurkat cells, or cells that had been transduced with GFP or HCV–GFP. For each condition, a CsA (cyclosporine A)-treated sample was also included. Values are represented as the difference in Ct values normalized to L32 for each sample as per the following equation: relative RNA expression = (2^-ΔΔCt)×100.

**Cell-surface receptor staining**

Staining of cell-surface receptors was performed with APC (allophycocyanin)-Cy7-labelled mouse anti-human CD25, PE (phycoerythrin)-Cy7-labelled mouse anti-human CD8, biotin-conjugated mouse anti-human CD4 and streptavidin-PE, APC-conjugated anti-(human CTLA-4) (all from BD Biosciences) and APC-labelled anti-(human GITR) (eBiosciences). For CD25, CTLA-4 and FOXP3 co-staining, cells were stimulated with anti-CD3 plus anti-CD28 by means of the T-cell activation/expansion kit (human Macs; Miltenyi Biotec), adding 5 × 10⁶ particles per 5 × 10⁶ cells and for 24 h. Staining of CD25 and CTLA-4 cell surface receptors was performed using PE-conjugated mouse anti-(human CD25) (BD Pharmingen) and APC-conjugated mouse anti-(human CD152) (BD Pharmingen) respectively, following the manufacturer’s procedure. For intracellular labelling, cells were fixed and permeabilized using the human FOXP3 buffer set (BD Pharmingen) and subsequently stained with V450 mouse anti-human CD25 and APC-labelled anti-FOXP3 antibodies (all from BD Pharmingen), following the manufacturer’s recommendations.

**Intracellular staining with anti-IL-2 or anti-FOXP3 antibodies**

Cells collected for IL-2 intracellular staining were previously stained with either CD4 or CD8 antibody. Intracellular IL-2 and FOXP3 staining was performed using the BD Cytotoxic/Cytoperm kit (BD Biosciences), following the manufacturer’s recommendations. For IL-2 labelling, 24 h before stimulation, CD4⁺ and
CD8+ T-cells were washed and resuspended in DMEM without IL-2 and left at 37°C, 10% CO2. Core- or GFP-transduced Jurkat cells were added to either CD4+ or CD8+ T-cells at two different Jurkat to primary cell ratios (1:1 and 2:1). Cells were stimulated for 8 h with 1 μg of immobilized mouse anti-(human CD3) and 1 μg of mouse anti-(human CD28) per 10^6 cells. After 4 h the intracellular protein transport inhibitor monensin (GolgiStop™; BD Biosciences) was added at 0.67 μg/ml, and the culture was incubated at 37°C for an additional 4 h. Cells were collected and labelled prior to permeabilization with either biotin-conjugated mouse anti-(human CD4) plus streptavidin-PE or PE-Cy7-conjugated mouse anti-(human CD8) (all from BD Biosciences) and were subsequently permeabilized. Intracellular staining was performed with an APC-labelled mouse anti-human IL-2 antibody (BD Biosciences). Primary CD4+ or CD8+ T-cells were incubated together with GFP-transduced Jurkat cells to control the effect of transduction, whereas CD4+ and CD8+ T-cells were cultured in the absence of Jurkat to determine basal IL-2 staining. For FOXP3 staining, HCV-core and GFP-expressing Jurkat cells were labelled with APC-conjugated anti-(human FOXP3) (eBiosciences) 72 h after transduction with the same kit, according to the manufacturer’s recommendations. Acquisition and data analysis was performed in a CyAN ADP MLETM cytometer (DakoCytomation) using the Summit™ R4.3 software.

**Proliferation and cell dead assays**

Untransduced CD8+ T-cells (0.15×10^6) were added to 0.15×10^6 GFP+ or HCV-core–GFP+ irradiated Jurkat cells (ratio 1:1 transduced:respondor), and resuspended at 2×10^6 cells/ml. Cells were stimulated with loaded anti-biotin MACSiBead Particles (T-cell activation/expansion kit human; Macs Miltenyi Biotech) by the addition of 5×10^6 particles per 5×10^6 cells. Cell proliferation was measured by means of the Click-it™ EdU Flow Cytometry Assay Kits (Invitrogen), following the manufacturer’s recommendations. Briefly, cells were incubated at 37°C, 5–10% CO2 with two 5 μM EdU (5-ethyl-2'-deoxyuridine) pulses (at time 0 and 24 h). At 48 h and 96 h, aliquots were stained with anti-CD8, and proliferation as well as cell death were analysed in parallel. For proliferation assays, cells were fixed, permeabilized and stained for EdU detection, following the manufacturer’s recommendations. Cell death was quantified by labelling with SYTOX® AADVanced™ Dead Cell Stain solution following manufacturer’s recommendations and using a final labelling concentration of 1 μM.

