厚生労働科学研究費
肝炎等克服緊急対策研究事業

小胞輸送 ESCRT 経路を利用したＣ型肝炎ウイルス排除

平成 23 年度
総括・分担研究報告書

研究代表者

玉井恵一

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小胞輸送ESCRT経路を利用したC型肝炎ウイルス排除

研究報告者　玉井恵一[1]
研究分担者　菅村和夫[2]
研究分担者　田中伸幸[1]

Keiichi TAMAI[1a], Kazuo SUGAMURA[2], and Nobuyuki TANAKA[1]

1 宮城県立がんセンター研究所　がん先進治療開発研究部
Division of Cancer Biology and Therapeutics, Miyagi Cancer Center Research Institute
2 宮城県立がんセンター研究所　発がん制御研究部
Division of Molecular and Cellular Oncology, Miyagi Cancer Center Research Institute
a) E-mail: tamaikeiichi@med.tohoku.ac.jp

要約

近年HCV培養系の確立によりライフサイクルの検討が行われている。我々は小胞輸送経路ESCRT分子であるHrsのノックアウト樹状細胞から産生されるエクソゾーム量が低下することを見いだした。エクソゾーム分泌とウイルス動態は酷似していることから、ESCRT経路とHCVとの関係を解析した。ESCRT構成分子の機能低下Huh7細胞を樹立し、JFH1を感染させたところ、上清中のJFH1-RNAはいずれの機能低下細胞においも有意に減少した。上清に放出される感染性HCV粒子も同様に減少した。細胞内のHCV-RNA には有意な変化は認めなかった。免疫電顕をおこなうと、HCVコアタンパクとmultivesicular body内のintraluminal vesicleは共局在していた。以上のことからHCVはHrs依存性エクソゾーム経路を利用してアセンブリを行っていることが示唆された。

キーワード　HCV ESCRT Hrs exosome

1. 背景

C型慢性肝炎は、C型肝炎ウイルス（HCV）を原因とする疾患であり、国内では約200万人が罹患しているとされている。HCVを排除する治療としてペグインターフェン・リバビリン・テラプレビル３剤併用療法が施行されているが、副作用により中止せざるを得ない等、未だ難治性の患者が存在する。HCVに対する抗ウイルス薬としては、現在使用されているものの他、複数の薬剤が臨床試験中である。HCVを標的とした抗ウイルス薬はプロテアーゼインヒビターやポリメラーゼインヒビター等、非構造タンパクを標的とした薬剤が主流であるが、HCVの細胞内ライフサイクルに着目した阻害剤はまだ報告がない。

これまで、HCV は培養系の確立が困難であったことから、そのライフサイクル・細胞内動態は不明な点が多かった。2005年に*in vitro*で感染・再感染可能なHCV株JFH-1が報告されてから[1]、HCVの細胞内動態の研究が可能になった。HCVはレセプターを介して細胞内にエンドサイトーシスされた後に、脱核・RNA複製・翻訳・プロセッシングを経てウイルス粒子が構築され、細胞外に放出される。ウイルスが構築される際にはエンドソーム上に集められた構造タンパクがエンドソーム内に出芽することで粒子を形成すると考えられているが、その詳細な機構は依然不明である。

HIVに対する精力的な研究の結果、ESCRT（Endosomal Sorting Complex Required for Transport）経路（図1）がウイルスのアセンブリや放出に関して重要な役割を担っていることが分かってきた。HBVやHTLV-Iにおいても、アセンブリおよび放出においてESCRT経路が重要であることが知られている。

