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G. 知的所有権の出願・登録状況  
なし

## HBVpseudotype 作製の試みとレセプターの分離・同定に関する研究

研究分担者 上田啓次 浜松医科大学・教授

研究要旨 HBV pseudotype の作製に成功した。これを用いて感染を指標にしたヒト肝細胞 cDNA ライブラリーの発現による HBV レセプターの同定を遂行中である。実験の経過上 cDNA ライブラリーの導入操作で本 pseudotype の感染性が著しく向上することが解った。本現象を見極め HBV レセプターの分離・同定に迫りたい。

### A. 研究目的

HBV レセプターは未だ不明で有効な感染系が樹立できずにいる。これまで試みられなかった HBV pseudotype (HBVp) による生物学的アッセイ法で本レセプターを分離・同定し感染系を構築し、病態解明、治療法の開発に役立てる。

### B. 研究方法

Gfp-hygR を含むレトロウイルスコア粒子を HBV 膜粒子で覆った HBVp の作製を試みた。本 HBVp を用いてヒト肝細胞 cDNA ライブラリーをスクリーニングした。

(倫理面への配慮)

現在のところ特に関係しないものと思われる。

### C. 研究結果

培養上清中に出現すると想定される HBVp を抗 HBs 抗体による免疫沈降し RNA 抽出、GFP 遺伝子を標的とした RT-PCR から HBVp 産生されたと考えられた。本 HBVp を用いてヒト肝細胞 cDNA ライブラリーのスクリーニング中にある処理で感染性が向上することが観察された。

### D. 考察

HBVp の作製は可能であり、本ウイルスを用いて HBV レセプターの分離・同定が生物学的アッセイで

可能になった。ある処理で感染性が向上することは HBV レセプターの性情を示唆すると思われた。

### E. 結論

HBVp を用いて感染性を指標に HBV レセプターの分離・同定を促進させ得る。

### F. 健康危険情報

なし

### G. 研究発表

#### 1. 論文発表

今のところなし

#### 2. 学会発表

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### H. 知的財産権の出願・登録状況

#### 1. 特許取得

なし

#### 2. 実用新案登録

なし

#### 3. その他

なし

### Ⅲ. 研究成果の刊行に関する一覧表

### III. 研究成果の刊行に関する一覧表

#### 書籍

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#### IV. 研究成果の刊行物・別冊

# Hepatitis C Virus Infection of T Cells Inhibits Proliferation and Enhances Fas-Mediated Apoptosis by Down-Regulating the Expression of CD44 Splicing Variant 6

Yasuteru Kondo,<sup>1,2</sup> Keigo Machida,<sup>1</sup> Helene Minyi Liu,<sup>1</sup> Yoshiyuki Ueno,<sup>2</sup> Koju Kobayashi,<sup>2</sup> Takaji Wakita,<sup>3</sup> Tooru Shimosegawa,<sup>2</sup> and Michael M. C. Lai<sup>1,4,5</sup>

<sup>1</sup>Department of Molecular Microbiology and Immunology, University of Southern California Keck School of Medicine, Los Angeles; <sup>2</sup>Division of Gastroenterology, Tohoku University, Sendai City, and <sup>3</sup>National Institute of Infectious Disease, Tokyo, Japan; <sup>4</sup>Institute of Molecular Biology, Academia Sinica, Taipei, and <sup>5</sup>National Cheng Kung University, Tainan, Taiwan

**Background.** A lymphotropic hepatitis C virus strain (HCV, SB strain, hereafter "SB-HCV") has been shown to infect established T cell lines (Molt-4 and Jurkat) and primary human naive CD4<sup>+</sup> T cells. During T cell development and activation, transient expression of CD44 splicing variant 6 (CD44v6) plays a significant role.

**Methods.** SB-HCV was used to infect Molt-4 cells, and their cellular proliferation and CD44 expression was examined.

**Results.** SB-HCV-infected Molt-4 cells expressed a significantly lower level of the CD44v6 isoform. The infected cells could be divided into 2 carboxyfluorescein succinimidyl ester (CFSE) groups, CFSE-high (indicating low proliferation activity; 34.2% of the cells) and CFSE-low (indicating high proliferation activity; 62.5% of the cells), whereas uninfected cells consisted of only a CFSE-low population. Of the CFSE-high cells, 82.4% were positive for the HCV protein NS5A, whereas only 1.2% of the CFSE-low cells were positive for this protein. Among the HCV proteins, NS5A alone caused the down-regulation of CD44v6 expression. After cells were stimulated with phorbol myristate acetate, the amount of phosphorylated mitogen-activated protein (MAP) kinase was significantly reduced in CFSE-high, SB-HCV-infected Molt-4 cells. After Fas ligand stimulation, SB-HCV-infected Molt-4 cells had increased cleavage of caspase 8 and 3 and enhanced apoptosis, compared with the rates of cleavage and apoptosis in control groups, indicating that SB-HCV infection increased Fas-mediated apoptosis.

**Conclusion.** HCV replication in T cells suppresses cellular proliferation and enhances susceptibility to Fas signaling by inhibiting CD44v6 signaling and expression.

Hepatitis C virus (HCV) infects about 170 million people worldwide and is a major cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC) [1]. HCV infection is often persistent and cellular immune response to HCV plays an important role in the pathogenesis of chronic hepatitis, cirrhosis, and HCC [2]. Several mechanisms have been proposed for the failure of

HCV-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cell response during HCV infection; these include deletion, anergy, cytotoxic T lymphocyte exhaustion, and suppression via regulatory CD4<sup>+</sup>CD25<sup>+</sup> T cells and interleukin-10 (IL-10)-secreting regulatory CD8<sup>+</sup> T cells [3, 4].

