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The J6JFH1 Strain of Hepatitis C Virus Infects Human B-Cells with Low Replication Efficacy

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Abstract

Hepatitis C virus (HCV) infection is a serious health problem worldwide that can lead to hepatocellular carcinoma or end-stage liver disease. Current treatment with pegylated interferon, ribavirin, and NS3/4A protease inhibitor would lead to a good prognosis in a large population of patients, but there is still no effective vaccine for HCV. HCV robustly infects hepatocytes in the liver. However, extrahepatic manifestations such as mixed cryoglobulinemia, a systemic immune complex-mediated disorder characterized by B-cell proliferation, which may evolve into overt B-cell non-Hodgkin's lymphoma, have been demonstrated. HCV-RNA is often found to be associated with peripheral blood lymphocytes, suggesting a possible interaction with peripheral blood mononuclear cells (PBMCs), especially B-cells with HCV. B-cell HCV infection was a matter of debate for a long time, and the new advance in HCV *in vitro* infectious systems suggest that exosome can transmit HCV genome to support "infection." We aimed to clarify the susceptibility of primary B-cells to HCV infection, and to study its functional effect. In this article, we found that the recombinant HCV J6JFH1 strain could infect human B-cells isolated from the peripheral blood of normal volunteers by the detection of both HCV-negative-strand RNA by reverse transcription polymerase chain reaction, and NS5A protein. We also show the blocking of HCV replication by type I interferon after B-cell HCV infection. Although HCV replication in B-lymphocytes showed lower efficiency, in comparison with hepatocyte line (Huh7) cells, our results clearly demonstrate that human B-lymphocytes without other non-B-cells can actually be infected with HCV, and that this interaction leads to the induction of B-cells' innate immune response, and change the response of these cells to apoptosis.

Introduction

Chronic infection by hepatitis C virus (HCV) is the major cause of liver cirrhosis and hepatocellular carcinoma. About 3.1% of the global population is infected with HCV (50). Historically, a combination therapy with pegylated interferon (IFN) and ribavirin was used for patients infected with genotype 1 HCV. NS3/4A protease inhibitors were recently developed in addition to pegylated IFN and ribavirin, and their combinations have been clinically tried for HCV treatment since then. Although >70% of patients with high viral loads of HCV genotype 1b have a sustained viral response by the therapy using simeprevir or telaplevir with pegylated IFN and ribavirin (17,22), the remaining patients fail to eliminate the virus, and drug resistance remains an issue that must be resolved. Recent development of direct-acting antiviral (DAA) drugs (such as daclatasvir, asunaprevir, and sofosbuvir) are a promising therapeutic option beyond IFN in the treatment of HCV patients (6,32).

HCV is a single-stranded, positive-sense RNA virus in the Hepacivirus genus of the Flaviviridae family. Although HCV is known to infect hepatocytes in the liver and induce hepatitis *in vivo*, *in vitro* cultured primary hepatocytes barely support the HCV life cycle: only hepatoma Huh7 cells and its subclones can efficiently maintain the HCV life cycle of a very limited number of HCV strains *in vitro* (53).

Chronic hepatitis patients with HCV sometimes show other extrahepatic complications such as lymphoproliferative diseases (LPD), including cryoglobulinemia and B-cell malignant lymphoma, autoimmune diseases, and dermatitis (1,12,15,16). Epidemiological analysis shows that chronic HCV patients have higher rates of LPDs than non-HCV-infected populations (36,48,52). Several reports suggested that some lymphotropic HCV strains effectively infected human

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lymphocytes (20,47), leading to the above-mentioned abnormalities. Infection of lymphocytes with HCV has been a matter of debate for a long time. More than one decade ago, several reports described the existence of HCV-RNA in peripheral blood mononucleated cells (PBMCs) (30,40). The detection rate of HCV-RNA in PBMCs was increased if patients were infected with human immunodeficiency virus (HIV) together with HCV (44). This phenomenon indicated that immune-suppressive circumstances and/or HIV antigen might enhance the replication activity of HCV in lymphoid cells (44). Moreover, it was reported that continuous release of HCV by PBMCs was detected in HCV-infected patients, especially in HIV co-infected patients (7). In addition to HCV-HIV co-infected patients, a low level of HCV replication could be detected in peripheral lymphoid cells from HCV mono-infected patients after antiviral treatment (34,45). Moreover, it was reported that HCV persisting at low levels long after therapy-induced resolution of chronic hepatitis C remained infectious (34). This continuous viral presence could present a risk of infection reactivation.

It has been reported that HCV replication was detected in various kinds of lymphoid cells. Many reports describing the existence of HCV in B-lymphocytes and B-cell lymphoma have been published (21,25,51). Among B-lymphocytes, CD27+ memory B-lymphocytes were more resistant to apoptosis than CD27− B-lymphocytes. CD27+ B-lymphocytes were reported as a candidate subset of the HCV reservoir in chronic hepatitis C (CH-C) (38). On the other hand, others claimed that distinguishing RNA association from true HCV replication was problematic, together with multiple artifacts complicated detection and quantitation of the replicative intermediate minus strand RNA (29,31), and also the failure of retroviral (37) and lentiviral (8) pseudoparticles bearing HCV envelope glycoproteins (HCVpp) to infect primary B-cells or B-cell lines. This led to continuous debate about HCV infection into B-lymphocytes, and the riddle remained unsolved.

Using the recent progress in HCV infection systems, we intended to clarify this debate and analyze HCV infection in human lymphocytes and its functional results. Here, albeit in a lower efficiency compared to HCV infection into Huh7 cells, we report that two different strains of recombinant HCV viruses could infect primary human lymphocytes not only by the detection of HCV-RNA positive and negative strands proliferation, but also NS5A protein detection, and the detection of the activity of luciferase reporter encoded by the recombinant HCV-genome. Blocking of HCV entry using anti-CD81 antibody (Ab), and replication by IFN-α or NS3/4A protease inhibitors successfully suppressed HCV infection. We also found that HCV infection into B-lymphocytes led to the initiation of host response including apoptosis resistance.

Materials and Methods

Cells and reagents

Huh7.5.1 cells were kindly provided by Dr. Francis V Chisari (The Scripps Research Institute, La Jolla, CA). Cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM ; Gibco/Invitrogen, Tokyo, Japan) supplemented with 2 mM L-Glutamine, 100 U of penicillin/mL, 100 μg of streptomycin/mL, 1 × MEM non-essential amino acid (Gibco/Invitrogen), and 10% fetal bovine serum (FBS).

Human peripheral blood mononuclear cells (PBMCs) were obtained from healthy volunteers by density gradient centrifugation using Ficoll Paque plus (GE-Healthcare, Waukesha, WI). CD19+ blood cells (representative of human primary B-cells) and CD19− cells (non-B-cells) were separated by MACS CD19 Beads (Milteny Biotec, Bergisch Gladbach, Germany). Purity of CD19+ B-cells was >95% after two-cycle separation. The cells were cultured in RPMI1640 (Gibco/Invitrogen) supplemented with 100 U of penicillin/mL, 100 μg of streptomycin/mL, and 10% FBS.

The following reagents were obtained as indicated: anti-CD81 Ab (BD Pharmingen, Franklin Lakes, NJ); PE anti-CD80 Ab, APC anti-CD86 Ab, and PE-labeled anti-CD19 Ab (eBioscience, San Diego, CA); recombinant IFN-α (Peprotech, Oak Park, CA); BILN2601 (Behringer, Willich, Germany); and Viaprobe 7AAD (BD Bioscience) and Annexin-V-Fluos (Roche, Mannheim, Germany).

Virus propagation

pJ6-N2X-JFH1 was kindly provided from Dr. Takaji Wakita (National Institute of Infectious Diseases, Tokyo) (2). pJc1-GLuc2A was gifted from Dr. Brett D. Lindenbach (Yale University, New Haven) (41). *In vitro* RNA transcription, gene transfection into Huh7.5.1 cells, and preparation of J6JFH1 and Jc1/GLuc2A viruses were performed as previously reported (53). Briefly, the HCV cDNA in plasmids were digested by XBaI and transcribed by T7 Megascript Kit (Invitrogen, Carlsbad, CA). RNA transfection into Huh7.5.1 was performed by electroporation using Gene Pulser II (Bio-Rad, Berkeley, CA) at 260 V and 950 Cap. Culture supernatant were collected on days 3, 5, 7, and 9 of postelectroporation, and concentrated with an Amicon Ultra-15 Centrifugal Filter unit (Millipore, Billerica, MA). The titer of HCVcc was checked by the immunofluoresence method using NS5A antibody when Huh7.5.1 was reinfected with these HCVcc.

Virus infection

Primary B-cells and non-B-cells were cultured with the J6JFH1 HCV strain at a multiplicity of infection (MOI) = 1–3 for 3 h, and cells were harvested after four extensive washes in culture medium. On days 1–6, cells were collected, washed with 0.25% trypsin-EDTA/saline, and incubated with 0.25% trypsin-EDTA for 5 min at 37°C. Then, suspended cells were collected as a source of total RNA. In some experiments, B-cells were infected with the Jc1/GLuc2A strain at MOI = 5 for 3 h. Cells were washed five times in 1 × phosphate buffered saline (PBS), and cultured until day 6 for determination of viral replication as GLuc activity with BioLux Gaussia luciferase assay kits (41).