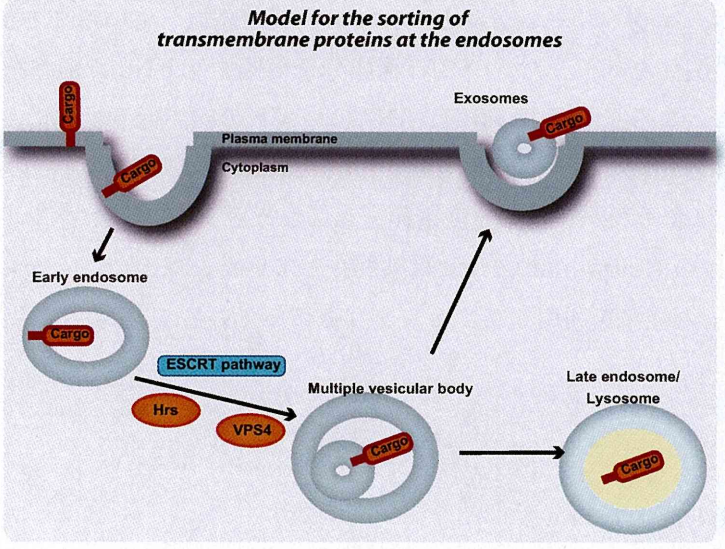

図1 ESCRT経路

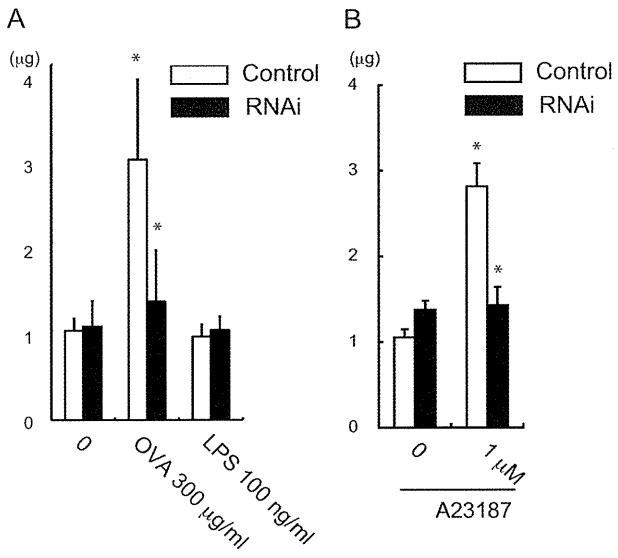

図2 Hrs はエクソゾーム放出に必須である

我々は、以前からレセプターのエンドサイトーシス経路に関して報告してきた[2] [3]。Epidermal growth factor (EGF) レセプターは、クラスリン依存性にエンドサイトーシスされたあと、EGF レセプターを含むエンドソームがESCRT（Endosomal Sorting Complex Required for Transport）経路を経てmultivesicular body（MVB）へと成熟した後に、リソソームでの分解を受ける。近年、MVB から内部の小胞をエクソゾームとして放出する機構が知られるようになった。エクソゾームは直径50-100 nm の微小小胞であり、B 細胞や樹状細胞から放出され、MHC class II 等を含み免疫反応に関わるとされているが[5]、近年はmRNAやmicroRNAを含んで細胞間で情報の受け渡しをするなど広い生理学的作用が知られている[9]。我々は最近、このエクソゾームの放出にはESCRT 経路の最上流で機能するHrsが必要であることを報告した[6]（図2）。エクソゾームはHCV エンベロープを含み、C型慢性肝炎の患者血清から分離されたエクソゾームからHCV RNAが検出されたとの報告がある[4]。我々は、エクソゾームの放出機構はHCVのアセンブリおよび出芽に酷似していることに着目し、小胞輸送経路ESCRTとHCVの関連を探索した。

2. 材料と方法

2. 1 供試した細胞と調整

Huh7 細胞は10%仔ウシ血清を含むDulbecco's Modfied Eagle培地で培養した。Huh7をHrs特異的にノックダウンするために、Hrs特異的なshRNAを発現するレトロウイルスベクターをトランスフェクションしたあと、2 ug/mL のピューロマイシンを用いて選択した。VPS4B野生型・ドミナントネガティブ（E235Q）（Dr. Sundquist より供与）は、1 日目にFuGene6 (Roche) を用いてトランスフェクションし、2 日目にJFH1を感染させて使用した（MOI =0.01）。

2. 2 Real-time PCR

HCV-RNA の定量は、以前に報告されているプライマーを用いてreal-time PCRを用いて行った[8]。

2. 3 エクソゾームの分離精製

培養上清中のエクソゾームを定量するために、10 cmシャーレに培養した細胞に1 mMのA23187 (Ca ionophore, Sigma) を用いて刺激し、48時間後に上清を回収した。培養上清に含まれるエクソゾームは、既報の手技を用いて精製した[6]。

2. 4 ショ糖密度勾配

ショ糖密度勾配はショ糖をHepes緩衝液に溶解し（10%および60%）、遠心管に2相に重層し、3時間水平に倒した後に使用した。精製したエクソゾームを加え、100,000 gで20時間遠心して分画を作成した[7]。

