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Lymphotropic HCV strain can infect human primary naïve CD4⁺ cells and affect their proliferation and IFN- γ secretion activity

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Abstract

Background—Lymphotropic hepatitis C virus (HCV) infection of B and T cells might play an important role in the pathogenesis of hepatitis C. Recently, we showed that a lymphotropic HCV (SB strain) could infect established T-cell lines and B-cell lines. However, whether HCV replication interferes with cell proliferation and function in primary T lymphocytes is still unclear.

Aim—The aim of this study was to analyze whether HCV replication in primary T lymphocytes affected their development, proliferation, and Th1 commitment.

Methods—SB strain cell culture supernatant (2×10^4 copies/ml HCV) was used to infect several kinds of primary lymphocyte subsets. Mock, UV-irradiated SB-HCV, JFH-1 strain, and JFH-1 NS5B mutant, which could not replicate in T cells, were included as negative controls. Carboxyfluorescein succinimidyl ester (CFSE) and CD45RA double staining was used to evaluate the proliferative activity of CD4⁺CD45RA⁺CD45RO⁻ naïve CD4⁺ cells. Interferon (IFN)- γ and interleukin (IL)-10 secretion assays magnetic cell sorting (MACS) were carried out.

Results—Negative strand HCV RNA was detected in CD4⁺, CD14⁺, and CD19⁺ cells. Among CD4⁺ cells, CD4⁺CD45RA⁺RO⁻ cells (naïve CD4⁺ cells) were most susceptible to replication of the SB strain. The levels of CFSE and CD45RA expression gradually declined during cell division in uninfected cells, while HCV-infected naïve CD4⁺ cells expressed higher levels of CFSE and CD45RA than Mock or UV-SB infected naïve CD4⁺ cells. Moreover, the production of IFN- γ was significantly suppressed in SB-infected naïve CD4⁺ cells.

Conclusions—Lymphotropic HCV replication suppressed proliferation and development, including that towards Th1 commitment, in human primary naïve CD4⁺ cells.

Keywords

HCV; Lymphotropic; Naïve CD4⁺ cell; Th1

Introduction

Hepatitis C virus (HCV) infects about 170 million people worldwide and is a major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) [1]. Cellular and humoral immune responses to HCV play an important role in the pathogenesis of chronic

hepatitis, liver cirrhosis, HCC, and B-lymphocyte proliferative disorders, including mixed cryoglobulinemia, a disorder characterized by the oligoclonal proliferation of B cells [2, 3].

Several mechanisms have been proposed for the failure of the cellular immune response, including anergy, cytotoxic T-lymphocyte (CTL) exhaustion, suppression via regulatory CD4⁺–CD25⁺ T cells interleukin-10 (IL-10)- secreting regulatory CD8⁺-T cells, and direct binding of HCV core antigen [4–7]. However, the influence of HCV replication in lymphoid cells on their functions is not fully understood. HCV replicates primarily in the liver, but HCV-RNA has been detected in other lymphoid cells, including B- and T-lymphocytes, monocytes, and dendritic cells [8–11]. Sung et al. [12] have previously reported a B-cell line (SB cells) that produces HCV particles that can further infect B lymphocytes in vitro. We have shown that the SB-HCV strain could infect and replicate in T-cell lines and that HCV replication could inhibit interferon (IFN)- γ /signal transducer and activator of transcription-1 (STAT-1)/T-bet signaling of the T cells [13]. Moreover, we reported that HCV replication in Molt-4 could affect the proliferation and FAS-mediated apoptosis of T cells by inhibiting CD44v6 expression and mitogen-activated protein kinase (MAPK) signaling in Molt-4 [14]. Most of these data came from studies using cell lines, since stable SB-HCV replication could be detected in lymphoid cell lines (Raji, Molt-4, etc.). However, the analysis of primary lymphocytes is preferable to determine the real effects of lymphotropic HCV strains on T-cell biology. In fact, the effects of low titers of HCV in primary T cells have not been clarified yet.

We first reported that, among T cells, CD4⁺CD45RA⁺ RO[–] naïve T cells were susceptible to SB-HCV infection [13]. Here we describe the functional and proliferative analysis of SB-HCV-infected naïve CD4⁺ T cells after short-term culture.