RNA purification, RT-PCR, and quantitative PCR

Total RNA was extracted by using Trizol Reagent (Invitrogen) according to the manufacturer's instructions. Using 100–400 ng of total RNA as a template, we performed RT-PCR and real-time RT-PCR as previously described (3,4). Primer sets are shown in Supplementary Table S1 and Table S2 (Supplementary Data are available online at www.liebertpub.com/vim).

Real-time PCR was used for quantification of positive-strand and negative-strand HCV RNA. Total Trizol-extracted

RNA was analyzed by RT-PCR with a modification of the previously described strand-specific rTth RT-PCR method (10,13). RT primers for complementary DNA synthesis of positive and negative strand HCV RNA are shown in Supplementary Table S1. Positive-strand and negative-strand HCV PCR amplifications were performed using Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK) with 200 nM of paired primers (Supplementary Table S1). The PCR conditions were 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min.

Virus production and releasing assay

Primary human B-cells were infected with J6JFH1 at MOI = 1. Six days postinfection, the supernatant was collected ("releasing samples"), cells were repeatedly frozen and thawed, and the supernatant was collected ("assembly samples"). Viral titers of "releasing samples" and "assembly samples" were determined with Huh7.5.1 cells using J6JFH1 virus (MOI = 0.001 and 0.01) as control. Total RNA was recovered from the cells on days 2, 4, and 6, and determined with HCV-RNA to check reinfectivity.

Indirect immunofluorescence

Indirect immunofluorescence (IF) expression of HCV proteins was detected in the infected cells using rabbit IgG anti-NS5A antibody (Cl-1) (3). Goat anti-rabbit Alexa 594 (Invitrogen) was used as secondary Ab. Fluorescence detection was performed on the Zeiss LSM 510 Meta confocal microscope (Zeiss, Jena, Germany) (13).

Luciferase assay

Primary B-cells were infected with Jc1/Gluc2A by using concentrated Medium or Mock Medium (PBS-electropolated Huh7.5.1 medium). Media were collected on days 0, 2, 4, and 6 postinfection, cleared by centrifugation (16,000 *g* for 5 min), and mixed with 0.25 volume of *Renilla* 5 lysis buffer (Promega, Madison, WI) to kill HCV infectivity. GLuc activity was measured on a Berthold Centro LB 960 luminescent plate reader (Berthold Technologies, Bad Wildbad, Germany) with each 20 μL sample injected with 50 μL BilLux Gaussia Luciferase Assay reagent (New England Biolabs, Ipswich, MA), integrated over 1 sec.

Cell survival assay

Apoptosis assay: Primary B cells were infected with J6JFH1 virus. Cells were collected 48 h after infection, stained by 7AAD Cell Viability assay kit and Annexin V, and analyzed by FACS Calibur (BD) (13).

ATP assay

Primary B-cells were infected with J6JFH1 virus or Mock concentrated medium. Cells were resuspended and cultured at Lumine plate (Berthold Technologies) postinfection. ATP activities were determined 72 h later using CellTiter-Glo® Luminescent Cell Viability Assay (Promega) according to the manufacturer's protocol.

miRNA detection

Total RNA was extracted by using Qiazol Reagent (Invitrogen). These RNA was purificated and reverse transcripted to cDNA by using the miScript II RT Kit. Synthesized cDNA was used to determine the expression levels of miR-122 (24). Total miRNA was prepared by using Qiazol and miScript II RT kit (Invitrogen), and miR-122 expression was determined by using miScript SYBR Green PCR Kit and miScript Primer Assay (Invitrogen) according to the manufacturer's protocol. U6 small nuclear RNA was used as an internal control.

Results

J6JFH1 infects and replicates in primary B-cells

To address HCV infectivity into primary B-cells, PBMC were isolated from the blood of healthy volunteers and were sorted into CD19+ cells (primary B-lymphocytes) and CD19− cells (non-B-cells). Their purities were >95%. These cells were then incubated with the J6JFH1 HCV. Total RNA was collected on days 2, 4, and 6. The Huh7.5.1 strain was used as positive control. Both Huh7.5.1 and primary B-cells, but not non-B-cells, showed an increase in intracellular HCV-RNA titer, albeit primary B-cells showed lower efficiency than Huh7.5.1 (Fig. 1A). We adjusted the HCV-RNA values using GAPDH as an internal control (Fig. 1B). To confirm J6JFH1 replication in primary B-cells using IF, we also measured the expression of HCV-NS5A, which is a nonstructural protein produced only by the virus secondary to replication. Although the expression was far lower than Huh7.5.1 cells, we managed to detect the NS5A expression in J6JFH1 infected primary B-cells (Fig. 1C).

We examined what kinds of HCV-entry receptors human primary B-cells expressed in our setting. Human CD81, SRB1, and NPC1L1 were expressed, but not the tight junction proteins claudin1 and occludin in mRNA levels (Supplementary Fig. S1). We could not detect miR122 in primary B-cells (Supplementary Fig. S2), expression of which makes the cells permissive to HCV (24). Human CD81 is a primary entry receptor for HCV in hepatocytes (42). Blocking human CD81 by its specific Ab resulted in blockage of HCV infection into primary B-cells, as shown by the suppression of HCV-RNA titer (Fig. 2), suggesting that HCVcc particles enter B-cells also using CD81 receptor. HCV-RNA titer was not suppressed by non-specific Ab (data not shown).

We then examined the effect of the different drugs used to suppress HCV replication (recombinant human IFN, and HCV protease inhibitor, BILN2601). Inhibition of HCV-RNA replication was observed when B-cells were treated with rhIFN-α or BILN2601 (Fig. 2) after infection. BILN2601 showed efficient inhibitory effect on replication of HCV RNA in Huh7.5.1 cells (Supplementary Fig. S3). As control studies, we confirmed that the production of HCV RNA was reduced in Huh7.5.1 cells by CD81 Ab, IFN-α, or BLIN2601 (Supplementary Fig. S4). In both Huh 7.5.1 and B-cells, BLIN2601 most effectively block HCV replication. These data reinforce that HCV is actually replicating in primary B-cells, and that activation of innate immunity by IFN treatment or blocking the NS3/4A protease function is a critical factor in blocking HCV replication in primary B-cells. These data suggest that our system can be used for screening the function of different inhibitors on HCV replication in B-cells.

HCV negative-strand RNA detected in human B-cells

To confirm HCV replication in primary B-cells further, we tested for an increase of negative-strand HCV-RNA after