Previously we have shown that a strain of HCV (strain SB, hereafter "SB-HCV") can infect and replicate in not only B cells but also T cells as well, and that HCV replication in T cells can inhibit signaling by interferon- $\gamma$ , signal transducer and activator of transcription factor 1 (STAT-1), and T-bet signaling of T cells [5]. However, the replication activity of SB-HCV in T cells is weaker than that in B cells, suggesting that the lymphotropic SB-HCV might preferentially replicate in B cells but nevertheless affect T cell development in local lymph nodes. In particular, among CD4<sup>+</sup> cells, CD45RA<sup>+</sup>-CD45RO<sup>-</sup>CD4<sup>+</sup> cells are the most susceptible to HCV

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Reprints or correspondence: Michael M. C. Lai, 2011 Zonal Ave. HMR500, Los Angeles, CA 90033 (michlai@usc.edu).

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infection [5]. It is known that during the activation that shifts T cells from naive to effector cells, T cells have to survive activation-induced cell death (AICD), which may contribute to the maintenance of an appropriate level of immune response. Fas–Fas ligand signaling is thought to be one of the mechanisms of AICD [6, 7].

There are several splicing variants of the immune molecules in T cells that contribute to proper immune response [8]. We have reported that SB-HCV could replicate in T cells during different temporal periods [5]. Therefore, we focused on CD44 splicing variants because the temporal regulation of alternative CD44 splicing in T lymphocytes plays a significant role in the maintenance of appropriate T cell development [8]. The CD44 family of transmembrane glycoproteins is present on a wide variety of cell types, including lymphocytes, neutrophils, endothelial cells, and fibroblasts [9, 10]. CD44 molecules display a multitude of functions involving a large array of signal transduction pathways, and CD44 is expressed into multiple splicing variants [11, 12]. During T cell development and activation, transient expression of CD44 splicing variant 6 (CD44v6) plays a significant role [13, 14]. The expression of CD44v6 provides a proliferative stimulus for T cells independent of the T cell receptor (TCR)–CD3 complex. Proliferative activity is accompanied by activation of the mitogen-activated protein kinase (MAPK) pathway [15]. Moreover, CD44v6 expression could interfere with Fas signaling [16].

Recently, the roles of individual HCV proteins have been studied extensively by using HCV-producing hepatocyte cell lines and the replicon system [17–21]. The processing of the HCV precursor polyprotein requires both host and viral proteases to produce structural and nonstructural proteins. Among the nonstructural proteins, NS5A interacts with a number of cellular proteins and may interfere with host cell signaling pathways [22, 23]. Several authors have reported that NS5A can inhibit the extracellular signal-regulated kinase (ERK)–MAPK pathway; however, several other HCV proteins, such as HCV core protein, have been reported to enhance this pathway [24, 25]. The mechanism of CD44v6 inclusion is still not clear, although it is known that the events upstream of this inclusion mechanism may include Ras-ERK activation [8, 26, 27]. Thus, HCV replication in T cells may affect proliferation and Fas-mediated apoptosis of T cells by altering CD44v6 expression or MAPK activation. During T cell development, T cells undergo vigorous proliferative activity which might facilitate HCV replication in T cells. However, extensive proliferation of HCV in T cells might interfere with proper T cell development.

Previously, our reports have indicated that SB-HCV, which was produced from an HCV-positive B-cell lymphoma cell line (SB cells), can infect and replicate in established B cell lines (Raji and Daudi), established T cell lines (Molt-4 and Jurkat), and primary lymphocytes [5, 28, 29]. HCV infection of these immune cells conceivably can perturb their functions. This study was conducted to analyze whether HCV replication in T lymphocytes might affect the proliferative activity and apoptosis of T cells as a result of signaling changes and aberrant expression of CD44 variants.

## MATERIALS AND METHODS

**Culture of cell lines.** SB cells, which continuously produce infectious HCV particles, were originally cultured from splenocytes obtained from an HCV-infected patient with type 2 mixed cryoglobulinemia and monocytoid B-cell lymphoma [29]. The cells were maintained in standard RPMI 1640 (Invitrogen) medium that contained 20% fetal bovine serum (FBS) without any supplement. Huh7.5 cells, which were kindly provided by C.M. Rice (Laboratory of Virology and Infectious Disease, Center for the Study of Hepatitis C, The Rockefeller University), were cultured at 37°C in Dulbecco's modified Eagle medium containing 10% FBS.