**Statistical analysis**

A two-sample Student’s t test was used, assuming unequal variances, when comparing replicates from the different treatments with the corresponding controls within each experiment.

**RESULTS**

**FOXP3 mRNA expression is up-regulated in HCV-core-expressing Jurkat cells**

We and others have previously shown that expression of HCV-core in CD4+ Jurkat T-cells leads to a condition akin to anergy with a decreased secretion of IL-2 and other cytokines upon stimulation [11,24]. Given the links between anergy and regulation [43–46] and the relevance of Treg+ cells in HCV persistence [25,26], we were interested in ascertaining the expression of FOXP3, the most relevant Treg-cell marker, in HCV-core expressing cells. Jurkat cells were transduced with a third-generation lentiviral system coding for HCV-core protein or GFP as a control, and after 72 h, the percentage of transduced cells was analysed by flow cytometry, being >90% in all the experiments (Supplementary Figure S1 at http://www.clinsci.org/cs/123/cs1230015add.htm). As shown in Figure 1(A) there is a 5.15 ± 1.3-fold increase in FOXP3 mRNA expression in HCV-core-transduced Jurkat cells when compared with control cells (P < 0.01). Since it has been shown that calcineurin inhibitors reduce FOXP3 mRNA expression in human Treg+ cells [47], CsA was added to the cultures 12 h before RNA purification. Addition of CsA to HCV-core-expressing Jurkat cells caused more than a 2.5-fold inhibition of FOXP3 expression (2.64 ± 1.13), whereas there was still a HCV-core-mediated CsA-resistant increase in FOXP3 expression that was apparent when CsA-treated HCV-core-expressing cells were compared with untreated GFP-expressing cells (Figure 1A). CsA did not affect FOXP3 expression in GFP-transduced Jurkat cells.

To evaluate whether the increased FOXP3 mRNA expression was transient, mRNA was analysed at days 3, 5 and 7 after transduction. As shown in Figure 1(B), the increased FOXP3 mRNA accumulation was maintained throughout the studied period.

To analyse whether the FOXP3 mRNA increase correlated with a concomitant increase in protein expression, Western blot assays were performed. As shown in Figure 1(C), FOXP3 was detectable in nuclear extracts from HCV-core-transduced Jurkat cells (lane 4), whereas it was undetectable in control cells transduced with a lentiviral vector expressing GFP (lane 3). To confirm these results, intracellular FOXP3 staining was performed in order to obtain the distribution of expression at a single-cell level (Figure 1D). HCV-core- and GFP-expressing Jurkat cells as well as untransduced cells (to rule out an effect due to transduction) were examined for FOXP3 expression.
Effects of HCV-core expression on Jurkat T-cells

Figure 1  HCV-core protein induces FOXP3 expression

(A) Total RNA was purified from HCV-core or GFP-expressing Jurkat cells and cDNA was synthesized to amplify FOXP3 by means of real-time PCR. L32 amplification was used as a control for normalization. Bars show relative accumulation of FOXP3 mRNA in HCV-core expressing cells compared with GFP expressing cells. The diagram shows means ± S.D. of three independent experiments. (**) P < 0.025).

(B) FOXP3 mRNA induction of HCV-core-transduced Jurkat cells compared to control (GFP-expressing Jurkat cells) at the indicated time points after transduction. Bar diagram represents means ± S.D. of four independent experiments.

(C) Western blots performed with nuclear extracts from HCV-core or GFP-transduced Jurkat cells labelled with anti-FOXP3 antibodies. Lane 1, total protein extracts from HEK-293-FT cells transfected with the FOXP3 gene in frame with V5 and a His6 tag; lane 2, molecular markers; lane 3, nuclear extracts from GFP-transduced Jurkat cells; lane 4, nuclear extracts from HCV-core-expressing Jurkat cells.