Materials and methods

Culture of cell lines

SB cells that continuously produce infectious HCV particles were originally established from splenocytes of an HCV-infected patient with type 2 mixed cryoglobulinemia and monocytoid B-cell lymphoma [12]. The cells were maintained in standard RPMI (Invitrogen, Carlsbad, CA, USA) medium with 20% fetal bovine serum (FBS) without any supplement. Every 5 days, the cells were sedimented by natural gravity for 30 min at 37°C.

In vitro infection of primary lymphoid cells

Supernatants from SB cells were purified by centrifugation and 0.2- μ m filter. SB culture supernatant (5 ml), which contained 2.2×10^4 copies/ml of HCV RNA, was used for the infection of several kinds of human primary lymphoid cells (1×10^5 cells). A control infection with UV-irradiated SB culture supernatant was included in every experiment. Supernatants of Huh7.5 cells transfected with JFH-1 strains [15–17] at 10 days post-transfection were used for several control experiments. Cells were washed 3 times at 2 days after infection. Then, a portion of the cells (3×10^5 to 5×10^5 cells) was harvested for analysis; the remaining cells (1×10^5 cells) were kept and incubated under the same condition.

Isolation of various kinds of lymphoid cells and naïve CD4⁺ T cells

We got informed consent from 5 healthy donors, from whom peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Paque centrifugation (Amersham Bioscience [Uppsala, Sweden]). Anti-CD3 phycoerythrin (PE), anti-CD4 (PE-Cy3), anti-CD8 (PE), anti-CD14 (PE), anti-CD19 (PE), anti-CD45RO (PE), and antiCD45RA (fluorescein isothiocyanate [FITC]) antibodies (BD Pharmingen) were used for the separation of different kinds of mononuclear cells by using fluorescence activated cell sorting (FACS) vantage (BD Pharmingen, San Jose, CA, USA). In some experiments, a naïve CD4⁺ T cell isolation kit II (Miltenyi Biotec [Bergish Gladbach, Germany]) was used to obtain more viable naïve CD4⁺ cells.

Strand-specific intracellular HCV RNA detection

Strand-specific intracellular HCV RNA was detected by using a recently established procedure that combined previously published methods [9, 18] with minor modifications [13]. Positive- and negative-strand-specific HCV RNAs were detected by a nested polymerase chain reaction (PCR) method. Reactions were performed with 2 µl of 10× reverse transcriptase (RT) buffer, 2 µl of 10-mmol/l magnesium chloride, 200-µmol/l each of deoxyadenosine triphosphate, deoxycytidine triphosphate, deoxyguanosine triphosphate, 100-µmol/l of thymidine triphosphate (dTTP), 0.2 U of uracil-*N* glycosylase (UNG; Perkin Elmer [Fremont, CA, USA]/Applied Biosystems), 5 U of rTth DNA Polymerase; and 50 pmol of strand-specific HCV primers (positions according to the 5' untranslated region), nt -285 to -256 (ACTGTCTTCACGCAGAAAGCGTCT AGCCAT) and -43 to -14 (CGAGACCTCCCGGGGCA CTCGCAAGCACCC) and template RNA. The RT mixture was incubated for 10 min at room temperature and then at 70°C for an additional 15 min. The cDNA product was subjected to the first PCR with 80 µl of PCR reaction buffer containing 50 pmol of HCV downstream strand-specific primer. The PCR amplification consisted of 5 min at 95°C, followed by 35 cycles (1 min at 94°C, followed by 1 min at 67°C, and then by 1 min at 72°C), and then 7-min extension at 72°C. For the second nested PCR, an aliquot (1/10) of the first PCR reaction mixture was re-amplified using 50 pmol of each of the two primers, nt -276 to -247 (ACGCAGAAAGCGTCTAGCCATGGCGTTAGT) and nt -21 to -50 (TCCCGGGGCACTCGCAAGCACCC ATCAGG), which span the 255-base pair region nt -276 to -21 (position according to the 5' untranslated region) of HCV RNA, and Taq polymerase (Applied Biosystems). The reaction was run for 35 cycles (1 min at 94°C, 1 min at 67°C, 1 min at 72°C), followed by 7 min at 72°C. Semiquantification was achieved by serial fourfold dilutions (in 10 µg/ml of *Escherichia coli* tRNA) of an initial amount of 200 ng of total RNA. The relative titer was expressed as the highest dilution giving a visible band of the appropriate size on a 2% agarose gel stained by ethidium bromide. For internal control, semi-quantification of β -actin mRNA was performed by using the same RNA extracts. To rule out false, random, and self-priming, extracted HCV RNA was run in every RT-PCR test without the addition of an upstream HCV primer.