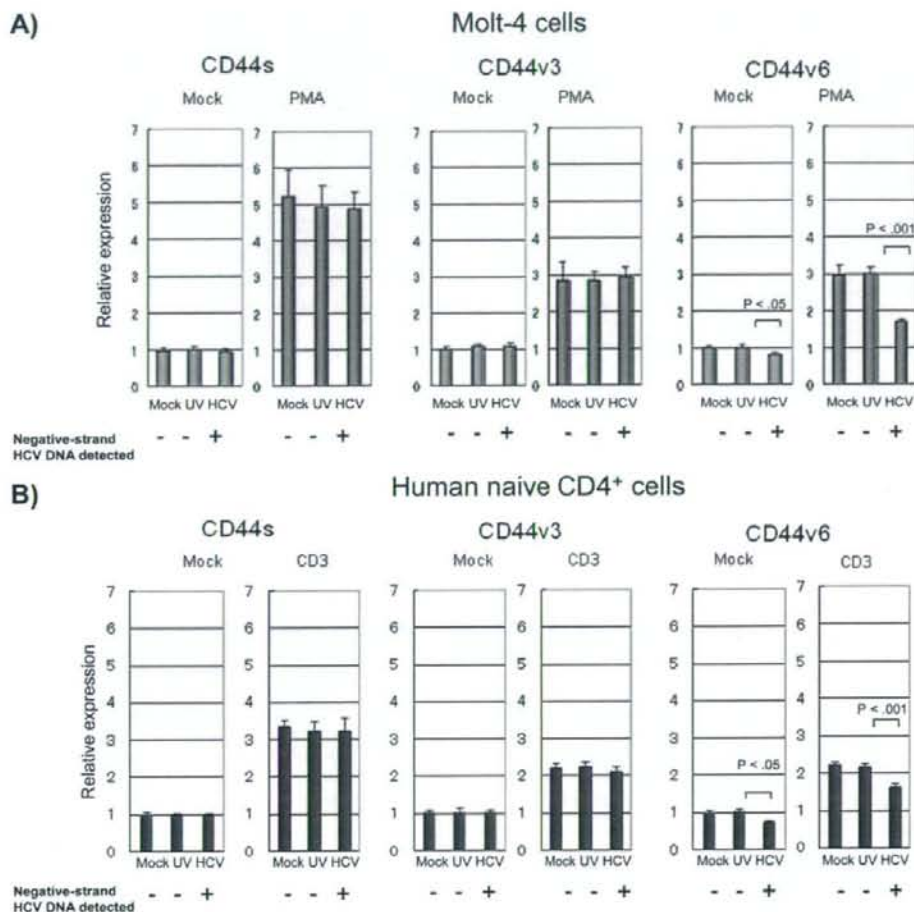
**In vitro infection of Molt-4 and primary naive (CD45RA<sup>+</sup>–CD45RO<sup>–</sup>) CD4<sup>+</sup> cells.** SB cell culture supernatant (5 mL), which contained  $2.2 \times 10^4$  copies/mL of HCV RNA, was used to infect Molt-4 and human naive CD4<sup>+</sup> cells ( $1 \times 10^5$  cells). Control cells infected with UV-irradiated SB cell culture supernatant (hereafter, "UV-SB-HCV-infected cells") were included in every experiment. Supernatant from Huh7.5 cells transfected with JFH-1 strains [19, 20, 30] at 10 days after transfection were used for several control experiments. Cells were washed 3 times at 5 days after infection. A portion of the cells ( $3 \times 10^5$ – $5 \times 10^5$  cells) was then harvested for analysis; the remaining cells ( $1 \times 10^5$  cells) were kept and incubated further.

**Strand-specific intracellular SB-HCV RNA detection and quantitative SB-HCV RNA detection in the culture supernatant.** Strand-specific intracellular SB-HCV RNA was detected by using a recently established procedure that combined methods published elsewhere [31, 32], with minor modifications [5]. Positive strand-specific and negative strand-specific SB-HCV RNA were detected by use of a nested polymerase chain reaction (PCR) method.

To quantify SB-HCV RNA in the culture supernatant, SB-HCV RNA extraction from culture supernatant (140  $\mu$ L) was carried out with QIAmp Viral RNA Minikit (Qiagen). Amplification was performed in 20- $\mu$ L reaction mixtures that contained AmpliTaq Gold DNA polymerase and optimized buffer (TaqMan Universal PCR Master Mix; Applied Biosystems) with the ABI prism 7900 sequence detector system. Forward primer (300 nmol/L, nucleotide 215–197, 5'-TCCCGGGAGAGCCATAG-TG-3'); reverse primer (600 nmol/L, nucleotide 158–140, 5'-TCCAAGAAAGGACCCRGT-3'); and 250 nmol/L TaqMan minor-groove-binding (MGB) probe labeled with 6-carboxy-fluorescein, nucleotide 195–181, 5'-FAM-TCTGCGGAACCGGTG-MGB-3') were also included in the PCR mix reaction. In vitro-transcribed full-length HCV RNA was used as a standard.







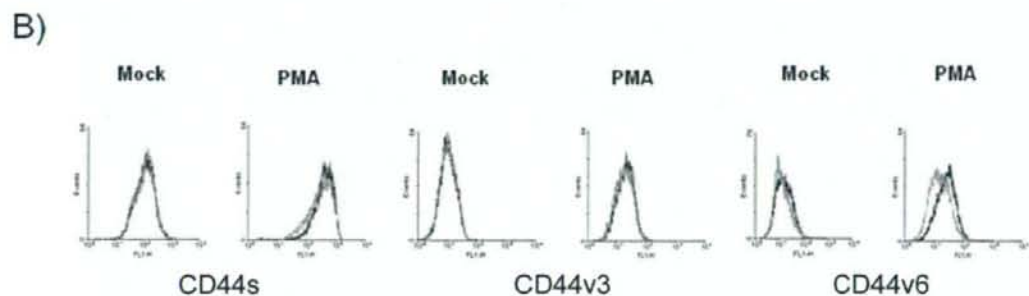
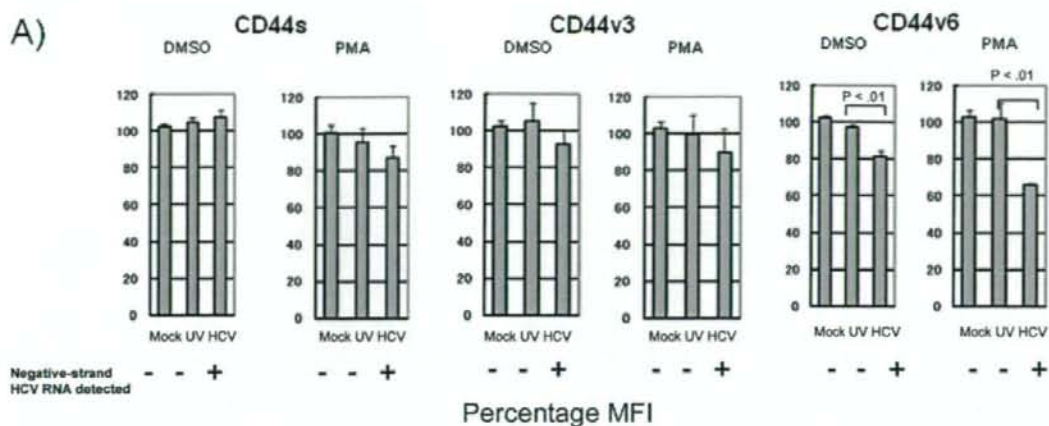
**Figure 1.** Quantification of standard CD44 (CD44s) mRNA as well as mRNA from splicing variants 3 and 6 (CD44v3 and CD44v6) in Molt-4 cells (A) and human CD45RA<sup>+</sup>CD45RO<sup>-</sup> naive T cells (B) (samples obtained from 1 healthy subject). Quantification of CD44s, CD44v3, and CD44v6 mRNA with or without stimulation (phorbol myristate acetate [PMA] or anti-CD3 antibody [CD3]) was carried out by real-time polymerase chain reaction with comparative cycle threshold methods. Five independent experiments yielded similar results. Mock, mock-infected cells; HCV, cells infected with hepatitis C virus, strain SB; UV, cells infected with UV-irradiated hepatitis C virus, strain SB.