(D) FACS analysis showing FOXP3 expression of HCV-core transduced cells (striped curve) compared with GFP-transduced (open black curve) as well as untransduced cells (open grey curve). (E) Median fluorescent intensity of FOXP3 curves in HCV-core expressing cells (striped bar) compared with GFP-expressing cells (open bar).

Core-expressing Jurkat cells have higher FOXP3 expression [MFI (mean fluorescence intensity) 8.03 ± 1.08] compared with untransduced Jurkat cells, as well as with GFP-expressing Jurkat cells (MFI, 4.63 ± 0.85) (Figure 1E). No significant differences were observed when GFP-transduced Jurkat cells were compared with untransduced cells.

Expression of CD25 on HCV-core-expressing cells

Although CD25 (IL-2 receptor α chain) is up-regulated not only in T_{reg}-cells, but also in activated T-cells, it could be considered a complementary T_{reg}-cell marker. Thus we next studied CD25 expression in Jurkat cells expressing HCV-core or GFP, before and after stimulation with anti-CD3 plus anti-CD28. Before stimulation, there were no significant differences in CD25 expression, whereas during the first 9 h after anti-CD3 plus anti-CD28 stimulation, HCV-core-expressing Jurkat cells showed a significant increase in CD25 expression (Figure 2). Differences disappeared after that period, and the expression of CD25 was similar on both HCV-core- and GFP-transduced Jurkat cells for the subsequent time points analysed (1, 3, 5 and 7 days after stimulation; results not shown).

CTLA-4 expression is increased on resting cells expressing HCV-core

We were interested in analysing the expression of other relevant T_{reg}-cell markers apart from FOXP3, such as CTLA-4 and GITR. Thus HCV-core- or GFP-expressing Jurkat cells were stained with the corresponding antibodies 72 h after transduction (Figure 3). There were no significant differences in GITR expression (Figure 3A), whereas resting HCV-core-expressing Jurkat cells showed an increase in CTLA-4 expression (MFI 7.24 ± 1.01 compared with 4.18 ± 0.43 in GFP-expressing Jurkat cells) (Figure 3B). This increase in CTLA-4 expression was maintained during the 7 days of analysis (Figure 3C). We then wanted to know whether CTLA-4 expression is associated with FOXP3 and/or CD25 expression in HCV-core-expressing Jurkat cells. Thus either HCV-core- or GFP-transduced Jurkat cells were stimulated and co-stained with antibodies against CD25, CTLA-4 and FOXP3. As shown in Figure 3(D), only a portion (3.98) of the 10 % cells that express CTLA-4, co-express FOXP3.

Core-expressing Jurkat cells inhibit primary CD4^{+} and CD8^{+} T-cell responses after anti-CD3 + anti-CD28 stimulation

As FOXP3 is also transiently expressed in activated T-cells [48,49], we wanted to perform functional experiments to analyse the regulatory function of HCV-core expressing cells. Thus, co-culture experiments were performed in order to determine whether HCV-core-expressing Jurkat cells were able to suppress primary T-cells after stimulation with anti-CD3 plus anti-CD28. Core- or GFP-transduced cells were added to primary CD4^{+} or CD8^{+} T-cell cultures at 1:1 or
Figure 2  Expression of CD25 in HCV-core-transduced Jurkat cells after stimulation with anti-CD3 plus anti-CD28
Core- or GFP-transduced Jurkat cells were stimulated with 1 μg/10^6 cells anti-CD3 and 1 μg/10^6 cells anti-CD28, and samples were taken at several time points to measure CD25 expression. (A) Traces represent CD25 intensity (APC-Cy7 Log) compared with cell count, in GFP (A, upper row) or HCV-core-expressing Jurkat cells (A, lower row). (B) Percentage of CD25+ cells at the time points shown, expressed as means ± S.D. of three independent experiments. Open bars correspond to GFP-transduced Jurkat cells and striped bars to HCV-core-expressing Jurkat cells (∗P < 0.05, ∗∗P < 0.025). Unstim, unstimulated.