CFSE staining

Cells were analyzed by using a CellTrace CFSE Cell Proliferation Kit (Invitrogen [Carlsbad, CA, USA]). The cell staining methods followed the manufacturer's protocol. Stained cells were washed three times and incubated for an additional 7 days. Cells were analyzed by flow cytometry with 510 nm excitation and emission filters. A proliferation index was calculated by FlowJo 7.5 (Tree Str Inc, Ashland, OR, USA), according to the manufacturer's protocol.

Annexin V and propidium iodide staining

Cells were stained with Annexin V and propidium iodide (PI) by using an apoptosis detection kit (R&D systems, Minneapolis, MN, USA). Staining methods were conducted according to the manufacturer's protocol. Briefly, collected cells were washed and gently re-suspended in the Annexin V incubation reagent at a concentration of 3×10^5 cells per 100 μ l. Then, re-suspended cells in binding buffer were stained by Streptavidin conjugate allophycocyanin (APC) and analyzed by flow cytometry within 1 h.

Transfection of HCV individual protein expression plasmids

The various expression plasmids were constructed by inserting HCV core, E1, E2, NS3, NS4B, NS5A, and NS5B cDNA of genotype 1a [19] behind the cytomegalovirus virus immediate-early promoter in pCDNA3.1 (Invitrogen). Primary CD4⁺ cells were transfected using Nucleofector I (Amaxa, Gaithersburg, Washington DC, USA) with a Human T cell Nucleofector kit (Amaxa), and various plasmids were purified using the EndFree plasmid kit (QIAGEN, Valencia, CA, USA). Viable transfected cells were isolated by Ficoll-Paque centrifugation (Amersham Bioscience) at 24 h post-transfection. Transfection and expression efficiency were analyzed by using intracellular staining of HCV individual proteins and flow cytometry analysis. Briefly, the cells were fixed and permeabilized with fixation/permeabilization solution (BD Bioscience) at 4°C for 25 min. The cells were then washed two times in BD Perm/Wash buffer (BD Bioscience) and resuspended in 50 μ l of BD Perm/Wash buffer containing pre-conjugating polyclonal anti-E1, E2, NS3, NS4B, NS5B, NS5A antibody (abcam, Cambridge, MA, USA) with a phycoerythrin (PE)-conjugated anti-mouse antibody.

Confocal laser microscopy

Primary lymphocytes (3×10^6 cells/ml) in suspension were fixed and permeabilized with fixation/permeabilization solution (BD Bioscience) at 4°C for 25 min. The cells were then washed two times in BD Perm/Wash buffer (BD Bioscience) and resuspended in 50 μ l of BD Perm/Wash buffer containing pre-conjugating polyclonal anti-NS5A antibody (Bioscience International, Saco, ME, USA) with an FITC-conjugated anti-mouse antibody.

Interferon- γ and interleukin 10 secretion assay

Cells were washed by adding 2 ml of cold phosphate-buffered saline (PBS) and resuspended in 90 μ l of cold RPMI 1640 medium. After the addition of 10 μ l of IL-10- or IFN- γ -Catch Reagent (Milenyi Biotec), cells were incubated for 5 min on ice. Then the cells were diluted with 1 ml of warm medium (37°C) and further incubated in a closed tube for 45 min at 37°C

under slow continuous rotation. Cells were washed and IL-10- or IFN- γ -secreting cells were stained by adding 10 μ L of IL-10- or IFN- γ - detection antibody (PE-conjugated) (Miltenyi Biotec) together with anti-CD4-PerCP.

Real-time PCR analysis

Cells were collected sequentially at various time points after the addition of recombinant human IFN- γ (500 ng/ml) (BD Biosciences, CA, USA). After the extraction of total RNA and the RT procedure, real-time PCR using a TaqMan Chemistry System was carried out. The readymade set of primers and probe for the amplification of T-bet (ID HS00203436) and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) were purchased from Perkin-Elmer/Applied Biosystems. The relative amount of target mRNA was obtained by using a comparative the threshold cycle (CT) method. The expression level of mRNAs of the non-stimulation sample of vector transfected-primary CD4⁺ cells was represented as 1.0 and the relative amount of target mRNA in a stimulated sample was calculated according to the manufacturer's protocol.