We first analyzed the time course of CD44s, CD44v3, and CD44v6 mRNA up-regulation in Molt-4 cells after PMA stimulation. We found that the expression of mRNA by these isoforms peaked at 8–12 h after stimulation, whereas the maximum up-regulation of surface expression of these CD44 variants was observed at 24 h after stimulation (data not shown). Therefore, to determine whether HCV could affect the expression of different splicing variants of CD44, we compared the expression level of these variants in cells that had been mock-infected, infected with UV-SB-HCV, or infected with SB-HCV; this comparison was performed at 8 h after activation for mRNA analysis and 24 h after activation for surface protein analysis. The status of HCV infection and replication was confirmed by detecting negative-strand HCV RNA by semiquantitative reverse-transcriptase

PCR (figure 4, which appears only in the electronic version of the *Journal*, and summarized in figure 1B).

All the CD44 variants examined showed a low level of mRNA expression, which was significantly increased after PMA stimulation (figure 1). We noted that CD44v6 mRNA expression in SB-HCV-infected Molt-4 cells was slightly but significantly lower than that observed in control groups, even without PMA stimulation ( $P < .05$ ), and this difference became more prominent after PMA stimulation ( $P < .001$ ) (figure 1). In contrast, the mRNA expression of CD44s and CD44v3 was not affected by HCV infection status. We further confirmed the suppression of CD44v6 mRNA by HCV infection in primary naive T cells (figure 1B). In this case, the primary CD4<sup>+</sup> cells were stimulated with anti-CD3 antibody, and the suppression of CD44v6 mRNA





**Figure 2.** Comparison of surface expression of standard CD44 (CD44s) and splicing variants 3 and 6 (CD44v3 and CD44v6) in Molt-4 cells. *A*, Comparison of mean fluorescence intensity (MFI) among the 3 groups with or without phorbol myristate acetate (PMA) stimulation was carried out with flow cytometry. The percentage MFI was calculated as follows: target mean fluorescence intensity divided by mean fluorescence intensity of mock-infected cells without PMA stimulation times 100. Three independent experiments yielded similar results. *B*, Fluorescence-activated cell sorter analyses of surface expression of different CD44 isoforms. *Red lines*, mock-infected Molt-4 cells; *black lines*, Molt-4 cells infected with UV-irradiated hepatitis C virus, strain SB (UV-SB-HCV); *green lines*, Molt-4 cells infected with SB-HCV.

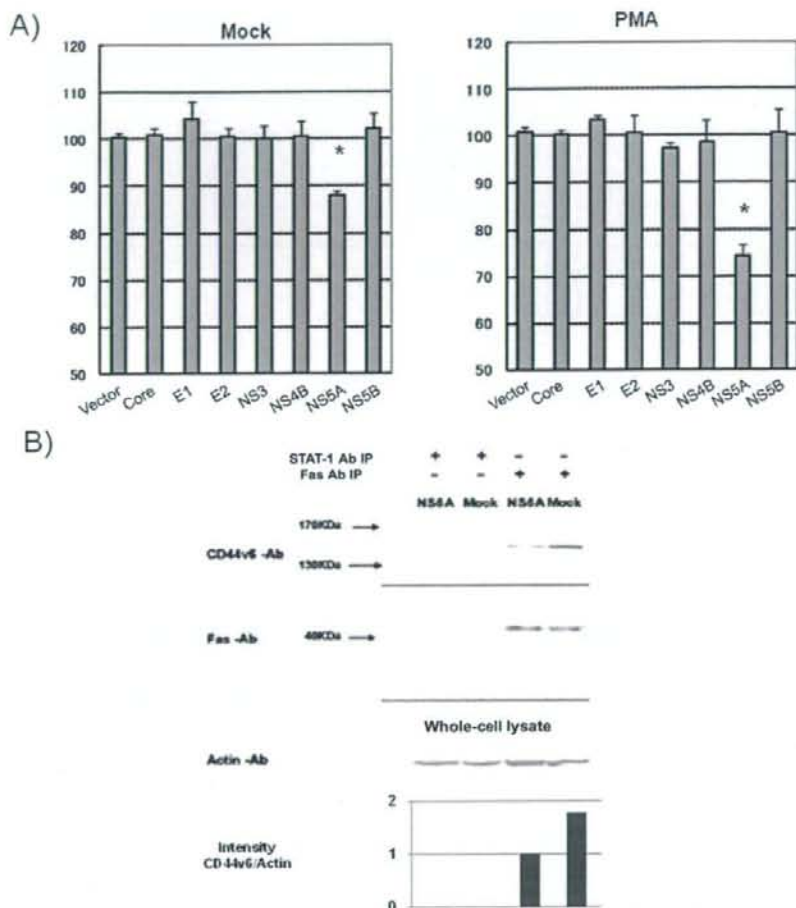
became more apparent after stimulation. Taken together, these data indicate that HCV replication in CD4<sup>+</sup> cells specifically suppressed CD44v6 mRNA inclusion.