3. 結果

3. 1 Hrs ノックダウンHuh7 細胞の樹立

Hrsに対するshRNA発現レトロウイルスベクターを導入したHuh7細胞はウェスタンブロットを用いてHrs特異的にノックアウトされていることを確認した。ノックダウン細胞およびコントロール細胞をCa ionophoreで刺激した後に上清のエクソゾームを回収し、ショ糖密度勾配にかけたところ、既報と同様のフラクションに存在することを確認し、電子顕微鏡による観察でも両者に形態学的な差はないことが確認された。

3. 2 Hrs はエクソゾーム放出に影響する

これらの細胞を使って、Ca ionophoreで刺激し上清中に放出されたエクソゾームを定量して、Hrsがエクソゾーム放出に影響するかどうかを調べた。刺激後48時間の時点での上清中のエクソゾームはHrsノックダウンHuh7細胞において著明に減少した（図3）。

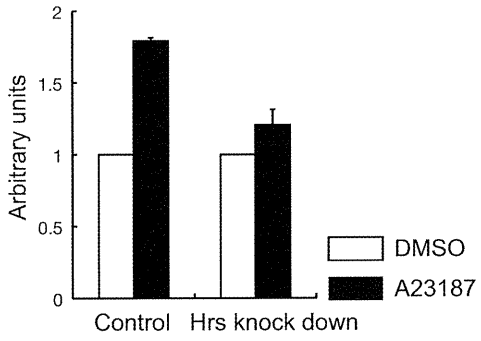

図３　上清中エクソゾームの定量

3. 3 HCV コアタンパクはエクソゾームと同じ分画に存在する

Huh7 細胞にJFH-1 を感染させ、その上清を回収し、エクソゾームを分離精製し、ショ糖密度勾配で分画した。エクソゾームマーカーCD63あるいはHCVコアタンパクで検出したところ、両者でみられるピークはほぼ同じ分画に存在した（図4）。

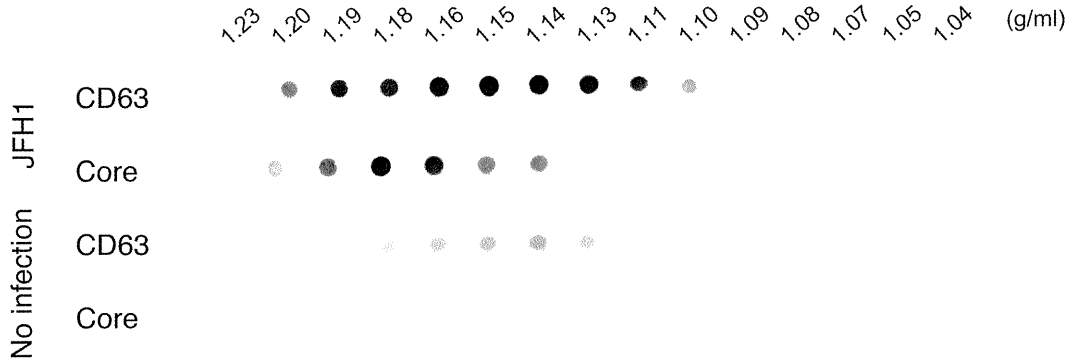

図4 培養上清中のHCV とエクソゾームのショ糖密度勾配による分離

3. 4 ESCRT 機能不全細胞では、JFH1の放出量が減少する。

Hrs ノックダウンHuh7 細胞にJFH1を感染させ、上清に放出されるHCV-RNA を経時的にreal-timePCR で定量した。Hrsノックダウン細胞では、コントロール細胞に比して約2 log(10) のJFH1-RNA の減少を認めた（図5）。同様の実験をVPS4Bドミナントネガティブ発現Huh7を用いて行ったが、同じ傾向を認めた。MVB形成に必須とされるRab27aのノックダウン細胞でも同様の傾向を認めた。ESCRT関連遺伝子だが、MVB 形成に必要とされないAMSHのノックダウン細胞では、HCV放出に差を認めなかった。細胞内のJFH1-RNA 量はHrsの有無あるいはVPS4Bの有無で明らかな差を認めなかった（図6）。