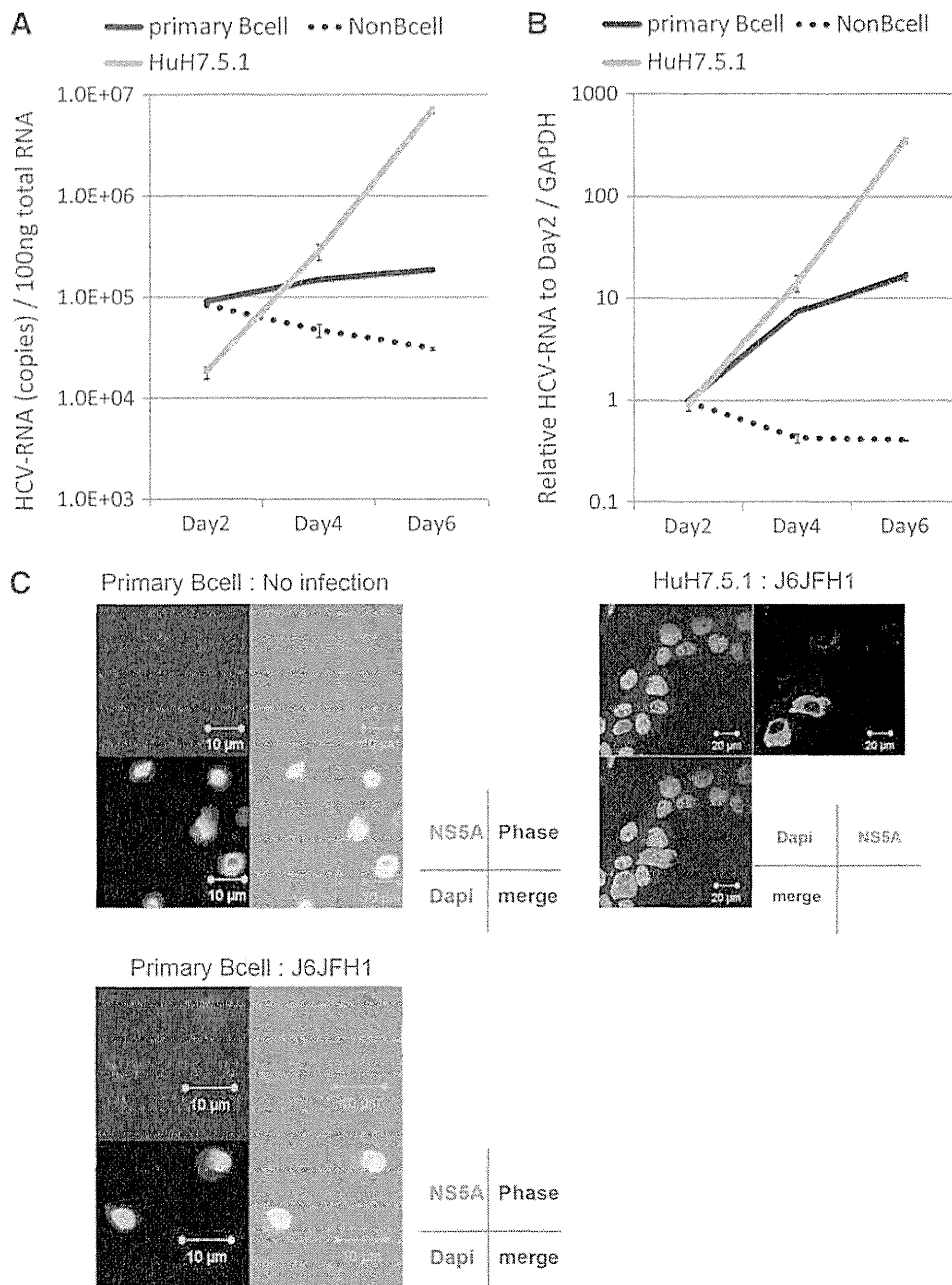

FIG. 1. J6JFH1 infects human peripheral blood B-cells. Human B-cells (CD19+ cells) and non-B-cells (CD19− cells) were separated by MACS as described in Materials and Methods. Primary B-cells, non-B-cells, and Huh7.5.1 cells were infected with J6JFH1 at MOI = 1 for 3 h. After infection, cells were washed twice with culture medium and continued culture. On days 2, 4, and 6, total RNA was collected and HCV-derived RNA was determined by reverse transcription polymerase chain reaction (RT-PCR). GAPDH was used as internal control. **(A)** HCV-RNA not adjusted by GAPDH. **(B)** HCV-RNA adjusted by GAPDH. **(C)** Immunofluorescence analysis of J6JFH1-infected human B-cells and Huh7.5.1 cells. Six days postinfection. Red, NS5A; blue: Dapi; phase: phase-shift microscope.

infection, since the negative-strand RNA is not yielded if HCV particles or RNA just adhere to the cell surface of human primary B-cells without internalization (9,14,19,35, 42,43). We measured the synthesis of plus-strand and minus-strand HCV-RNA separately using strand-specific RT primers and rTth polymerase as previously described (4). The titer increase of minus-strand HCV-RNA indicates HCV-RNA replication. As shown in Figure 3, both minus- and plus-strand HCV-RNA increased time dependently in primary B-cells, and both types of RNA concomitantly decreased in non-B-cells (Fig. 3A and B). Plus- and minus-strand RNA were exponentially increased in Huh7.5.1 cells infected with J6JFH1 (Fig. 3C). These results indicated that primary human B-cells supported J6JFH1 infection and replication, although viral replication levels in B-cells were modest compared with those in Huh7.5.1 cells. These results

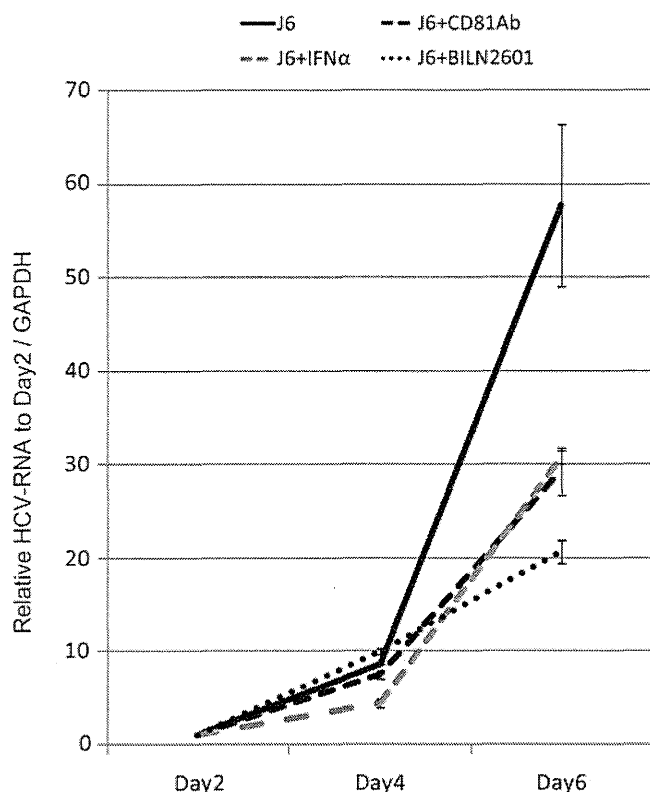

FIG. 2. J6JFH1 B-cell infection is blocked by anti-CD81 Ab, IFN-α, or an NS3/4A inhibitor. Anti-CD81 neutralizing Ab (20 μg/mL) was added to the B-cell culture 1 h before infection. Otherwise, recombinant IFN-α rhIFN-α, 200 IU/mL) or BLIN2601 (250 nM, which is IC75; see Supplementary Fig. S3) was added 1 h after infection. On days 2, 4, and 6, total RNA was extracted, and HCV-RNA was determined by RT-PCR. The values were adjusted by GAPDH.

may reflect the fact that the NS5A protein is difficult to detect in infected B-cells using IF assay.

B-cells can be infected with different HCV strains

We next used the Jc1/GLuc2A strain to investigate whether different HCV strains infect primary B-cells. Primary B-cells, non-B-cells (data not shown), and Huh7.5.1 cells were infected with the Jc1/GLuc2A strain. After five washes, supernatant was collected (day 0 samples). On days 2, 4, and 6, medium was collected. Luciferase activity was determined for all samples by luminescence (GLuc). GLuc activity and detection of RNA increased exponentially in Huh7.5.1 cells infected with the Jc1/GLuc2A strain (Fig. 4A). GLuc activity on day 4 to day 6 increased more in primary B-cells than in non-B-cells (Fig. 4B). These results suggest that HCV replication is substantial, but low in the HCV line Jc1/GLuc2A.

B-cells neither produce nor release detectable level of HCV infectious particles

We collected supernatants of J6JFH1-infected primary human B-cells to measure productive infection in B-cells. The supernatant was then added to culture of Huh7.5.1 cells, and we compared infection with control Huh7.5.1 cells, whose cells were infected with a low MOI (0.01 and 0.001) of J6JFH1 collected from media of the infected Huh7.5.1 cells. HCV-RNA titer in the Huh7.5.1 titrating cells was decreased over time after co-culture with B-cell supernatants obtained from either "releasing samples" "assembly samples." In contrast, HCV-RNA titers were slightly increased over time in the Huh7.5.1 titrating cells that had been infected with medium collected from low MOI-J6JFH1-infected Huh7.5.1 cells (Fig. 5). These results indicated that primary human B-cells were infected with J6JFH1 but failed to assemble or produce particles into the supernatant.

Host response to HCV infection into primary B-cells

Next, we determined whether B-cell activation was induced in HCV-infected B-cells that survived under HCV infection. We measured induction of CD80 and CD86 as B-cell activation markers. After 2–3 days of infection, the CD80/86 levels on B-cells treated with J6JFH1 were compared with those treated with medium from mock-infected cells (concentrated Huh7.5.1 medium) by FACS analysis (Fig. 6A). We found that CD80/86 were upregulated in infected cells compared to mock-infected cells.

Since B-cell lymphoma is a known complication of chronic HCV infection (20,36) and acquiring apoptotic resistance is essential for the development of cancer (21,51,38), we measured the ability of B-cells to escape apoptosis after HCV infection. B-cell apoptosis spontaneously occurs during culture at 37°C. The percent of apoptosis of primary B-cells was decreased in FACS analysis using 7AAD viaprobe + annexinV (Fig. 6B) and ATP assays postinfection (Fig. 6C). These results suggest that primary B-cells are protected from apoptosis by infection with HCVcc. It has been reported that B-cells were vulnerable to apoptotic cell death at various stages of peripheral differentiation and during signal responses (18). Thus, the results infer that HCV stimulation interferes with B-cell apoptotic signal in human B-cells.

Discussion

We show evidence suggesting that human peripheral B-cells can be infected with HCV strains. Establishment of J6JFH1 infection was evaluated by minus-strand PCR amplification, production of core and NS5A proteins, and protection from apoptosis. An increase in HCV RNA in B-cells was inhibited by an exogenously added antibody against CD81 that blocked HCV receptor function. Furthermore, blocking HCV replication in B-cells by type I IFN and NS3/4A protease inhibitor confirmed the presence of HCV infection/replication in human B-cells. The results were corroborated with another HCV strain, Jc1/GLuc2A. Although we failed to establish an EBV-transformed B-cell line to reproduce HCV infection of B-cells, peripheral blood B-cells were infected with J6JFH1 in 12 independent experiments.