To corroborate this finding, we then analyzed surface expression of CD44s, CD44v3, and CD44v6 among the 3 groups of cells (mock infected, UV-SB-HCV infected, and SB-HCV infected) (figure 2). The mean fluorescence intensity (MFI) of CD44v6 with or without PMA stimulation was significantly suppressed in SB-HCV-infected Molt-4 cells, compared with Molt-4 cells that had been mock infected or infected with UV-SB-HCV ( $P < .01$ ) (figure 2A). CD44s and CD44v3 also showed slightly lower levels of expression after PMA stimulation in HCV-infected cells; however, these differences were not statistically significant. The lower surface expression of CD44v6 in SB-HCV-infected cells was further confirmed by fluorescence-activated cell sorter analysis (figure 2B). These data suggested that SB-HCV infection of T cells caused down-regulation of CD44v6 and that HCV replication in T cells, not merely the binding of HCV to T cells, is required for this effect.

To further demonstrate that HCV replication was required for the down-regulation of CD44v6 expression in T cells, we used JFH-1 HCV [20] to infect Molt-4 cells. We have previously shown that JFH-1-HCV cannot infect or replicate in lymphocytes (Machida et al., unpublished data). Molt-4 cells infected with JFH-1 HCV and Molt-4 cells infected with UV-irradiated JFH-1 HCV showed no difference in mRNA or surface protein expression for CD44s, CD44v3, or CD44v6 (data not shown). Therefore, HCV replication might be necessary for the down-regulation of CD44v6 inclusion in T cells. However, we could not rule out the possibility that SB-HCV has a unique ability to down-regulate CD44v6 inclusion because we have so far only been able to use the SB strain to infect T cells.

**SB-HCV infection of T cells and decreased proliferation.** Since CD44v6 expression is associated with cellular proliferation, we analyzed the proliferative activity of SB-HCV-infected Molt-4 cells [15]. The results showed that SB-HCV-infected Molt-4 cells could be clearly separated into 2 groups (CFSE high





**Figure 3.** NS5A protein and suppression of CD44v6 in T cells. *A*, Effects of individual hepatitis C virus (HCV) proteins on surface expression of CD44 splicing variant 6 (CD44v6). Mean fluorescence intensity (MFI) of CD44v6 on Molt-4 cells transfected with individual HCV proteins was normalized by the MFI of CD44v6 on mock-infected Molt-4 cells. Five independent experiments yielded similar results. *B*, Coimmunoprecipitation of CD44v6 with Fas. Cell lysates were precipitated with anti-Fas antibody (Fas Ab) and detected by immunoblotting with different antibodies. Band intensity was analyzed with Image J (National Institutes of Health). The bar graph shows the relative intensity of CD44v6 and actin. Three independent experiments yielded similar results. IP, immunoprecipitation; STAT-1 Ab, anti-signal transducer and activator of transcription factor 1 antibody.

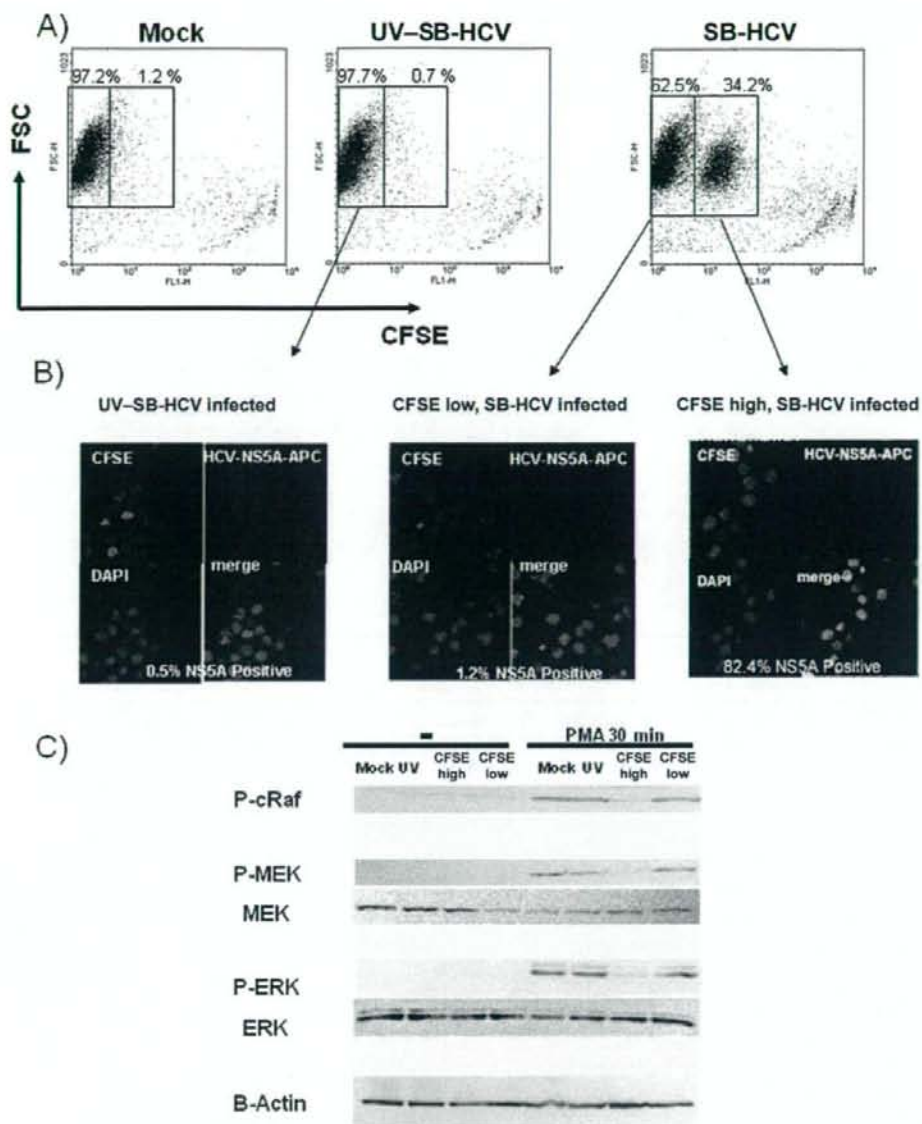
and CFSE low), which were sorted by use of a flow cytometry cell sorter (FACSVantage; Becton Dickinson), whereas mock-infected cells or cells infected with UV-SB-HCV formed only 1 group (figure 5A). The CFSE-high and CFSE-low groups were separated and analyzed for NS5A expression. The data showed that 82.4% of the CFSE-high group expressed NS5A protein,

whereas only 1.2% of the CFSE-low group were positive for NS5A (figure 5B). These data indicated that most cells in the CFSE-high group (i.e., the slow-growing group) were infected with SB-HCV, whereas the uninfected cells were mostly fast growing. Therefore, SB-HCV infection suppressed the proliferation activity of Molt-4 cells.