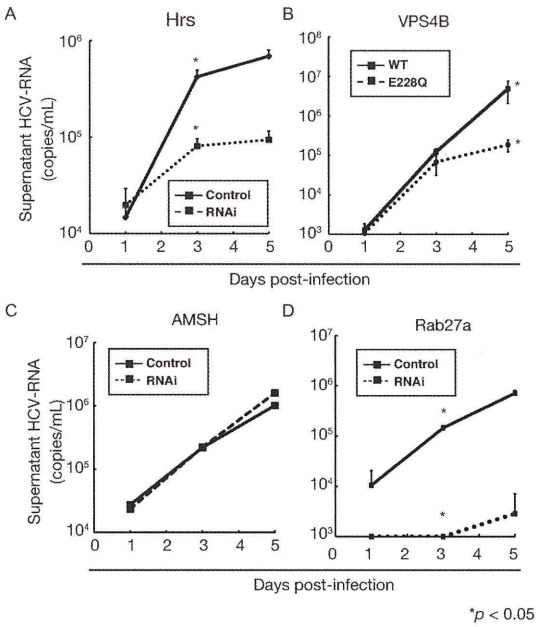

図5 培養上清中のHCV-RNA 定量

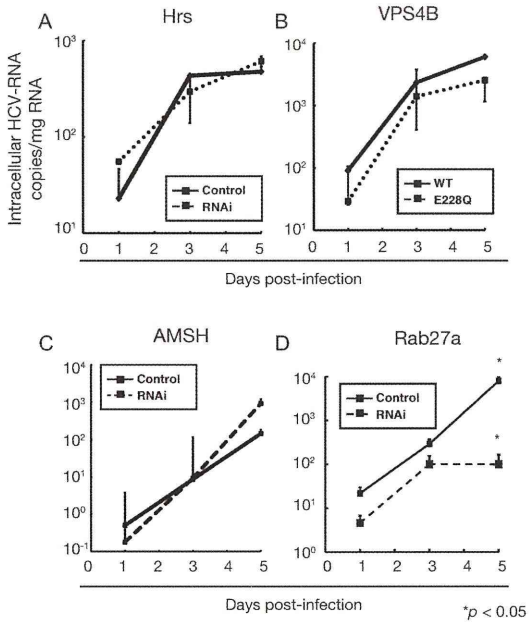

図6 細胞内のHCV-RNA 定量

3. 5 HCV-coreとMVB内のintraluminal vesicleは共局在する

我々はHCV 粒子が細胞内のどのコンパートメントに局在するかを更に調べるために、免疫電顕を用いてHCV感染Huh7細胞をHCV-core抗体とMVBマーカーである抗CD63 抗体で染色した。その結果、HCVコア抗原は、MVB内のintraluminal vesicle と共局在していた（図７）。これらのことから、HCVは、Hrs依存性エクソゾーム経路を利用していることが示唆された。