One of the well-known complications of chronic HCV infection is LPD, including cryoglobulinemia and B-cell malignant lymphoma, indicating the involvement of B-cells in the course of the disease (1,12,15,16). However, many reports describing the existence of the HCV genome in B-cells and lymphomas (21,25,51) and HCV replication in B-cells have been controversial due to multiple artifacts complicated in detection and quantitation of the replicative

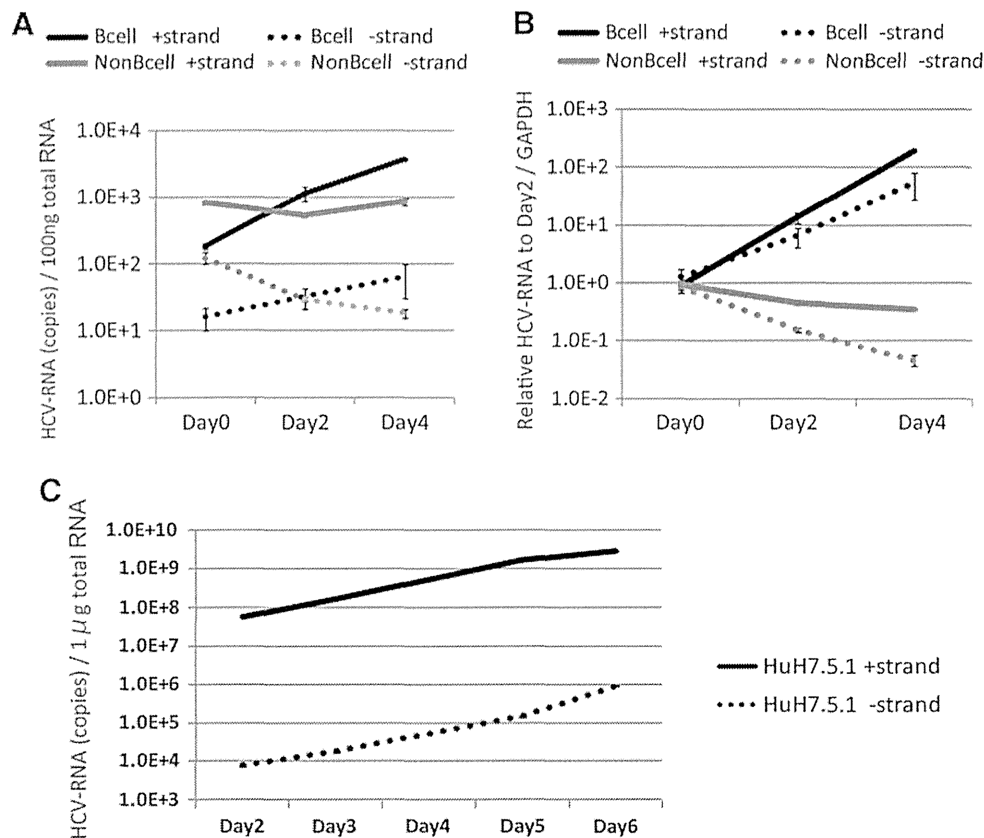

FIG. 3. HCV negative strand RNA is detected in human B-cells. By using rTth methods, HCV strand-specific RNA was determined in J6JFH1-infected human B-cells. (**A**) Not only plus strand HCV-RNA but also minus strand HCV-RNA were increased in a time-dependent manner in human B-cells. (**B**) When HCV-RNA was adjusted by GAPDH that was used as an internal control, HCV-RNAs in B-cells were substantially increased compared with those in non-B-cells. (**C**) Plus and minus strand HCV-RNAs were efficiently amplified in J6JFH1-infected Huh 7.5.1 cells. The level of HCV-RNA exponentially increased in this hepatocyte line.

intermediate minus strand RNA (29,31). This has led to a continuous debate about HCV infection in B-lymphocytes.

HCV entry into B-cells has also been previously reported to be absent because retroviral (37) and lentiviral (8) pseudoparticles bearing HCV envelope glycoproteins (HCVpp) did not infect primary B-cells or B-cell lines. In our study, while we succeeded in infecting Huh7.5.1 cells efficiently with retroviral pseudoparticles for expressing both HCV E1/E2 and the control VSV-G, we failed to establish the same infection in B-cells, suggesting that the block of pseudoparticle entry into B-cells is not related to HCV glycoproteins alone.

Total PBMCs reportedly facilitate HCV attachment but not internalization (42), so HCV infection of B-cells is abrogated in total PBMCs (35). The cause of HCV absorption is unclear, but incomplete sets of HCV receptors in non-B PBMC cells permit attachment of HCV without internalization. B-cells possess CD81, SRBI, LDL-R, and NPC1L1. Because B-cells are not adherent cells, they do not express claudin 1 and occludin, which forms a receptor complex for HCV (9,14,19,43). Claudin 1 and occludin are components of tight junctions and serve as HCV receptors in human hepatocytes. In infection studies using cells expressing these proteins, however, claudin 1 and occludin only upgrade infection efficacy and are dispensable to infection (5), although CD81 is essential for establishment of infection (42). Lack of claudin 1 and occludin or miR122 might be a cause of the low HCV infection efficiency observed in human B-cells. Function blocking of CD81 by its specific antibody suppressed HCV infection in primary B-lymphocytes, which imply that HCV entry into primary B-lymphocyte is dependent on the direct interaction phenomenon between HCV virus particles and CD81 receptor and is not mediated by other nonspecific (CD81 independent) pathways such as exosomal transfer of HCV from Huh7 cells to nonhepatic cells, such as dendritic cells (46).

Previous report using *in vitro* prepared recombinant HCV JFH1 particles (HCVcc) failed to establish HCV infection in B-lymphocyte cell lines (39). While HCV is known to infect human hepatocytes *in vivo* leading to chronic viral hepatitis, in the *in vitro* conditions, only the combination between Huh7 cells and its derived clones supported robust replication and infection with only JFH1 or its derived chimeras (5). Neither hepatocyte cell lines including primary hepatocytes nor other HCV strains could reproduce HCV infection efficiently *in vitro* (5). These data suggest that the clonal selection of HCV quasispecies by hepatoma Huh7 cells is essential for this robust infection *in vitro*. The situation would be similar to the JFH1 story in B-cell HCV infection.

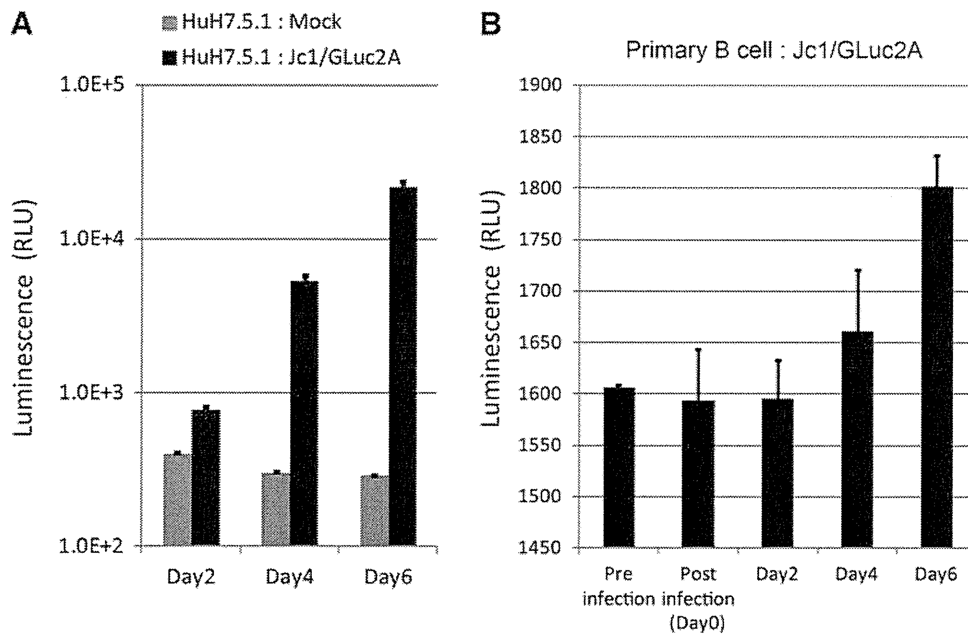

FIG. 4. Jc1/GLuc2A strain infects human B-cells with an increase of Gluc activity. Human B-cells and Huh7.5.1 cells were infected with the JC1/GLuc2A strain that contains secretory luciferase derived from *Gaussia* (GLuc) at MOI = 5. Huh7.5.1 cells were used as control. GLuc activity was increased as time cultured. The GLuc activity was saturated in Huh7.5.1 (**A**). On the other hand, GLuc activity was increased from day 4 in human B-cells (**B**).