**SB-HCV infection and suppression of Ras-MEK-ERK activation and CD44v6 up-regulation in T cells.** We next analyzed the Ras-MEK-ERK signaling that may induce proliferation-related genes, since it has been reported that this signaling is the upstream mechanism of CD44 variant inclusion and that CD44v6 can enhance this signaling, acting

The figure is available in its entirety in the online edition of the *Journal of Infectious Diseases*.

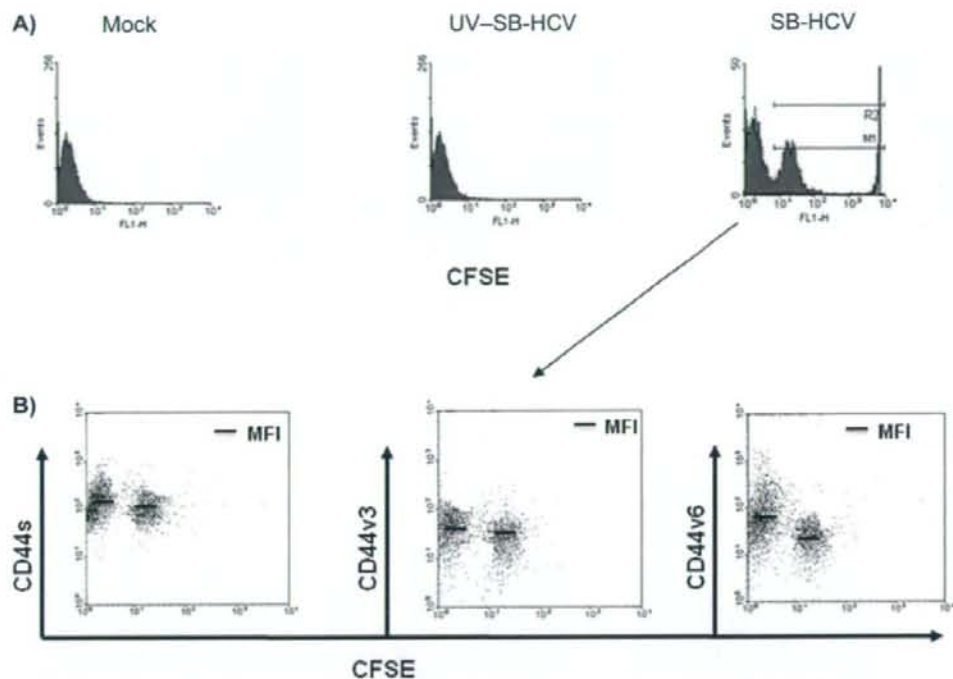
**Figure 4.** Detection of positive- and negative-strand hepatitis C virus, SB strain (SB-HCV), RNA by semiquantitative reverse-transcriptase polymerase chain reaction.



**Figure 5.** Effects of hepatitis C virus, strain SB (SB-HCV), infection on cell proliferation in Molt-4 cells. *A*, Representative fluorescence-activated cell sorter (FACS) analysis of cell proliferation. *B*, Carboxyfluorescein succinimidyl ester (CFSE)-high and CFSE-low groups were separated by FACS analysis and analyzed for NS5A expression by immunofluorescence analysis. Green stain, CFSE; red stain, NS5A; blue stain, 4',6-diamidino-phenylindole dihydrochloride (DAPI). The percentage of infectivity was determined by counting numbers of positively stained cells per 400 cells. For the negative control, cells treated with UV-irradiated HCV and detection antibody alone were used. *C*, Analysis of Ras/mitogen-activated protein (MAP) kinase/extracellular signal-regulated kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) signaling. For details about immunoblotting analysis, see Methods. Three independent experiments yielded similar results. FSC, forward scatter characteristics; P-cRaf, phospho-cRaf; P-ERK, phospho-ERK; P-MEK, phospho-MEK.

like a positive loop [27]. The CFSE-high group (i.e., the enriched SB-HCV-infected Molt-4 cells) showed significantly lower levels of phospho-cRaf, phospho-MEK, and phospho-ERK after PMA stimulation than did cells in the control

groups (mock-infected cells, cells infected with UV-SB-HCV, and cells in the CFSE-low group) (figure 5C). These data indicated that HCV replication in T cells suppressed the PMA-induced activation of Ras-MEK-ERK signaling.



**Figure 6.** Comparison of carboxyfluorescein succinimidyl ester (CFSE)-low cells and CFSE-high cells (i.e., cells highly infected with hepatitis C virus, strain SB [SB-HCV]) with respect to expression of standard CD44 (CD44s) and the splicing variants 3 and 6 (CD44v3 and CD44v6). *A*, Representative graphs show CFSE intensity among mock-infected cells, cells infected with UV-irradiated SB-HCV (UV-SB-HCV), and cells infected with SB-HCV. *B*, CD44s and CD44v3 were only weakly suppressed in the SB-HCV-infected CFSE-high cells, compared with the CFSE-low group. Three independent experiments yielded similar results. MFI, mean fluorescence intensity.