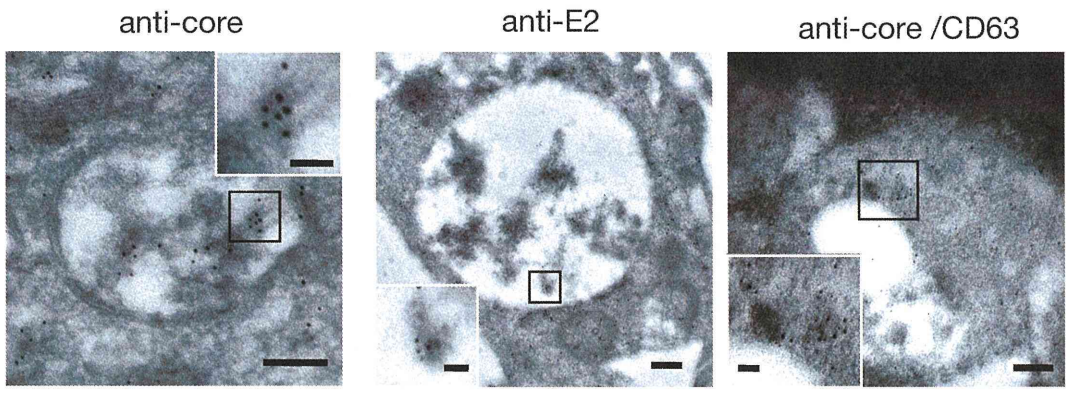

図７ JFH1感染細胞の免疫電顕像

4. 考案

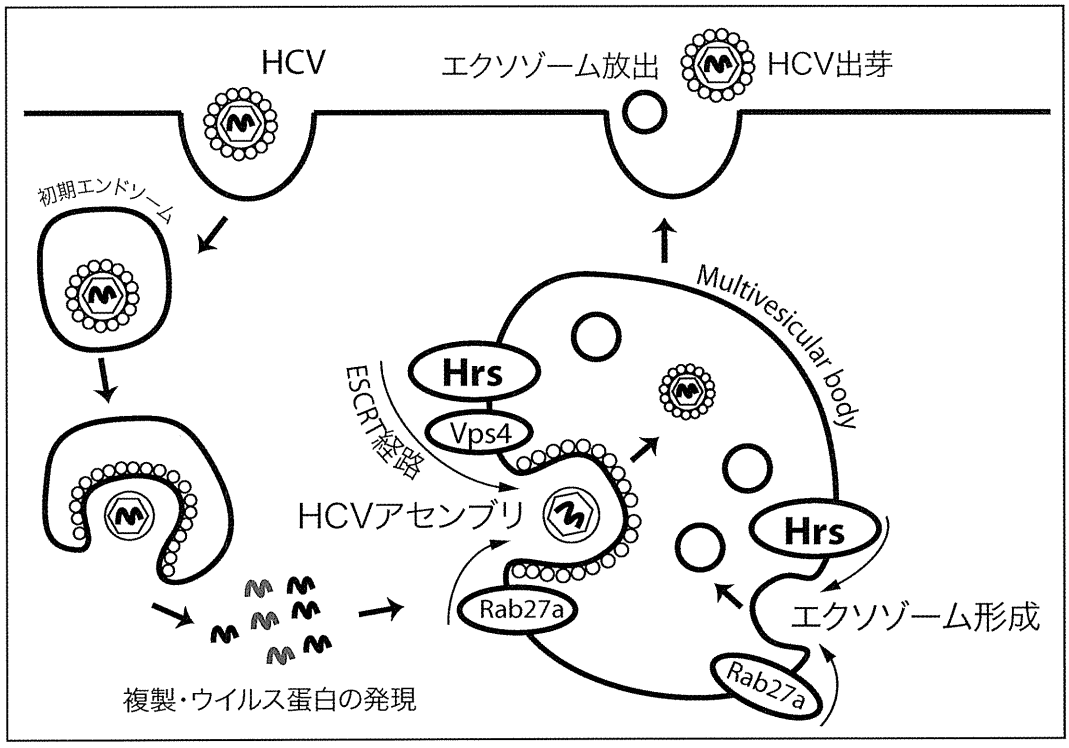

図7 研究結果の模式図

本研究では、Huh7細胞を用いて、ESCRT経路を阻害することでHCV放出が抑制されることを示した。同時にCa ionophore刺激で放出されるエクソゾーム量も減少していた。エクソゾームにはHCV が含まれるとされており[3]、本研究でもHCVとエクソゾームはショ糖密度勾配において同じ分画に存在することから、HCVとエクソゾームは同じESCRT経路を介して放出されている可能性が考えられた。更に、免疫電子顕微鏡を用いて細胞内外の微小粒子を観察した結果、HCV粒子はMVB内に存在することが明らかとなった。また、Hrs/VPS4/Rab27aノックダウンによるMVB形成不全細胞においては、上清中の感染性HCV粒子の酸性低下が見られた。以上のことから、HCVはHrs依存性エクソゾーム経路を介してアセンブリ・放出されていることが明らかとなった。

ESCRT経路はMVB形成に必須の経路であり、エクソゾームはMVBの内部小胞が放出されたものとされているが[6]、エクソゾームとMVB との関連性には議論が多い。エクソゾームの形成にはセラミドを必要とするが、ESCRT経路には依存していなかったとの報告もある[7]。放出するエクソゾームの内容物によって依存する経路が違うことも予想され、今後詳細な検討が必要である。

本研究からは、Hrs依存性エクソゾーム経路を利用してHCVが放出されている可能性が明らかになった[10]（図7）。本研究はHCV ライフサイクルの一端を明らかにするものであり、この経路を阻害することが出来れば、新たな抗ウイルス薬の開発につながるものと考えられる。