Immunoblot assay

Proteins were resolved by electrophoresis in sodium dodecyl sulfate–polyacrylamide gels and electrophoretically transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA). The membrane was incubated with anti-STAT-1 α , or anti-p-STAT-1 antibodies (Cell Signaling, Danver, MA, USA) and then reacted with peroxidase-conjugated secondary antibody. Immunoreactivity was visualized by an enhanced chemiluminescence detection system (Amersham Bioscience).

Statistical analysis

Statistical analyses of the data in Figs. 1c, 2c, 3b, and 4 were performed by the analysis of variance (ANOVA) test (SPSS10.0, SPSS Inc, Chicago, IL, USA). Values of $p < 0.05$ were considered to be statistically significant.

Results

Detection of negative-strand HCV-RNA among lymphoid cells

Strand-specific rTth based nested PCR was carried out to analyze the susceptibility to HCV infection among the various kinds of lymphoid cells with or without short term culture (7 days). Isolated lymphoid cells were infected with SB-HCV, UV-irradiated-HCV, or JFH-1 strain and were cultured with appropriate cytokines and/or antibody stimulation (Table 1). We needed to add different kinds of cytokines to maintain the cell proliferation and viability. Negative-strand HCV-RNA could be detected in CD4⁺, CD14⁺, and CD19⁺ cells and in CD8⁺ cell-depleted PBMCs (PBMC-CD8⁺) after short-term culture (Table 2). However, negative- and positive-strand HCV-RNA could not be detected in any kinds of lymphoid cells infected with the supernatant of JFH-1 and JFH-1 GND mutant (data not shown). Undetectable negative-strand HCV-RNA at 2 days post-infection indicated that HCV-RNA was replicated after inoculation. We found that depletion of CD8⁺ cells from PBMCs was favorable to replication in lymphoid cells. In this study, we used CD3CD28 beads and IL2 stimulation that could stimulate more efficiently than CD3 and IL2 stimulation. However,

among the CD4⁺ cells, CD4⁺ CD45RA⁺RO⁻ naïve CD4⁺ cells were most susceptible to infection, as we previously demonstrated (Table 2) (Suppl. Fig. 1) [13]. These data indicate that CD4⁺CD45RA⁺RO⁻ naïve CD4⁺ cells could be infected with SB-HCV during T-cell development. CD81 was one of the main candidates of HCV receptors for the infection of the cells [20–22]. We tried to analyze whether anti-CD81 antibody might block the SB-HCV infection of primarily naïve CD4⁺ cells. HCV-NS5A protein could be detected in 12.2% of SB-HCV-inoculated naïve CD4⁺ cells at 10 days post-infection. However, the pretreatment of anti-CD81 antibody reduced the frequency of NS5A detection among the SB-HCV-inoculated naïve CD4⁺ cells (4.7%) (Suppl. Fig. 2). The sensitivity of NS5A immunostaining was lower than that of the strand-specific nested PCR method [13].

Suppression of proliferation activity in SB-HCV-infected naïve CD4⁺ cells

The purity of CD45RA⁺RO⁻ naïve CD4⁺ cells after isolation was around 92% (Fig. 1a). CFSE staining was carried out at 5 days post-infection in SB-HCV, UV-irradiated HCV, and Mock. Stained cells were washed three times and incubated for an additional 7 days with T-cell expander (CD3CD28 coated beads and IL2 stimulation). Cells were analyzed by flow cytometry with 510 nm excitation and emission filters. The proliferation index was calculated by FlowJo 7.5 software according to the manufacturer's protocol. The proliferation index of SB-HCV-infected naïve CD4⁺ cells was significantly lower than that of controls ($p<0.01$) (Fig. 1b, c). These data indicate that lymphotropic SB-HCV suppresses the proliferation activity of T cells.