Figure 3  GITR, CD25, CTLA-4 and FOXP3 expression
HCV-core- or GFP-transduced Jurkat cells were stained for GITR (A) or CTLA-4 (B) expression 72 h post-transduction. Striped curves correspond to HCV-core-expressing Jurkat cells, open black curves to GFP-transduced Jurkat cells and open grey curves to isotype controls. The histograms (right) represents mean ± S.D. MFI for four independent experiments (∗P < 0.05). (C) Time course of CTLA-4 expression. (D) HCV-core- or GFP-transduced Jurkat cells were co-stained for CD25, CTLA-4 and FOXP3 expression. Dot plots show double staining for CTLA-4+/FOXP3+, CTLA-4+/CD25+ or FOXP3+/CD25+ for GFP- or HCV-core-transduced Jurkat cells. Numbers in quadrants indicate the percentage of positive cells in each quadrant.
Effects of HCV-core expression on Jurkat T-cells

Figure 4 Core-transduced Jurkat cells inhibit CD4+ T-cell IL-2 production upon anti-CD3 plus anti-CD28 stimulation

CD4+ T-cells were incubated with HCV-core or GFP-expressing Jurkat cells at two different Jurkat/responder ratios (1:1 and 2:1) and stimulated with anti-CD3 + anti-CD28 for 8 h. Golgi Stop was added for the last 4 h. Cells were stained with anti-CD4 to distinguish Jurkat cells from primary CD4 T-cells, and anti-IL-2 to detect IL-2-producing CD4+ T-cells. (A) Traces represent IL-2 production by CD4+ T-cells, identified by electronically gating for FSC (forward scatter) and SSC (side scatter) as well as for stronger CD4 staining and negative GFP expression. (B) Histograms from four independent experiments expressed as means ± S.D. of the percentage of IL-2-producing CD4+ T-cells co-cultured with HCV-core (striped bars) or GFP-transduced Jurkat cells (white bars). Stimulated CD4 T-cells alone are also shown in black (***P < 0.005, ****P < 0.0005).

(C) Percentage of inhibition on IL-2 secretion caused by co-culturing with HCV-core-expressing Jurkat cells.

2:1 Jurkat:responder ratios, and cells were stimulated with antibodies against CD3 and CD28 to measure IL-2 production by primary T-cells (Figures 4 and 5). Four hours after stimulation, monensin (Golgi Stop™, BD Biosciences) was added to inhibit protein secretion and cells were incubated for an additional 4 h before performing an intracellular cytokine staining with APC-labelled anti-IL-2. Jurkat cells were distinguished from primary CD4+ T-cells by means of their difference in cell size as well as the presence of HCV-core–GFP in Jurkat cells and the higher intensity of CD4 expression in primary cells. GFP-transduced Jurkat cells as well as CD4+ and CD8+ T-cells were cultured separately as individual controls. As shown in Figure 4, HCV-core-expressing Jurkat cells were able to inhibit IL-2 production by CD4+ T-cells at both ratios tested. In Figure 4(A) a representative experiment corresponding to each ratio is displayed, whereas Figure 4(B) shows means ± S.D. of four independent experiments and C shows the percentage of IL-2 inhibition. The percentage of CD4+ T-cells that produce IL-2 in the presence of HCV-core-transduced Jurkat cells is 30.78 ± 1.42 % at a Jurkat:responder ratio of 1:1, compared with 36.41 ± 2 % in those CD4+ T-cells in contact with GFP-transduced Jurkat cells. Jurkat cells did not alter IL-2 production in CD4+ T-cells, as purified CD4+ T-cells stimulated in the absence of Jurkat cells gave similar results to those in contact with GFP-expressing Jurkat cells (results not shown).

When a Jurkat/responder ratio of 2:1 was used, the decrease observed in IL-2-producing primary cells co-cultured with HCV-core-expressing Jurkat cells, was even more apparent, approximately one third of that seen in cells co-cultured with GFP-expressing cells (25.08 ± 2.88 % of IL-2-producing CD4+ T-cells in the presence of HCV-core-transduced Jurkat cells compared with 38.73 ± 1.86 % in those CD4+ T-cells incubated with control Jurkat cells).