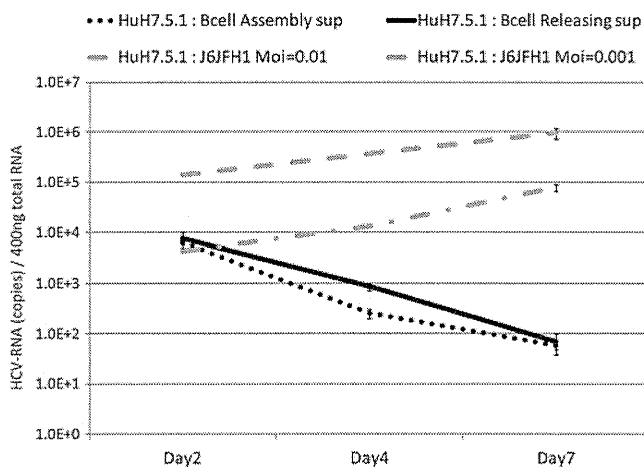

FIG. 5. B-cells infected with J6JFH1 fail to produce virus particles. Human B-cells were infected with J6JFH1 for 3 h, washed twice with phosphate buffered saline (PBS), and cultured. Six days after infection, the supernatant was collected ("releasing samples"). Cells were periodically frozen and thawed five times, and the supernatant was collected ("assembly samples"). For evaluation of the infectious virions, Huh7.5.1 cells were treated with these "releasing samples" or "assembly samples." Similarly, Huh7.5.1 cells were treated with J6JFH1 at low MOI (MOI = 0.01 and 0.001) in parallel. After the treatment, cells were washed and cultured. On days 2, 4, and 7, cells were harvested to collect HCV-RNA. Total RNA was extracted from each samples, and HCV-RNA was determined by RT-PCR methods.

B-cell apoptosis spontaneously occurs during culture at 37°C. We found that B-cell apoptosis was blocked by J6JFH1 infection, as reported previously using Raji cells (11). B-cell apoptosis usually occurs secondary to viral infection, but HCV is particular since apoptotic signaling interferes with infection, leading to protection from cell death. However, B-cell survival was not due to primary infection, because the percent of cells circumventing apoptosis was usually higher than cells infected with HCV. We could not define the pathways that participated in apoptosis regulation by HCV, although a previous report (11) suggested that E2-CD81 engagement was related to B-lymphocyte disorders and weak neutralizing antibody response in HCV patients. Since B-cell lymphoma is a known complication of chronic HCV infection (27), the inability of infected cells to undergo apoptosis can be associated with the development of cancer (28,33,49). In this context, B-cell lymphoma often occurs in mice with Cre-initiated HCV transgenes (26). It is notable that anti-apoptotic effect of HCV core gene was reported in genotype 3a in Huh7 cells (23) and, here, genotype 2a in B-cells. In another report (51), HCV strains established from B-cell lymphoma persistently infected with HCV were genotype 2b. B-cell HCV infection might not be linked to some specific genotypes of HCV.

We believe that our report shows that human primary B-cells can be infected *in vitro* with HCV, and that this infection is dependent on HCV particles binding with its receptor CD81 and is not nonspecific entry (e.g., exosomal mediated). We also show that this infection could be blocked with antibodies interfering with this binding, or with drugs that suppress HCV replication. Although no virion was generated from B-cells in HCV infection, it is still likely that B-cells serve as a temporal reservoir of HCV in the blood circulation. If B-cells permit HCV infection, RNA sensors

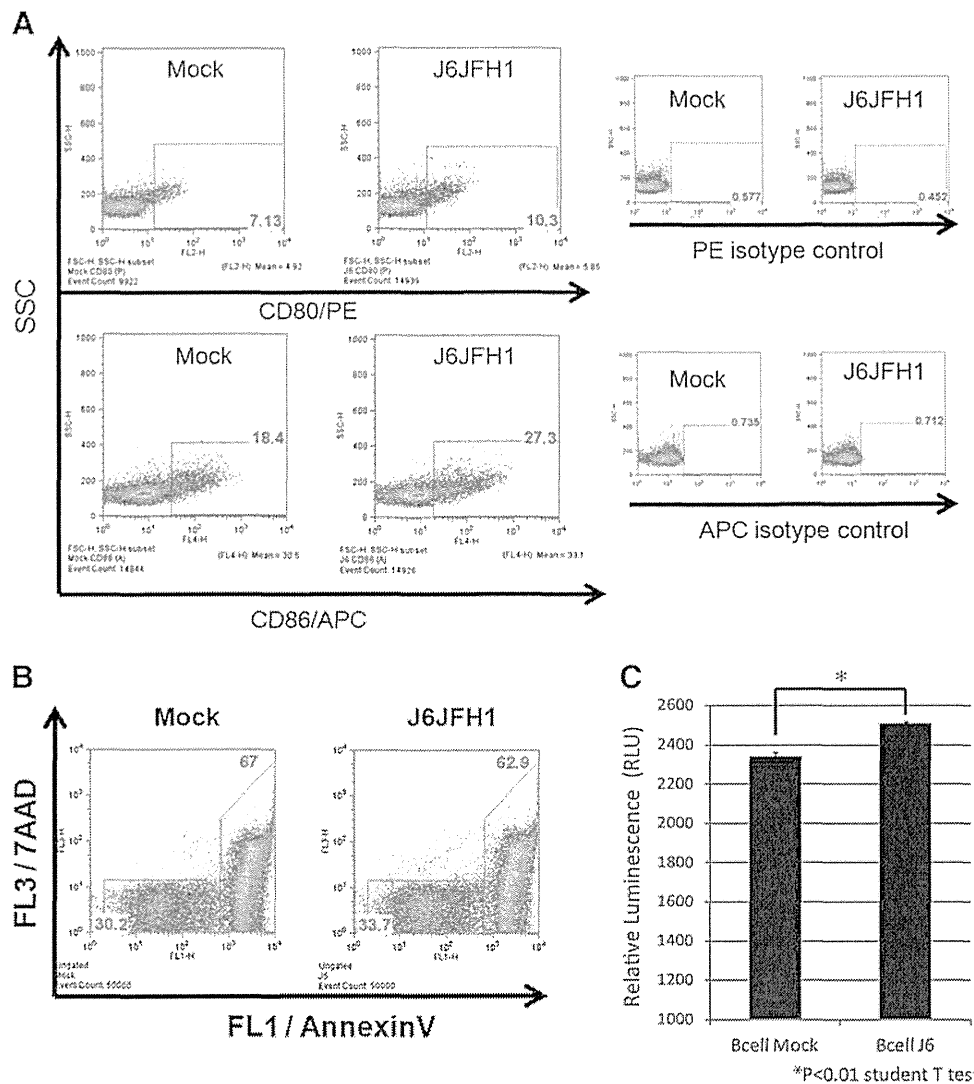

FIG. 6. J6JFH1 infection activates B-cells and protects the cells from apoptosis. Human B-cells were infected with J6JFH1 at MOI = 1 for 3 h, washed twice with PBS, and cultured. Two days after inoculation, cells were washed and suspended with FACS buffer. (**A**) The cells were incubated with PE-conjugated anti-human CD80 antibody, APC-conjugated CD86 antibody, or PE/APC-conjugated mouse IgG1 isotype control for 30 min. Then, the cells were washed and resuspended in FACS buffer. Cells were analyzed by FACS. (**B**) Annexin V and 7AAD viaprobe were added and cultured at 18°C for 10 min. Then, cells were analyzed by FACS. (**C**) 2×10^5 human B-cells were infected with J6JFH1- or Mock-concentrated medium for 3 h. Cells were then washed, resuspended, and cultured in a 96-well white microwell plate. Two days later, ATP activity was determined with a CellTiter-Glo® Luminescent Cell Viability Assay Kit (Promega). ATP activity was adjusted by day 0 ATP activity.

RIG-I and MDA5 in B-cells might recognize HCV RNA and evoke intracellular signaling, including by transcription factors NF-κB and IRF-3/7 (5). Activation of the cytokine network is triggered in human B-cells in response to HCV RNA. In fact, host factors liberated by HCV-infecting B-cells have been previously reported in HCV patients (1,12,15,16,52). Although patients' outcomes would be more than we can be predicted from our results, this system would actually benefit the future study on B-cell–virus interaction.

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Author Disclosure Statement

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HCV Infection Enhances Th17 Commitment, Which Could Affect the Pathogenesis of Autoimmune Diseases

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Abstract

Background: Various kinds of autoimmune diseases have been reported to have a significant relationship with persistent hepatitis c virus (HCV) infection and Th17 cells. Previously, our group reported that the existence of HCV in T lymphocytes could affect the development of $CD4^+$ helper T cells and their proliferation, in addition to the induction of immunoglobulin hyper-mutation.

Methods: Therefore, we analyzed the relationship between persistent infection of HCV and the mechanism of Th17 cell induction *ex vivo* and *in vitro*.

Results: The prevalence of autoimmune-related diseases in chronic hepatitis c patients (CH-C) was significantly higher than in other types of chronic hepatitis (hepatitis B and NASH). A significantly higher frequency of IL6 and TGF-β double-high patients was detected in CH-C than in other liver diseases. Moreover, these double-high patients had significantly higher positivity of anti-nuclear antibody, cryoglobulinemia, and lymphotropic HCV and higher amounts of IL1-β, IL21, IL23. In addition to the previously reported lymphotropic SB-HCV strain, we found a novel, genotype 1b lymphotropic HCV (Ly-HCV), by deep sequencing analysis. Lymphotropic-HCV replication could be detected in the lymphoid cells with various kinds of cytokine-conditions including IL1β, IL23, IL6 and TGF-β in vitro. Infection by HCV could significantly enhance the development of Th17 cells. The HCV protein responsible for inducing the Th17 cells was HCV-Core protein, which could enhance the STAT-3 signaling and up-regulate the expression of RORγt as a Th17 master gene.