Disturbance of cell development and IFN- γ -secreting activity

CD45RA and CFSE double staining was carried out to analyze the cell development. The expression level of CD45RA on naïve CD4⁺ cells had gradually declined during cell proliferation. However, the CD45RA expression level of SB-HCV-infected naïve CD4⁺ cells remained higher than those of the control groups (Fig. 2a). Moreover, the frequency of IFN- γ -secreting cells among SB-HCV-infected CD4⁺ cells was significantly lower than those of the control groups ($p<0.01$) (Fig. 2b, c). On the other hand, the frequency of IL10-secreting cells was comparable in the three groups (Fig. 2b, c). These data indicate that HCV infection could interrupt cell development, especially Th1 development.

SB-HCV infection could induce apoptosis of naïve CD4⁺ cells

Annexin V and PI double staining were carried out to detect early apoptotic cells. The frequency of Annexin-V-positive PI-negative early apoptotic cells in SB-HCV-infected naïve T cells was significantly higher than those in the control groups ($p<0.01$) (Fig. 3a, b). UV-irradiated SB-HCV did not enhance the induction of apoptosis in naïve T cells with CD3CD28 stimulation. During T-cell activation, apoptosis is easily induced in order to maintain an appropriate immune response. In line with this feature, 3.04% of early apoptotic cells were detected in naïve T cells with CD3CD28 beads stimulation and Mock serum. These data indicate that SB-HCV replication could induce apoptosis, as seen in Molt-4 cells [14].

HCV core and NS5A proteins could suppress IFN- γ secretion from primary CD4⁺ cells

We investigated the HCV proteins responsible for the suppression of IFN- γ secretion. HCV E1, E2, Core, NS3, NS4B, NS5A, and NS5B expression plasmids were used to transfect into primary CD4⁺ lymphocytes by Nucleofector. The intracellular staining of these proteins was carried out and the transfection efficiency was about 35–55% (Suppl. Fig. 3). Among these proteins, HCV core and NS5A could significantly suppress the IFN- γ secretion ($p < 0.05$) (Fig. 4). HCV core and NS5A transfected primary CD4⁺ lymphocytes were stimulated with IFN- γ . The relative expression of T-bet-mRNA was sequentially analyzed by real-time PCR. T-bet-mRNA expression in HCV core or NS5A transfected primary CD4⁺ T lymphocytes was significantly suppressed at 90 and 180 min post-transfection in comparison to vector-transfected primary CD4⁺ T lymphocytes. Moreover, the amount of STAT-1 protein in HCV-Core-expressing CD4⁺ cells was remarkably lower than the amounts in vector and HCV-E2 transfected CD4⁺ cells (Fig. 4c). The amount of phosphorylated STAT-1 (p-STAT-1) after IFN- γ stimulation was also analyzed. The amount of p-STAT-1 in HCV-Core and NS5A expressing CD4⁺ cells was remarkably lower than that in the vector control.

Discussion

There are many reports about the existence of extrahepatic HCV replication that might contribute to immune dysfunction [13, 14, 23–25]. We have reported that a specific SB-HCV strain could replicate in B- and T-cell lines and affect various immune systems [13, 14, 25]. However, the results of these studies were not definitely conclusive, since the cell lines were inappropriate to investigate the development and commitment of the lymphocytes. In the present study, we demonstrated that the SB-HCV strain could replicate in primary CD19⁺ B cells, CD4⁺ T cells, and CD14⁺ monocytes with cytokine stimulation. Among the CD4⁺ T cells, CD4⁺CD45RA⁺RO⁻ naïve CD4⁺ cells were the most susceptible to SB-HCV infection. One of the speculated reasons to explain why naïve CD4⁺ cells with stimulation were most susceptible to SB-HCV infection is that T cells might temporarily express various kinds of molecules which may contribute to the HCV infection during T-cell development. The infectivity of naïve CD4⁺ T cells was not as high as that of Molt-4 cells. However, significant suppression of cell development and IFN- γ secretion were seen in SB-HCV-infected naïve T cells with CD3, CD28, and IL2 stimulation. UV-irradiated-HCV that could not replicate in the cells suppressed the IFN- γ secretion slightly. These data indicate that not only the effect of HCV replication but also the direct binding effects of HCV structured proteins might contribute to the suppression of IFN- γ secretion. One report indicated that HCV-core protein could interact with the complement receptor gC1qR and upregulate suppressor of cytokine signaling-1 (SOCS-1), accompanied by downregulation of signal transducer and activator of transcription-1 (STAT-1) phosphorylation in T cells [7]. Another possible explanation of the discrepancies between HCV infectivity and suppression of proliferation and IFN- γ secretion might be the low sensitivity of HCV antigen-immunostaining, since lower sensitivity of immunostaining in comparison to the nested PCR method was found in our previous study [13].