Inhibition of CD8+ T-cell responses by HCV-core-expressing Jurkat cells was even more pronounced (Figure 5). When cultured in the presence of HCV-core-expressing Jurkat cells at a 1:1 ratio, the percentage of CD8+ cells producing IL-2 declined from 12.75 ± 0.91 % to 9.97 ± 1.06 % (P < 0.005). When a ratio of 2:1 was used, HCV-core-transduced Jurkat cells inhibited IL-2 production by CD8+ T-cells by approximately 50 %, compared with GFP-transduced Jurkat cells (6.33 ± 2.58 % of CD8+ T-cells produce IL-2 when incubated with HCV-core-transduced Jurkat cells compared with 12.78 ± 1.78 % when in contact with GFP-expressing Jurkat cells). Figures 4(C) and 5(C) show bar diagrams where the percentage of IL-2 inhibition is represented, considering IL-2 production corresponding to T-cells in contact with GFP-transduced Jurkat cells as 100 %. The former results suggest that HCV-core mediated FOXP3 up-regulation confers regulatory-like function on Jurkat cells, as is demonstrated by its ability to inhibit CD4+ and CD8+ IL-2 production upon stimulation with anti-CD3 and anti-CD28.
HCV-core-transduced Jurkat cells inhibit CD8+ T-cell proliferation while only slightly affecting apoptosis

As the most frequent method used to analyse the suppressive capacity of Treg-cells is the inhibition of T-cell proliferation and as CD8+ T-cells show the most dramatic inhibition in IL-2 production, we were interested in analysing the effect of HCV-core-expressing Jurkat cell addition on CD8 proliferation. HCV-core or GFP-transduced Jurkat cells were co-cultured with purified CD8+ T-cells at 1:1 Jurkat:responder ratio, stimulated with anti-CD3 plus anti-CD28 and stained with EdU. EdU incorporation was measured at 48 and 96 h (Figure 6A). As shown in Figure 6, at 48 h after stimulation, the percentage of proliferating CD8+ T-cells co-cultured in the presence of transduced Jurkat cells declined from 11.29% for CD8+ T-cells cultured with GFP-transduced Jurkat cells to 9.59% (Figure 6A). At 96 h post stimulation, inhibition of CD8+ T-cell proliferation by HCV-core-expressing Jurkat cells was more pronounced as the percentage of proliferating CD8+ T-cells declined from 11.87% for CD8+ T-cells co-cultured in the presence of GFP-transduced Jurkat cells to 3.98% for those co-cultured with HCV-core-expressing Jurkat cells (Figure 6A). We then wanted to ascertain whether the observed inhibition in IL-2 production and proliferation was mediated by apoptosis. Thus we analysed aliquots from the proliferation experiments for cell death using the SYTOX® Advanced Dead Cell Stain kit. As shown in Figure 6(B), the percentage of SYTOX+ cells only slightly increased, 1.41-fold, in CD8 cells co-cultured with HCV-core-expressing Jurkat cells, whereas proliferation decreased 2.98-fold.

DISCUSSION

Among the mechanisms that have been suggested as being responsible for HCV-mediated immune tolerance, regulation is emerging as the most important. There are several reports showing an increase in CD4+CD25+ Treg-cell frequency in liver infiltrates [32], as well as in peripheral blood of chronically infected patients when compared with those that spontaneously recovered or to healthy controls [26]. It has been shown that Treg-cells from chronically infected subjects not only suppress proliferation and function of HCV-specific CD4+ and CD8+ T-cells, but also T-cell responses against other viruses such as influenza or EBV (Epstein–Barr virus), suggesting a possible non-specific pattern of suppression [31,42]. In the present study, we show that HCV-core protein is able to induce de novo generation of CD4+FOXP3+ T-cells with a modest regulatory function, suggesting that the increase in Treg-cells found in peripheral blood from chronically infected patients may develop at least partially in the periphery upon internalization of viral particles or proteins.

FOXP3 expression is, to date, the most reliable marker for Treg-cell function [39], although several groups have shown that this transcription factor is also transiently expressed in activated T-cells, where it does not confer a regulatory phenotype [49]. Our results show that
Effects of HCV-core expression on Jurkat T-cells

HCV-core protein is sufficient to induce FOXP3 expression in Jurkat cells.