5. 謝辞

本研究に協力頂いた、技術員　中村真央・小山杏子に感謝する。

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9. 知的財産権の出願・登録状況

9. 1 特許取得

該当なし

9. 2 実用新案登録

該当なし

9. 3 その他

該当なし

10 研究成果の刊行物・印刷物

J Gastroenterol (2011) 46:232–241
DOI 10.1007/s00535-010-0297-2

ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

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

Yasuteru Kondo · Yoshiyuki Ueno · Eiji Kakazu · Koju Kobayashi · Masaaki Shiina · Keiichi Tamai · Keigo Machida · Jun Inoue · Yuta Wakui · Koji Fukushima · Noriyuki Obara · Osamu Kimura · Tooru Shimosegawa

Received: 9 May 2010 / Accepted: 11 July 2010 / Published online: 17 August 2010

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

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Y. Kondo · Y. Ueno (✉) · E. Kakazu · M. Shiina · K. Tamai · J. Inoue · Y. Wakui · K. Fukushima · N. Obara · O. Kimura · T. Shimosegawa
Division of Gastroenterology,
Tohoku University Graduate School of Medicine,
1-1 Seiryo, Aobaku, Sendai 980-8574, Japan
e-mail: yueno@med.tohoku.ac.jp

K. Machida
Department of Molecular Microbiology and Immunology,
University of Southern California Keck School of Medicine,
2011 Zonal Avenue, Los Angeles, CA 90033, USA

K. Kobayashi
School of Health Science, Tohoku University, 1-1 Seiryo,
Aobaku, Sendai 980-8574, Japan

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-μm 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 Parmingen, 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 [Fremount, 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 (TCCCGGGGCACTCGCAAGCACCCT 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. Semi-quantification 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 μl. 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 μl 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 μl of BD Perm/Wash buffer containing pre-conjugating polyclonal anti-NS5A antibody (Biodesign 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 μl of cold RPMI 1640 medium. After the addition of 10 μl of IL-10- or IFN-γ-Catch Reagent (Miltenyi 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 μL 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 ready-made 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-1α, 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)