HCV-Core and -NS5A proteins were the proteins responsible for the suppression of IFN- γ secretion from T cells. Lin et al. [26] have documented that HCV-core protein causes the

degradation of STAT-1 protein and suppresses the Jak-STAT pathway in hepatocytes. In our previous study, reduction of STAT-1 protein was detected in HCV-core transfected primary naïve T cells and HCV-replicating Molt-4 cells [13]. Moreover, inhibition of intrahepatic gamma interferon production by HCV-NS5A in transgenic mice was recently reported [27]. Recently, detection of HCV replicative intermediate RNA in perihepatic lymph nodes was reported [28]. The disturbance of Th1 commitment might influence the development of HCV-specific CTL in perihepatic lymph nodes. The selective infection of certain T cells by HCV in vivo may explain why there is only relative HCV-specific T-cell suppression without general immune suppression.

Suppression of proliferation activity was seen in HCV-infected naïve T cells as well as HCV-infected Molt-4 cells [14]. The expression level of CD45RA, which is a surface marker of T-cell development, gradually declined along with cell proliferation. However, HCV-infected naïve T cells expressed significantly higher levels of CD45RA than the control groups. We previously reported that HCV replication could suppress Ras/MEK/ERK signaling of Molt-4 [14]. During T-cell development, T cells showed strong proliferation activity that might facilitate HCV replication in T cells. However, extensive proliferation of HCV in T cells might interfere with the proper development of T cells.

The induction of apoptosis was seen in SB-HCV-infected naïve T lymphocytes with CD3CD28 and IL2 stimulation. It is known that, during T-cell activation from naïve to effector cells, T cells have to survive activation-induced cell death (AICD), which may contribute to the maintenance of an appropriate level of the immune response [29, 30]. However, some groups reported that HCV replication could inhibit apoptosis in hepatoma cell lines [31, 32]. The developmental stages and characteristics of naïve T cells might explain these contradictory results. During T-cell activation, apoptosis is easily induced in order to maintain an appropriate immune response.

In conclusion, HCV replication in human naïve T cells might affect their proliferation activity and Th1 development, as was shown in the cell lines used in a previous study. The results suggest that the infectivity of HCV in human naïve T lymphocytes is low, although the biological effect of this infection might be significant because of its bystander effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Suppression of proliferation activity in hepatitis C virus (*HCV*)-infected human naïve T lymphocytes.

a A representative dot plot of $CD4^+CD45RA^+RO^-$ cells is shown. Cells are stained with CD4-PerCP-antibody (Ab), CD45RA-fluorescein isothiocyanate (FITC)-Ab, and CD45RO-APC-Ab. The purity of isolated $CD4^+CD45RA^+RO^-$ naïve T lymphocytes is over 92%. **b, c** Carboxyfluorescein succinimidyl ester (CFSE) staining was carried out at 5 days post-infection in SB-HCV, UV-irradiated HCV, and Mock. Stained cells were washed three times and incubated for an additional 7 days with T-cell expander. Cells were analyzed by flow cytometry with 510 nm excitation and emission filters. *Numbers* in the representative histogram indicate numbers of cell divisions. The proliferation index was calculated by FlowJo 7.5 software according to the manufacturer's protocol. The proliferation index is

shown in this *bar graph*. Three independent experiments were carried out. *Error bars* indicate the standard deviation.