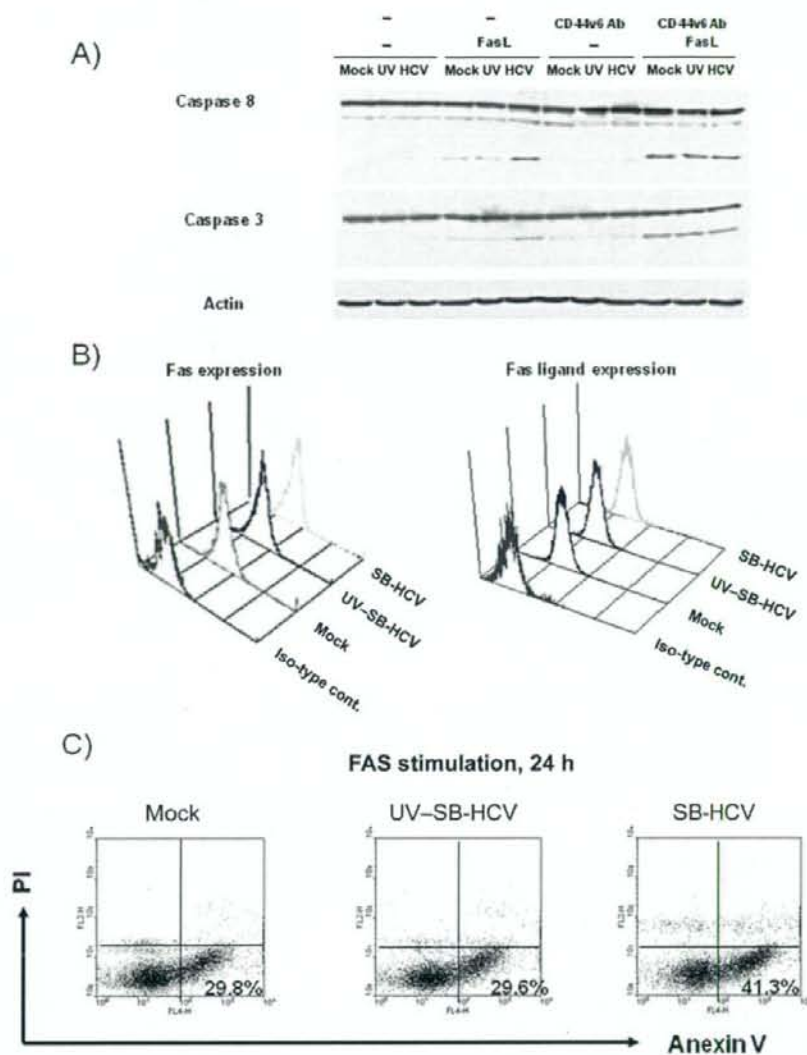
We next carried out double staining analysis of CD44 variants and CFSE to further confirm the correlation between low CD44v6 expression and high CFSE staining. The result showed that the CFSE-high group (the cells infected with SB-HCV) had a significantly lower level of CD44v6 expression than did the cells in the CFSE-low group ( $P < .01$ ) (figure 6A). CD44s and CD44v3 were only weakly suppressed in the SB-HCV-infected CFSE-high cells, compared with the CFSE-low group (figure 6B).

**SB-HCV infection and enhanced Fas-dependent apoptosis by suppression of CD44v6.** We next analyzed Fas-mediated apoptosis because CD44v6 has been reported to cause suppression of Fas-mediated apoptosis [16]. Immunoblotting analysis showed that, after treatment with Fas ligand (FasL), Molt-4 cells infected with SB-HCV had a higher extent of cleavage of caspases 8 and 3 than did cells that had been mock infected or infected with UV-SB-HCV (figure 7A), whereas the surface expression levels of Fas and FasL were almost the same among the 3 groups (figure 7B). Moreover, pretreatment of cells with anti-CD44v6 antibody that could block the interaction of CD44v6 with Fas [15] eliminated the differences in caspase activation among the 3 groups (figure 7A). The number of early apoptotic cells, which

were represented as annexin V-positive, PI-negative cells, was significantly increased among the SB-HCV-infected Molt-4 cells after Fas stimulation (the percentage of early apoptotic cells in each group was as follows: mock infected, 29.8%; UV-SB-HCV infected cells, 29.6%; and SB-HCV infected cells, 41.3%) (figure 7C). These results indicated that SB-HCV infection caused suppression of the CD44v6 isoform, leading to the enhancement of Fas-mediated apoptosis.

**NS5A protein and suppression of CD44v6 in T cells.** To determine which viral protein was responsible for CD44v6 suppression, we expressed each HCV protein in Molt-4 cells and then studied its effects on CD44v6 expression. The transfection efficiency of each plasmid was ~50%, and cell viability after transfection was >80% after purification by Ficoll-Paque centrifugation. The results showed that, among all the viral proteins, only NS5A could suppress CD44v6 significantly ( $P < .01$ ) (figure 3A). The NS5A-induced CD44v6 suppression was particularly evident after PMA stimulation. Immunoprecipitation by Western blot analysis showed that CD44v6 could coprecipitate with Fas, and the amount of CD44v6-associated Fas was reduced in NS5A-expressing Molt-4 cells, compared with Molt-4 cells without NS5A (figure 3B). As a control, STAT-1 antibody did not coprecipitate CD44v6.