Conclusion: Infection by lymphotropic HCV might enhance the Th17 development and contribute to understanding the pathogenesis of autoimmune-related diseases.

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Introduction

Cellular and humoral immune responses to HCV play an important role in the pathogenesis of chronic hepatitis, HCC and B-lymphocyte proliferative disorders including mixed cryoglobulinemia, a disorder characterized by the oligoclonal proliferation of B cells [1–5]. B cell activation and/or dis-regulation could originate as a result of HCV binding to CD81 tetraspanin molecule or as a consequence of its ability to replicate in B lymphocytes[6]. It has been reported that HCV could infect B lymphocytes[7–9]. We previously reported that HCV-replication in B lymphocytes could induce immunoglobulin hypermutation and reduce the affinity and neutralizing activities of antibodies against HCV envelope protein[5]. On the other hand, the hypermutation of immunoglobulin might induce autoantibodies that contribute to the immunopathogenesis of autoimmune diseases, since various kinds of autoimmune diseases were reported to have a significant relationship with persistent HCV infection [10–12].

Previously, our group reported that the existence of HCV in T lymphocytes could affect the development and proliferation of type 1 T helper (Th1) cells[3,4,13]. Other groups have also reported the existence of HCV in T lymphocytes[14,15]. HCV replication in T lymphocytes could suppress Interferon-γ (IFN-γ)/signal transducers and activators of transcription factor 1 (STAT-1) signaling that might affect signal transducers and activators of transcription factor 3 (STAT-3) signaling[4,13].

It has been reported that a subset of type 17 T helper (Th17) cells might be involved in various kinds of autoimmune diseases[16–19]. The orphan nuclear receptor RORγt (RORγt) is the key transcription factor that induces the transcription of the genes encoding Interleukin (IL)-17 in naïve $CD4^+$ T helper cells[20]. Moreover, the activation of STAT-3 signaling could contribute to the induction of Th17 development[21–23]. Previously, Machida et al. reported that HCV replication in B lymphocytes could enhance the production of IL-6 from B lymphocyte[24]. In addition to TGF-β1, the existence of IL-6 could enhance the development of Th17 cells. IL17A-producing T lymphocytes have been recently shown to comprise a distinct lineage of pro-inflammatory T helper cells, termed Th17 cells, that are major contributors to autoimmune disease[20]. IL17A stimulates the secretion of a wide range of proinflammatory chemokines and cytokines. As its receptor is widely expressed, various kinds of immune cells as well as other cell types can respond to it[25]. Recently, we reported that the frequency of Th17 cells was remarkably high in a difficult-to-treat case of pyoderma gangrenosum-like lesion in a patient with lymphotropic HCV infection[26].

In this study, we clarified the relationship between Th17 cells and the biological significance of lymphotropic HCV.

Material and Methods

Study design and Patients

Two hundred-fifty patients with HCV persistent infection who were treated in Tohoku University Hospital were enrolled in this study. None of the patients had liver disease due to other causes, such as alcohol, drug, or congestive heart failure. Permission for the study was obtained from the Ethics Committee at Tohoku University Graduate School of Medicine (permission no. 2006–194) following ethical guidelines of the 1975 Declaration of Helsinki. Written informed consent was obtained from all the participants enrolled in this study. Participants were monitored for 6 months and peripheral blood samples were obtained from selected patients. We collected the peripheral blood before the treatment (treatment naïve). The concurrent diseases were diagnosed by specialized physicians belonging to the department of hematology and rheumatology. Patients were evaluated for serum levels of HCV-RNA, blood chemistry and hematology.

Quantification of IL1β, IL6, Transforming growth factor 1 (TGF-β1) and IL17A, IL21, IL23 in the serum

The amounts of IL1β, IL6, TGF-β1, IL17A, IL21 and IL23 were quantified using IL1β, IL6, TGF-β, IL17A, IL21 and IL23 enzyme-linked immunosorbent assay (ELISA) kits (eBioscience). The serum samples from patients were collected at sampling points and stored at −20°C. The ELISA procedure was performed according to the manufacturer's protocol.

Isolation of peripheral blood mononuclear cells (PBMCs), $CD4^+$ cells, $CD19^+$ cells and $CD45RA^+$ naïve $CD4^+$ cells

PBMCs were isolated from fresh heparinized blood by means of Ficoll-Paque (Amersham Bioscience) density gradient centrifugation. $CD4^+$ T cells and $CD19^+$ B cells were positively isolated by dynabeads (Dynal) to carry out the analysis of strand-specific HCV RNA detection. Naïve $CD4^+$ cells were isolated by the MACS beads system (Miltenyi Biotec).

Strand-specific intracellular HCV RNA detection

Strand-specific intracellular HCV RNA was detected using a recently established procedure that combined previously published methods [27,28] with minor modifications [4,13]. Positive- and negative-strand-specific HCV RNAs were detected by a nested polymerase chain reaction (PCR) method. 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 the internal control, semi-quantification of β-actin mRNA was performed 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.

The deep-sequencing analysis of Ly-HCV

Serum samples and PBMCs were collected from a patient with para-aortic lymph node enlargement with chronic HCV infection. Serum samples were stored at −20°C until testing. Total RNA was extracted from 800 μl of serum and 1.0×10^7 of PBMC using Trizol LS (Invitrogen). Each library was prepared using TruSeq RNA sample preparation kits v2 (Illumina). Libraries were clonally amplified on the flow cell and sequenced on an Illumina HiSeq 2000 (HiSeq Control Software 1.5, Illumina) with a 101-mer paired end sequence. Image analysis and base calling were performed using Real Time Analysis (RTA) 1.13. In the first mapping analysis, sequence reads not of human origin were aligned with 27675 reference virus sequences registered at the Hepatitis virus database server (HVDB) (http://s2as02.genes.nig.ac.jp/index.html) and the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) using bwa (0.5.9-r26) and allowing mismatches of within 10 nucleotide bases. Based on the highest homology to the reference virus genome in the first mapping analysis, the tentative consensus HCV full genome sequence was created. The second mapping analysis was conducted using the tentative consensus HCV full genome sequence and bwa, allowing mismatches of within 5 nucleotide bases. The result of the analysis was displayed using Integrative Genomics Viewer (IGV; 2,0,17). Sequence analysis was performed using Genetyx-Mac ver.12. A phylogenetic tree was constructed by the unweighted pair group method with the arithmetic mean. The reliability of the phylogenetic results was assessed using 100 bootstrap replicate.

Inoculation of lymphotropic HCV strains in various kinds of lymphoid cell lines and human primary lymphocyte with stimulation

We used two different lymphotropic HCV strains. One was the SB-HCV strain that was previously reported by Sung et al[29]. The other one was Ly-HCV that was identified in this study by our group. The almost full-length sequence (95.9% coverage) of Ly-HCV was determined using a deep-sequence Hi-Seq 2000 system (illumina) (Fig S1A and B). These two-lymphotropic HCV strains were used for the experiments of HCV infection into lymphoid cells. Previously, we reported Raji, Molt-4 and primary human lymphoid cells were susceptible to the SB-HCV strain. In addition to these cells, we used miR122-transduced RIG-1/MDA-knock-down Raji cells provided by Machida K et al, since this cell line was most susceptible to SB-HCV replication (ongoing project, data not shown). These lymphotropic HCV strains were inoculated at day 0. SB cell culture supernatant and diluted serum from the patient with Ly-HCV, which contained 2×10^5 copies/ml of HCV-