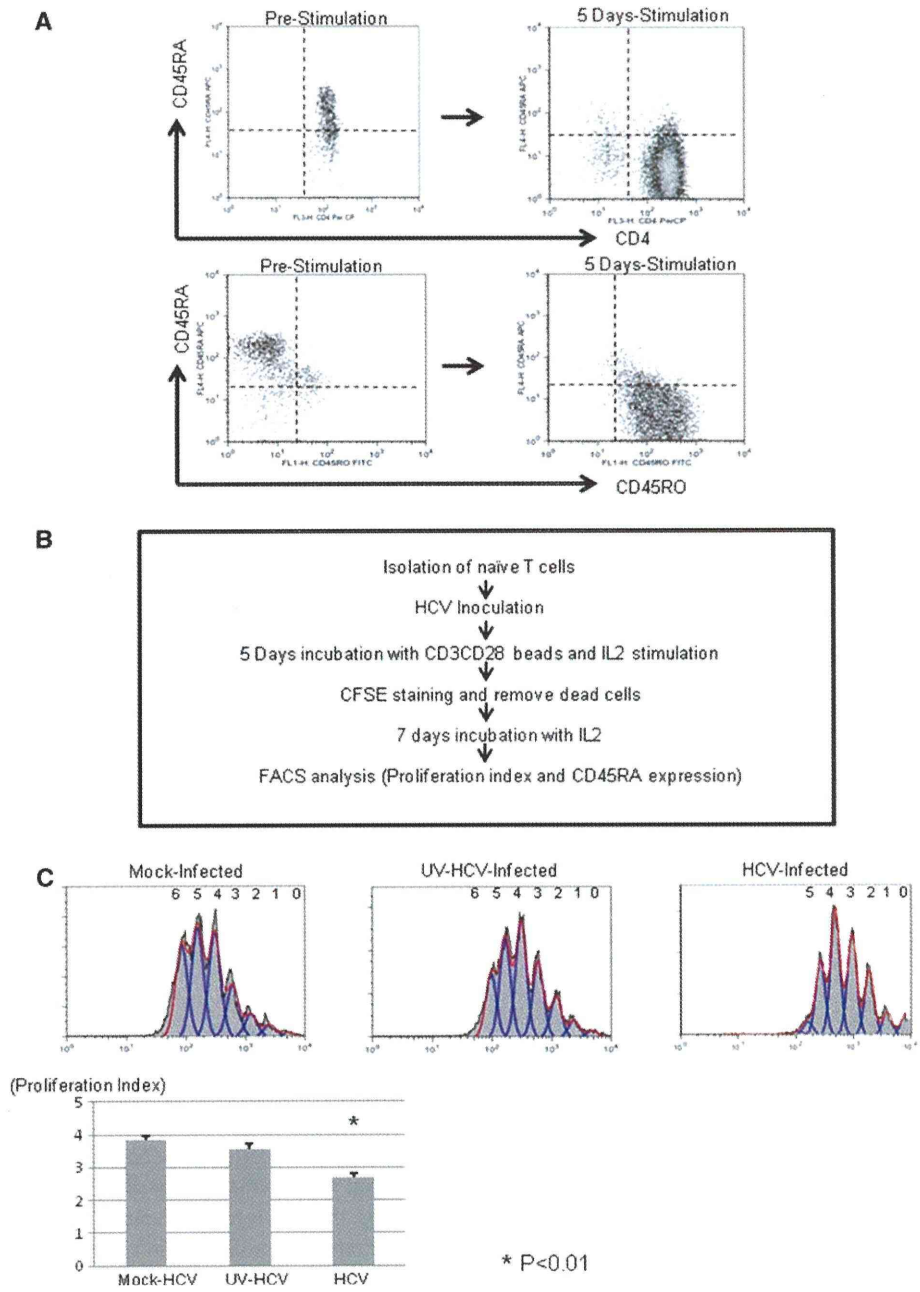

Fig. 1 Suppression of proliferation activity in hepatitis C virus (*HCV*)-infected human naïve T lymphocytes. **a** A representative dot plot of $CD4^+CD45RA^+RO^-$ cells is shown. Cells are stained with CD4-PerCP-antibody (Ab), CD45RA-fluorescein isothiocyanate (FITC)-Ab, and CD45RO-APC-Ab. The purity 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