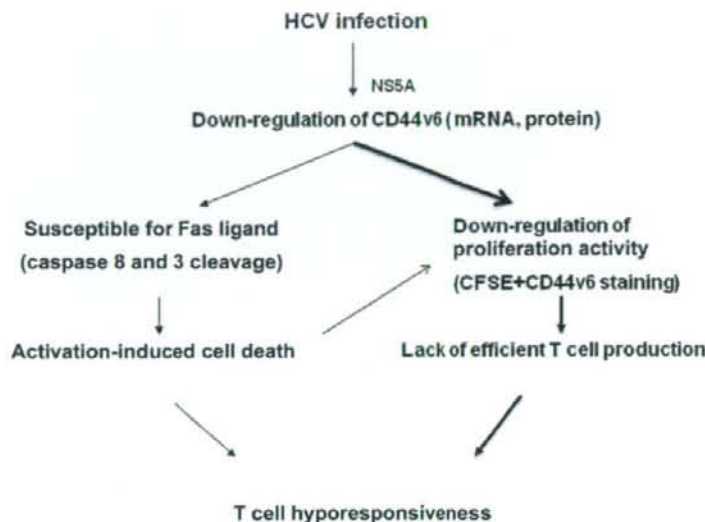


**Figure 7.** Susceptibility of different cells to Fas stimulation. *A*, Immunoblotting analysis was carried out by using anti-caspase 8, anti-caspase 3, and anti-actin antibodies, and the membrane was then subjected to reaction with peroxidase-conjugated secondary antibody. Immunoreactivity was visualized by use of enhanced chemiluminescence detection. Loading proteins were obtained from the following cell groups: mock-infected Molt-4 cells; Molt-4 cells infected with UV-irradiated hepatitis C virus, strain B (UV-SB-HCV); SB-HCV-infected Molt-4 cells from the carboxyfluorescein succinimidyl ester (CFSE)-high group; and SB-HCV-infected Molt-4 cells from the CFSE-low group, with or without Fas-ligand (FasL) stimulation. Pretreatment with CD44 splicing variant 6-blocking antibody (CD44v6 Ab) was carried out for some samples, as indicated. *B*, Surface expression of Fas and FasL as measured by flow cytometry. *C*, Fluorescence-activated cell sorter analysis of annexin V and propidium iodide (PI) staining. Mock-infected cells, Molt-4 cells infected with UV-SB-HCV, and Molt-4 cells infected with SB-HCV were stimulated with FasL for 24 h and analyzed to detect annexin V-positive, PI-negative early apoptotic cells. The percentages of early apoptotic cells in each group are indicated in the dot plots. Iso-type cont., isotype control antibodies.

## DISCUSSION

The SB cell line, which was derived from an HCV-positive B-cell lymphoma, produces lymphotropic HCV particles that can infect and replicate in B cell lines, such as Raji and Daudi cells, as well as PBMCs [28, 29]. Most recently, we also demonstrated

that SB-HCV could infect T cell lines, such as Molt-4 cells, and that this system could be used for signaling analysis of T cells [5]. Although there has been accumulating evidence indicating that HCV could replicate in both established T cell lines and primary T lymphocytes, so far little is known about the extent and biological significance of T cell infection [39-41]. The site of infec-



**Figure 8.** A proposed mechanism for T cell hyporesponsiveness induced by hepatitis C virus, strain SB (SB-HCV). T cell proliferation activity is strongly suppressed in SB-HCV infected T cells (*thick arrows*). A second effect is the induction of apoptosis through Fas. Both pathways lead to T cell hyporesponsiveness. The relative effects of these 2 pathways is not clear. CFSE, carboxyfluorescein succinimidyl ester.

tion, as well as proliferative activity and the subset of CD4<sup>+</sup> cells involved may be important factors for HCV replication in vivo [42]. Some authors have suggested that coinfection with human T cell lymphotropic virus type 1 or HIV might induce HCV replication in T lymphocytes [43–45]. Previously, our data indicated that with CD3 and IL-2 stimulation, naive CD4<sup>+</sup> T cells could be one of the target cells for HCV [5]. As a result of HCV infection, changes in the development and activation of apoptosis-related molecules in T cells may contribute partially to T cell hyporesponsiveness in some patients with hepatitis C.

The temporal expression of CD44v6 is important for proliferation, activation, and apoptosis in T cells [13, 16]. The suppression of CD44v6 expression or Ras-MEK-ERK signaling by HCV replication disrupted the positive loop of proliferation in T cells [27]. We could not conclude that CD44v6 suppression was either the result or the cause of Ras-MEK-ERK suppression, since Ras-MEK-ERK have been reported as upstream regulatory molecules for CD44v6 expression and CD44v6 could enhance Ras-MEK-ERK suppression [27]. However, unexpectedly, the other CD44 splicing variants, for example CD44v3, could not be suppressed by Ras-MEK-ERK signaling in this Molt-4 cell infection system [13, 14]. Our studies further showed that the protein NS5A is responsible for CD44v6 suppression. One of the possible mechanisms for this suppression is the stimulation of phosphatase 2A activity that can suppress MAPK signaling [24]. However, many individual HCV proteins have been reported to affect MAPK signaling and apoptotic signaling in diverse ways [25]; our Molt-4 cell HCV replication system showed that HCV replication, and NS5A protein alone, could suppress CD44v6

expression and MAPK signaling in T cells. Moreover, the results of Fas signaling experiments showed that suppression of CD44v6 might contribute to the apoptosis of T cells. However, some authors have reported that NS5A can inhibit apoptosis in hepatoma cell lines [46, 47]. One of the explanations for these contradictory results probably lies in the developmental stages and characteristics of the naive T cells. During T cell activation, apoptosis is easily induced, in order to maintain an appropriate immune response. During this stage, suppression of CD44v6 might strongly affect apoptosis signaling in T cells.

We have found that our results apply not only to T cell lines, but also to primary naive T cells. We could also detect significant suppression of proliferation after SB-HCV infection (data not shown). Furthermore, in our ongoing clinical study some clinical samples (PBMCs) from patients with chronic hepatitis C showed a significant, albeit small, degree of CD44v6 down-regulation with CD3 stimulation (data not shown).

We conclude that HCV replication in T cells may play a role in the regulation of proliferation and apoptosis during T cell activation. The results suggest that NS5A expression induces the suppression of MAPK signaling and CD44v6 expression in T cells. Suppression of CD44v6 could enhance susceptibility to Fas signaling by reducing the binding of Fas and CD44v6. These biological effects may contribute to the disturbance of T cell proliferation and activation in individuals with persistent lymphotropic HCV infection (figure 8). A key issue for future research will be determining what percentage of T cells are infected with HCV during natural T cell infection.



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