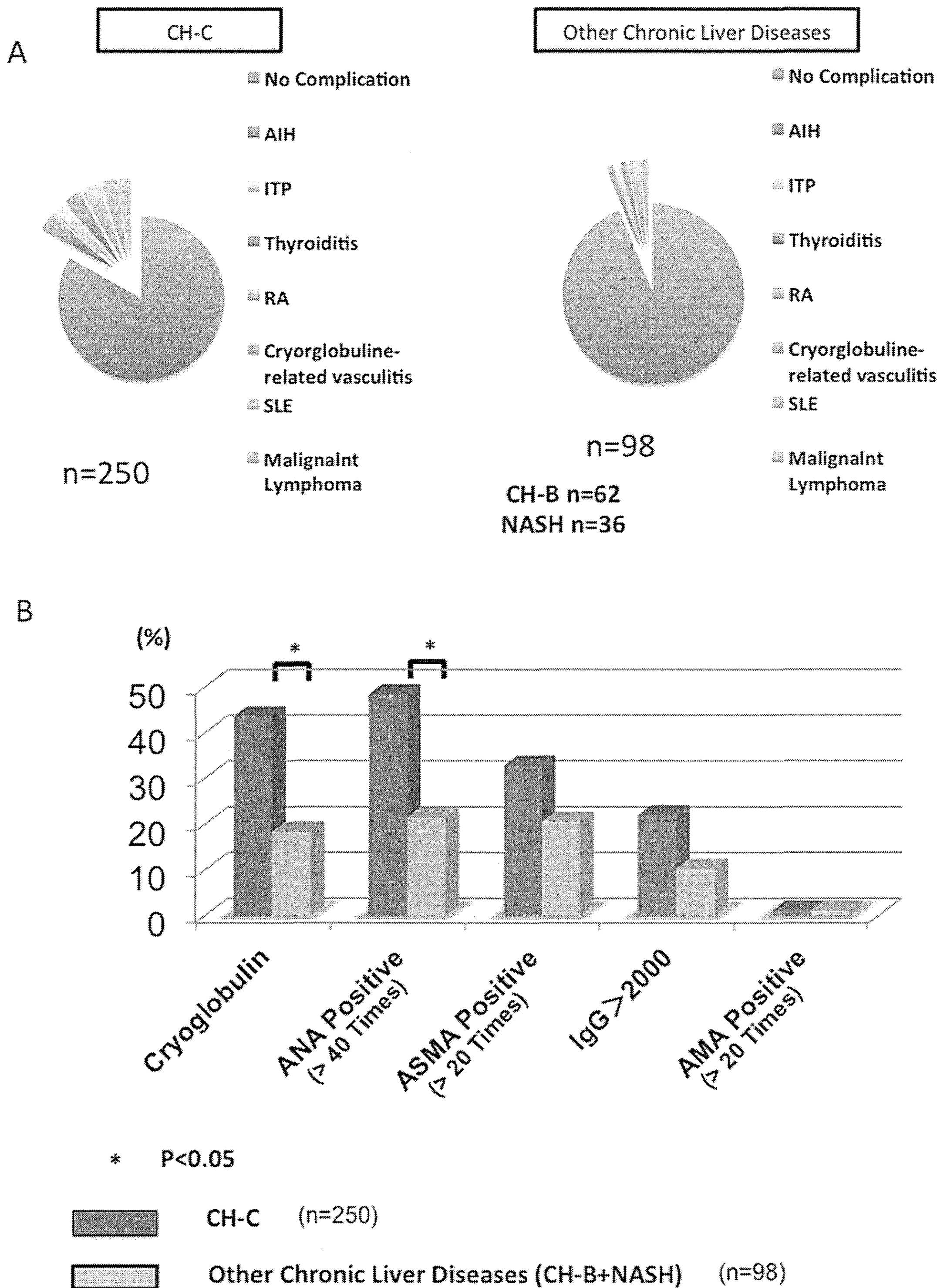

Figure 1. The relation between CH-C and the phenotype of autoimmune- diseases. The prevalence of these diseases in CH-C (n = 250) was significantly higher than in other chronic liver diseases (n = 98) (p = 0.0011) (A). The prevalence of these diseases and the positive rate of cryoglobulin, ANA (>40 times), ASMA (>20 times) and AMA (>20 times), and the amount of IgG (>2000mg/dl) are shown (B).
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RNA, were used for the infection of several kinds of human primary lymphoid cells (1×10^5 cells). A control infection with UV-irradiated HCV was included in every experiment. The supernatant of Huh-7 cells transfected with JFH-1 strains at 10 days post-transfection was used for several control experiments. The HCV-1T strain obtained from a CH-C patient without extrahepatic diseases and lymphoproliferative diseases was also used for several control experiments.

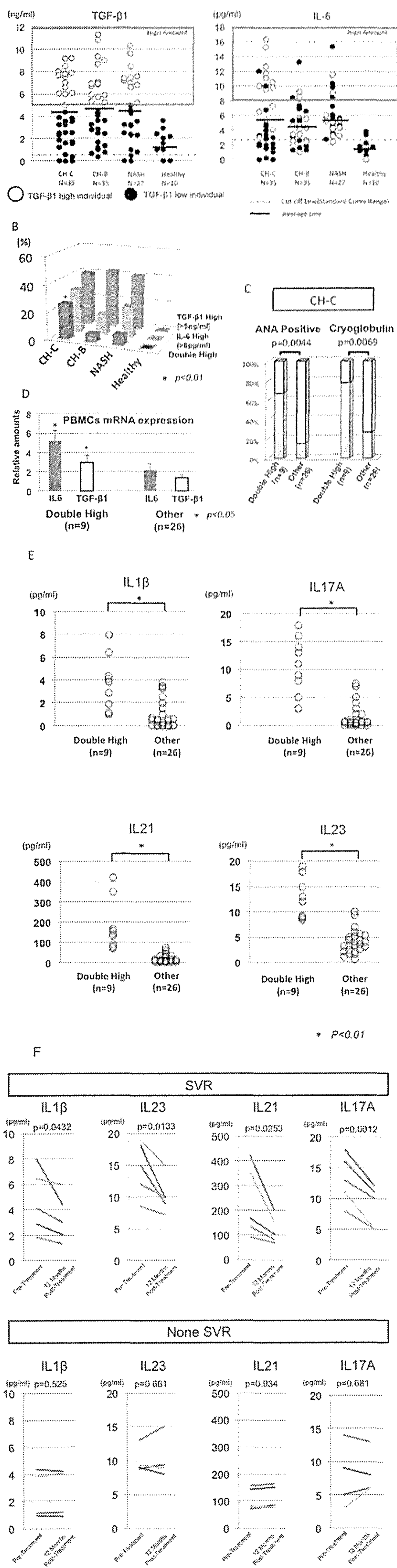

Figure 2. The cytokine conditions affecting the positivity of ANA and Cryoglobulin, and Th17 development. A comparison of the amounts of IL6 and TGF-β among the CH-C, CH-B, NASH and healthy subjects is shown (A). The bar indicates the mean cytokine amounts. The frequency of TGF-β1 high, IL6 high, and TGF-β1 and IL6 double high patients among the 4 groups (CH-C, CH-B, NASH, and healthy subjects) is shown (B). The positive rate of ANA and Cryoglobulin in the double high CH-C patients (n = 9) and the other CH-C patients (n = 26) is shown(C). The IL6 and TGF-β1 mRNA expression of PBMCs in the double-high patients (n = 9) and other patients (n = 26) is shown in the bar graphs (D). The amounts of IL1β, IL17A, IL21 and IL23 in the serum were compared between double high CH-C patients (n = 9) and the other CH-C patients (n = 26) (E). The comparisons of serum cytokines before and after the Peg-interferon/Ribavirin treatment are shown (F). Serum samples were collected at just before the treatment and twelve month after the end of treatment. SVR indicates sustained virological treatment (n = 5).
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The analysis of IL17-secreting CD4$^+$ T cells

Naïve CD4$^+$ cells were negatively isolated by using a naïve CD4$^+$ T cells isolation kit II (Miltenyi Biotec). Isolated naïve CD4$^+$ cells were exposed to SB-HCV, Ly-HCV, UV-irradiated-SB-HCV, UV-irradiated-Ly-HCV or Mock. Then, CD3$^+$CD28$^+$ coated beads and various kinds of cytokines were added to the culture medium to analyze the Th17 commitment and development (Table S1). The cytokine conditions for Th17 commitment and development included IL-1β (10 ng/ml), and IL23 (1 ng/ml), which are important for the Th17 development in human, because the differentiation of Th17 cells is very difficult without these cytokines when using human PBMCs[30]. The cells were harvested at 7 days post-inoculation and IL17A-secreting cells were analyzed by MACS cytokine secretion assay (Miltenyi Biotec).

Transwell co-culture system

The trans-membrane with 0.4 um pore size was used for the analysis of soluble factor-inducing Th17 cells, especially IL6 and TGF-β1. The upper chamber included PBMCs (2×10^6 cells/ml) of CH-C patients (Ly-HCV or HCV-1T). The lower chamber included naïve CD4$^+$ cells (2×10^5 cells/ml) of a healthy individual and CD3CD28 coated beads with or without IL6 (40 ng/ml)(abcam) and TGF-β1 (40 ng/ml)(abcam) neutralizing antibodies. After five days incubation, the total RNA was isolated from cells of the lower chamber. The expression levels of RORγt were analyzed by real time PCR.

Construction of Lenti-virus expressing HCV-Core antigen

HCV core cDNA cloned in pcDNA3 was kindly provided by Dr. K. Takeuchi [31]. The full length HCV core cDNA was cloned into lentiviral vector, pCSII-EF plasmid, to create the pCSII-EF-HCV core[32]. The pCSII-EF-HCV core or control pCSII-EF-IRES-GFP plasmid was transfected into HEK293T cells together with two packaging plasmids, pCAG-HIVgp and pCMV-VSV-G/RSV-Rev (provided by the RIKEN Bio-resource Center), using the calcium phosphate method. The supernatants containing the recombinant lenti-virus were used for the infection of human primary lymphocyte.

Transfection of HCV individual protein expression plasmids

Various expression plasmids were constructed by inserting HCV-core, E1, E2, NS3, NS4B, NS5A and NS5B cDNA of genotype 1a behind the cytomegalovirus immediate-early promoter in pCDNA3.1 (Invitrogen). Primary CD4$^+$ cells were transfect-

Table 1. The frequency of Strand specific-HCV-RNA positive CD4+ T cells and CD19+ B cells.