It is noteworthy that FOXP3 up-regulation could be abrogated by CsA, in agreement with reports showing that calcineurin inhibitors and more specifically CsA reduce FOXP3 gene transcription in murine [50] and human [47] T<sub>reg</sub>-cells. In our present study, FOXP3 mRNA expression was up-regulated in HCV-core-transduced Jurkat cells, with mRNA accumulation being concordant with an increased presence of FOXP3 protein in the nucleus, as well as with results obtained by intracellular staining. In the latter, cells that express HCV-core showed increased FOXP3 expression as a population, but with considerable overlap with the control, suggesting that a threshold HCV-core expression is needed to boost FOXP3 expression above the threshold needed to induce regulation. On the other hand, a maintained FOXP3 expression may be needed to induce a regulatory function, as has been suggested based on the sustained high expression of FOXP3 required for a full suppressive activity of human nT<sub>reg</sub>-cells as well as for FOXP3-induced T<sub>reg</sub>-cell like cells [51].

It is well known that NFAT (nuclear factor of activated T-cells) plays a central role in T-cell activation and anergy induction, as well as regulatory T-cell function. In this regard, Wu et al. [52] have shown that NFAT co-operates with FOXP3 in the function of T<sub>reg</sub>-cells. We and others have previously shown that HCV-core protein expression was sufficient to activate NFAT and cause its translocation to the nucleus in Jurkat cells, as well as to convert Jurkat cells into an ‘anergic-like’ phenotype [11,24]. Induction of FOXP3 gene expression requires binding of NFAT to its promoter, and it has been reported that mutations in the binding region of NFAT to the FOXP3 promoter are sufficient to induce a decrease in FOXP3 expression [53]. Taking into account that CsA treatment blocks NFAT dephosphorylation and translocation to the nucleus, the decrease in FOXP3 mRNA expression due to CsA treatment in HCV-core-expressing Jurkat cells suggests that HCV-core is responsible for the up-regulation of FOXP3 mostly by means of NFAT activation. The partial inhibition observed in the presence of CsA suggests that there are additional calcineurin-independent mechanisms utilized by the virus to induce FOXP3 expression.

According to published data, CD25 cannot be considered a ‘bona fide’ marker for T<sub>reg</sub>-cells, since CD4<sup>+</sup> FOXP3<sup>+</sup> CD25<sup>−</sup> with suppressive function have been described and CD25 has been shown to be expressed also by activated T-cells in mice [54]. Nonetheless, CD25 expression is up-regulated in HCV-core-transduced Jurkat cells when compared with controls, during the first 9 h after anti-CD3 and anti-CD28 stimulation, but this increased expression was not maintained and, after 24 h, CD25 levels were indistinguishable between HCV-core- and GFP-transduced cells.

Several other molecules in addition to CD25 and FOXP3 have been proposed as T<sub>reg</sub>-cell markers, such as...
as CTLA-4 [35] and GITR [36]. In our present study, HCV-core protein did not induce any increase in GITR expression, whereas CTLA-4 was up-regulated in resting as well as stimulated HCV-core-transduced Jurkat cells. CTLA-4 is a co-stimulatory receptor that delivers negative signals to T-cells, and thus contributes to the unresponsive phenotype induced by the virus. The implication of NFAT1 in CTLA-4 expression [55] is consistent with previous results by others, showing an activation of NFAT1 by HCV-core [11, 24] and an implication of NFAT in anergy induction [56, 57].

Our results show that HCV-core-transduced Jurkat cells are able to inhibit T-cell activation, measured by IL-2 production. This inhibition, although modest, is more dramatic in CD8+ T-cells, where cells that produce IL-2 show a 50% decrease at a Jurkat/responder ratio of 2:1 with a decrease in proliferation.

The finding that expression of HCV-core protein confers regulatory properties even on a CD4+ T-cell line such as Jurkat sheds new light on the development of cells with regulatory-like function in the periphery during chronic infections.

Our results are also in agreement with findings showing that Jurkat cells ectopically expressing FOXP3 acquire a regulatory-like phenotype, and have functional and biochemical characteristics of anergic cells [51]. Both mechanisms (anergy and regulation) are important means, poised to maintain peripheral tolerance, and there are several reports in the literature showing that these two mechanisms share many biochemical and phenotypical features, with several tolerizing stimuli co-inducing both phenomena [58].