IL interleukin

Fig. 2. Suppression of development and Th1 commitment in HCV-infected human naïve T lymphocytes. **a** Representative dot plots of CD45RA and carboxyfluorescein succinimidyl ester (CFSE) double staining are shown. *Numbers* in the representative dot plots indicate the numbers of cell divisions. *Bar graphs* indicate the mean fluorescence intensity (MFI) of cell clusters. **b** Representative dot plots of interferon- γ (IFN- γ) and interleukin (IL) 10 secretion assays are shown. The *numbers* in the quadrant indicate IFN- γ and/or IL10-secreting cells among CD4⁺ cells. **c** The frequencies of IFN- γ - and IL10- secreting cells among the three groups are shown in these *bar graphs*. Three independent experiments were carried out. *Error bars* indicate the standard deviation

Fig. 3. HCV replication induces apoptosis of naive CD4⁺ cells. **a** Representative dot plots of Annexin V and propidium iodide (PI) staining are shown. The *numbers* in the quadrants indicate the frequencies of early apoptotic cells (Annexin V⁺ and PI⁻) and dead cells (Annexin V⁺ and PI⁺ cells). **b** The frequencies of early apoptotic cells are shown in this *bar graph*. Three independent experiments were carried out. *Error bars* indicate the standard deviations

Fig. 4.

HCV-Core and NS5A proteins are the proteins that contribute to the suppression of IFN- γ secretion. **a** HCV E1, E2, Core, NS3, NS4B, NS5A, and NS5B expression plasmids were used to transfect into primary CD4⁺ lymphocytes by Nucleofector. The frequencies of IFN- γ secreting cells among the samples' CD4⁺ cells/the frequencies of IFN- γ secreting cells among the vector's CD4⁺ cells $\times 100$ are shown in this *bar graph*. **b** HCV core and NS5A transfected primary CD4⁺ lymphocytes were stimulated with IFN- γ (500 ng/ml). The relative expression of T-bet-mRNA was sequentially analyzed by real-time polymerase chain reaction (PCR). The relative amount of target mRNA was obtained by using a comparative the threshold cycle (CT) method. The expression level of mRNAs of the nonstimulation sample of vector transfected-primary CD4⁺ cells is represented as 1.0 and the relative amount of target mRNA in a stimulated sample was calculated. Three independent experiments were carried out. *Error bars* indicate the standard deviation. **c** Immunoblotting assay was carried out to detect the protein of signal transducer and activator of transcription-1 (STAT-1), phospho-STAT-1 (p-STAT-1), and actin in the HCV-core, NS5A, and vector-plasmid transfected human primary CD4⁺ cells with or without IFN- γ stimulation (30 min)

Table 1

Cytokine conditions for various kinds of lymphoid cell culture

Cells	Cytokine condition	Other stimulant	Cell viability (%)
PBMC	IL2 (50 ng/ml) + IL6 (20 ng/ml) + CSF (250 ng/ml)	None	80
PBMC-CD8	IL2 (50 ng/ml) + IL6 (20 ng/ml) + CSF (250 ng/ml)	None	80
CD3	IL2 (50 ng/ml)	CD3CD28 coated beads	70
CD4	IL2 (50 ng/ml)	CD3CD28 coated beads	70
CD8	IL2 (50 ng/ml)	CD3CD28 coated beads	70
CD14	CSF (250 ng/ml)	None	60
CD19	IL-6 (20 ng/ml)	None	70

The conditions of the cell culture are shown. Peripheral blood mononuclear cell (PBMC)-CD8 indicates CD8 cell-depleted PBMCs

IL interleukin, *CSF* colony stimulating factor

Table 2

Strand-specific hepatitis C virus (HCV)-RNA detection in various kinds of lymphoid cells

Subset	PBMC	PBMC-CD8	CD3	CD4	CD8	CD14	CD19	Whole CD4 ⁺	CD4 ⁺ CD45RA ⁺ RO ⁻	CD4 ⁺ CD45RA ⁻ RO ⁺
Positive strand										
2 days	+	+	-	+	-	-	+	+	+	+
7 days	++	++	+	++	-	++	+++	++	+++	+
7 days UV-irradiated	-	-	-	-	-	-	-	-	-	-
Negative strand										
2 days	-	-	-	-	-	-	-	-	-	-
7 days	-	+	+/-	+	-	+	++	+	++	+/-
7 days UV-irradiated	-	-	-	-	-	-	-	-	-	-

Positive- and negative-strand-specific HCV-RNA was detected by semiquantitative nested polymerase chain reaction (PCR) methods

-, negative detection; +, positive detection without dilution; ++, positive detection with 4 times dilution; +++, positive detection with 16 times dilution; ±, only one detection in three independent experiments. Three independent experiments were carried out. Similar results were obtained three times