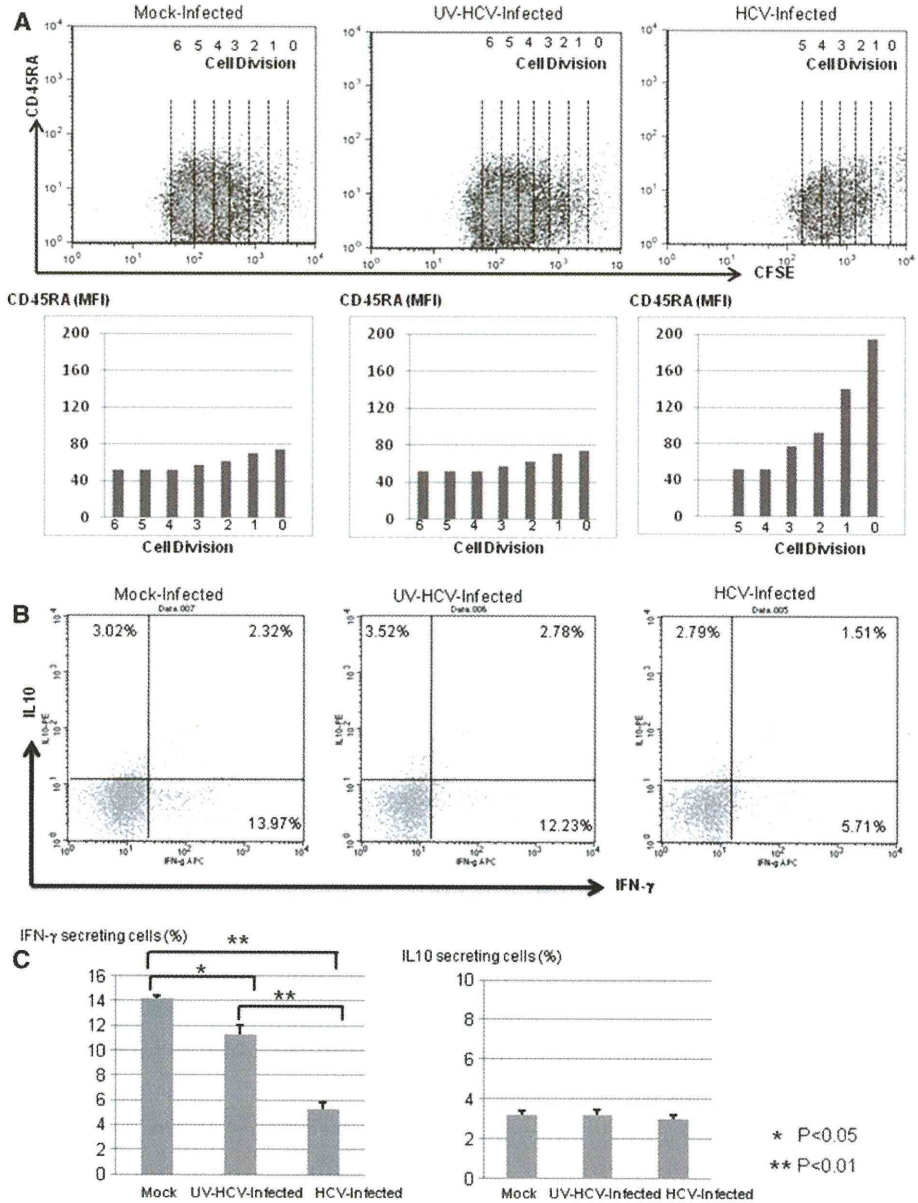

Fig. 2 Suppression of development and Th1 commitment in HCV-infected human naïve T lymphocytes. **a** Representative dot plots of CD45RA and carboxyfluorescein succinimidyl ester (CFSE) double staining are shown. *Numbers* in the representative dot plots indicate the numbers of cell divisions. *Bar graphs* 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

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

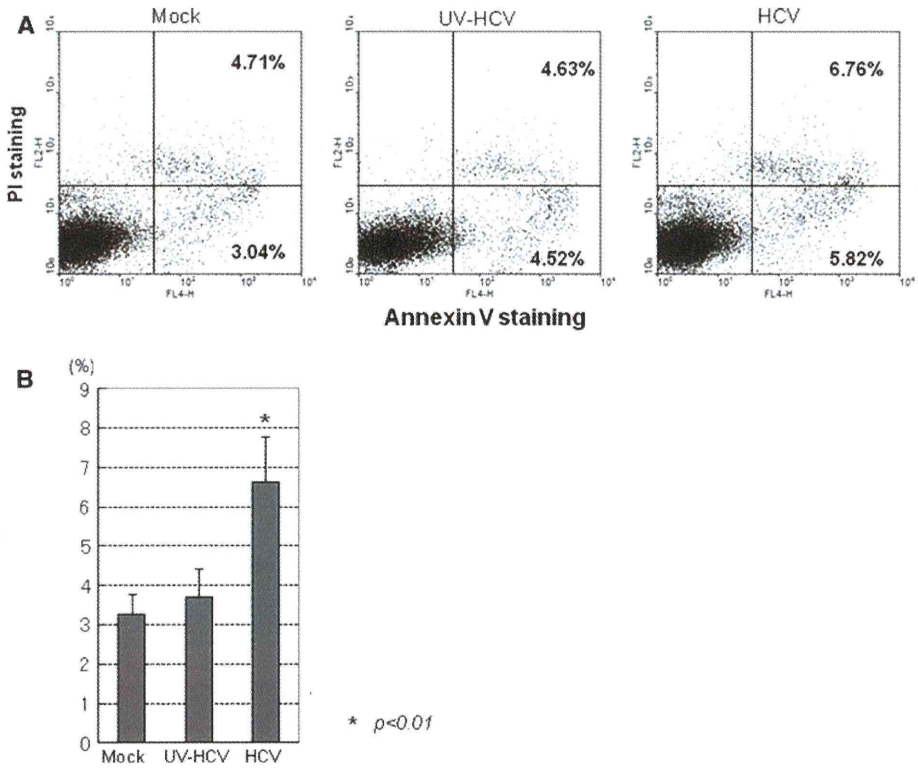

Fig. 3 HCV replication induces apoptosis of naive $CD4^+$ cells. **a** Representative dot plots of Annexin V and propidium iodide (*PI*) staining are shown. The *numbers* in the quadrants indicate the frequencies of early apoptotic cells (Annexin V^+ and PI^-) and dead cells (Annexin V^+ and PI^+ cells). **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

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.