HCV-core protein has already been implicated by us and others in anergy induction in CD4+ T-cells [11, 24], and in the present study we show that HCV-core-expressing Jurkat cells acquire a suppressive activity, being capable of inhibiting not only CD4+, but even more strongly CD8+ T-cell activation, thus favouring viral persistence.

Several studies have shown a decrease in CD8+ T-cell function [18, 59–67], many of them invoking CD4+ T-cell regulation and/or a decrease in CD4+ T-cell help [21, 68, 70].

Our results are relevant for the pathophysiology of HCV as CD4+ FOXP3+ T-cell have been shown to be predominantly localized in piecemeal and lobular necrosis, in contact with CD8+ T-cells [71]. Thus Treg- cell cells, within HCV-infected livers, have direct access to CD8 T-cells in vivo.

Although, in the context of HCV liver fibrosis a total increase in CD8 number [72] or a relative increase when compared with CD4 [73] has been reported [72], others have shown that differences in the periphery were not significant being mainly confined to the intrahepatic lymphocyte composition with detection of lymphocyte subsets being negative in normal livers [73]. It has also been shown that CD8+ T-cells can survive years in the persistently infected liver [68].

It has also been reported that CD8+ T-cells show a higher expression of activation markers in HCV patients [74]. Nonetheless, they co-express inhibitory markers, are severely exhausted [59, 74] and stunned, with impaired proliferation, IFNγ (interferon γ) production, and cytotoxicity [67]. Moreover, half of CD8+ T-cells in HCV are CD161+ CD8+ T-cells with type 17 differentiation and inhibitory capacity [74] that appear to be low in perforin and granzyme B [60].

Both situations, namely an increase in CD8+ T-cell number and/or activation related to their role in fibrosis and an inability of CD8+ T-cells to clear the virus due to CD4+ T-cell regulation and/or lack of CD4+ T-cell help are not necessarily contradictory: CD8+ T-cells do not have to be functionally active or even healthy to induce fibrosis, nor do they directly participate in fibrogenesis, but rather through activation of stellate cells upon phagocytosis of CD8 cells [75] or CD8 cells microparticles [76].

Moreover, TGF-β (transforming growth factor-β), which is mainly produced by T-cells with regulatory or suppressive function, also contribute to hepatic stellate cell activation and induction of fibrosis [26, 32, 71].

The presence of core cells in CD4 T-cells in vivo could be explained either by direct infection or alternatively the HCV-core protein could enter CD4+ T-cells directly as has already been suggested [77]. Results showing that HCV-core protein is present in non-parenchymal liver cells including lymphocytes [78] and is found in the serum of infected patients [79], together with data showing that it can enter hepatoma cell lines [80], further reinforces such a hypothesis.

Mechanisms involved in peripheral tolerance are essential to prevent autoimmune diseases and maintain immune homoeostasis. Nonetheless, induction of immune tolerance by pathogens, such as HCV, favours viral persistence and has to be neutralized in the clinic.

On the other hand understanding the means by which the virus has evolved to induce immune suppression can be capitalized on in clinical settings where the induction of tolerance is a goal. The results provided in the present paper show for the first time a direct effect of a viral protein, in the ‘de novo’ generation of ‘regulatory-like’ T-cells from the CD4+ T-cell line Jurkat.

**AUTHOR CONTRIBUTION**

All authors contributed to the discussions of the experimental design and data analysis. Margarita Dominguez-Villar performed of all of the experimental studies; Alba Munoz-Suano, Cecilia Fernandez-Ponce and Esperanza Gomez participated in the experiments, provided technical assistance and/or specific reagents;
Manuel Rodriguez-Iglesias provided suggestions. Francisco Garcia-Cozar directed the study.

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SUPPLEMENTARY ONLINE DATA

Up-regulation of FOXP3 and induction of suppressive function in CD4\(^+\) Jurkat T-cells expressing hepatitis C virus core protein

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Figure S1  Jurkat cells were efficiently transduced with lentiviral vectors expressing HCV-core protein or GFP as a control
Traces represent GFP intensity (logarithmic scale) against cell count for untransduced control cells (grey curve) and HCV-core–GFP- or GFP-transduced Jurkat cells (black striped curve). In all cases, the percentage of transduction was higher than 90%.

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