		Negative-st-positive		Positive-st-positive	
		%(n: positive/total)		%(n: postive/total)	
	Double High (n = 9)	33.3 (3/9)		44.4 (4/9)	
CD4+ T cell			p = 0.0166		p = 0.033
	Other (n = 26)	3.8 (1/26)		11.5 (3/26)	
	Double High (n = 9)	44.4 (4/9)		66.6 (6/9)	
CD19+ B cell			p = 0.0027		p = 0.0003
	Other (n = 26)	3.8 (1/26)		7.6 (2/26)	

St-specific HCV-RNA were detected by nested PCR with rTth polymerase.
Double high indicates that the amount of IL6 and TGF-β are high.
doi:10.1371/journal.pone.0098521.t001

ed using 4D-Nucleofector II (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 hour post-transfection. The transfection and expression efficiencies were analyzed using intracellular staining of individual proteins of HCV and flow cytometry analysis.

Real-time PCR analysis

Cells were collected before the inoculation of lenti-virus and 10 days after the inoculation of lenti-virus. Total RNA was isolated using a column isolation kit (QIAGEN). After the isolation of RNA, one-step real-time PCR using a TaqMan Chemistry System was carried out. The ready-made set of primers and probe for the amplification of IL-6 (Hs00985639_m1), TGF-β1 (Hs00998133_m1), T-bet (Hs00203436_m1), GATA-3 (Hs00231122_m1), RORC (Hs01076112_m1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Hs03929097_g1) were purchased from Applied Biosystems. The relative amount of target mRNA was obtained using a comparative threshold cycle (CT) method. The expression level of mRNAs of the non-stimulation sample of mock infected $CD4^{+}$ cells was represented as 1.0 and the relative amounts of target mRNA were calculated according to the manufacturer's protocol.

The analysis of STAT-1 and STAT-3 signaling

STAT-1 and STAT-3 signaling was analyzed by phospho-STAT-1 (Tyr701) and phosphor-STAT-3 (Tyr705) sandwich ELISA kit (Cell Signaling Technology). Briefly, naïve $CD4^{+}$ cells transfected with or without HCV-core expressing plasmid were incubated with IL6 and TGF-β1. The cells were harvested at various time points. Then, the cell lysates were used for the quantification of phosphor-STAT-1 and phosphor-STAT-3.

Statistical analysis

The data in Figure 1A, 1B, 2B and 2C were analyzed by χ^2 test. The data in Figure 2D and 2E were analyzed by independent Students t test. Figure 3A, 3C, 4A, 4B and 4C were analyzed by Mann-Whitney U test. All statistical analyses were carried out using JMP Pro version 9.

Accession Numbers

Accession number EntryID
AB779562 51027b2b6a8011fb860007e4.LyHCVserumSR
Accession number EntryID
AB779679 51029c6f6a8011fb8600093e.LyHCVpbmcSR

Results

Prevalence of autoimmune-related diseases in the CH-C patients

The prevalence of autoimmune-related disease in the CH-C patients was significantly higher than in the subjects with other chronic liver diseases in Tohoku University Hospital (p = 0.0011) (Fig.1A). In addition to the prevalence of autoimmune-related diseases, we analyzed the immunological laboratory tests including cryoglobulin, anti-nuclear antibody (ANA), anti-smooth muscle antibodies (ASMA), Immunoglobulin G (IgG), anti-mitochondrial antibody (AMA). The frequency of ANA positive or cryoglobulin positive patients in CH-C patients was significantly higher than in those with other chronic liver diseases ($p<0.05$) (Fig.1B).

The amount of IL6 and TGF-β1 in the peripheral blood of CH-C patients

The average amounts of IL6 and TGF-β1 were comparable among healthy subjects, CH-C, CH-B and NASH (IL6: 1.77, 5.83, 4.84 and 5.99 pg/ml), (TGF-β: 1.45, 4.18, 4.68 and 4.5 mg/ml), (average amount) (Fig. 2A). However, the frequency of patients with high amounts of IL6 (over 8 pg/ml) and TGF-β1 (over 5 ng/ml) (double-high) was significantly higher than in those with other chronic liver diseases ($p<0.05$)(Fig. 2B). The cut-off levels of high amount of IL6 (over 8 pg/ml) and TGF-β1 (over 5 ng/ml) were determined by the appearance of two clusters(high and low) in the CH-C samples. Interestingly, Most of the TGF-β1 high CH-C patients had high amounts of IL6 (Fig. 2B). Moreover, the amount of IL6 were significantly correlated with the amount of TGF-β1(data not shown). The serum amounts of IL6 and TGFβ1 were analyzed at 6 months after the sampling points. The serum amount of IL6 and TGF-β1 in the high amount of IL6 and TGF-β1 both (double-high) patients remained doubly high (data not shown). It has been reported that the combination of IL6 and TGF-β1 cytokines could induce Th17 cells[20]. Therefore, we compared the frequency of ANA-positive or cryoglobulin-positive patients between double-high patients and the other patients with HCV persistent infection. The frequency of ANA-positive or cryoglobulin-positive patients among the double-high patients was significantly higher than among the other CH-C patients ($p<0.01$)(Fig. 2C). The expression of IL-6 and TGF-β1-mRNA in PBMCs of double-high patients was significantly higher than in other CH-C patients ($p<0.05$)(Fig. 2D). Moreover, the serum amounts of IL1-β, IL17A, IL21 and IL23 in the double-high

Table 2. The frequency of different nucleotide bases between LyHCVserumSR and LyHCVpbmcSR.

Nucleo tide Position	LyHCVserumSR										LyHCVpbmcSR									
	Max base	No. of nucleotide	A (%)		C (%)		G (%)		T (%)		Max base	No. of nucleotide	A (%)		C (%)		G (%)		T (%)	
538	G	1835	482	(26.27)	3	(0.16)	1349	(73.51)	1	(0.05)	A	1	1	(100.0)	0	(0.00)	0	(0.00)	0	(0.00)
659	G	1821	484	(26.58)	1	(0.05)	1334	(73.26)	2	(0.11)	A	1	1	(100.0)	0	(0.00)	0	(0.00)	0	(0.00)
1,026	A	2041	1098	(53.80)	2	(0.10)	939	(46.01)	2	(0.10)	G	3	1	(33.33)	0	(0.00)	2	(66.67)	0	(0.00)
1,034	C	2120	4	(0.19)	1263	(59.58)	0	(0.00)	853	(40.24)	T	3	0	(0.00)	1	(33.33)	0	(0.00)	2	(66.67)
1,280	C	2050	2	(0.10)	1785	(87.07)	2	(0.10)	261	(12.73)	T	7	0	(0.00)	2	(28.57)	0	(0.00)	5	(71.43)
2,050	C	1595	11	(0.69)	930	(58.31)	652	(40.88)	2	(0.13)	G	4	0	(0.00)	1	(25.00)	3	(75.00)	0	(0.00)
2,105	T	1852	1	(0.05)	587	(31.70)	0	(0.00)	1264	(68.25)	C	6	0	(0.00)	4	(66.67)	0	(0.00)	2	(33.33)
2,114	A	1814	1261	(69.51)	525	(28.94)	22	(1.21)	6	(0.33)	C	7	3	(42.86)	4	(57.14)	0	(0.00)	0	(0.00)
2,136	T	1813	0	(0.00)	527	(29.07)	1	(0.06)	1285	(70.88)	C	7	0	(0.00)	4	(57.14)	0	(0.00)	3	(42.86)
2,159	T	1904	0	(0.00)	505	(26.52)	1	(0.05)	1398	(73.42)	C	7	0	(0.00)	4	(57.14)	0	(0.00)	3	(42.86)
2,234	C	2023	5	(0.25)	1128	(55.76)	3	(0.15)	887	(43.85)	T	9	0	(0.00)	3	(33.33)	0	(0.00)	6	(66.67)
2,249	C	2030	5	(0.25)	1471	(72.46)	0	(0.00)	554	(27.29)	T	8	0	(0.00)	3	(37.50)	0	(0.00)	5	(62.50)
2,717	G	1841	471	(25.58)	1	(0.05)	1365	(74.14)	4	(0.22)	A	3	2	(66.67)	0	(0.00)	1	(33.33)	0	(0.00)
3,878	T	2449	3	(0.12)	37	(1.51)	1	(0.04)	2408	(98.33)	C	3	0	(0.00)	2	(66.67)	0	(0.00)	1	(33.33)
4,043	C	1894	14	(0.74)	1836	(96.94)	0	(0.00)	44	(2.32)	T	3	0	(0.00)	1	(33.33)	0	(0.00)	2	(66.67)
4,473	C	2105	811	(38.53)	1292	(61.38)	1	(0.05)	1	(0.05)	A	5	4	(80.00)	1	(20.00)	0	(0.00)	0	(0.00)
4,661	C	2263	2	(0.09)	1301	(57.49)	1	(0.04)	959	(42.38)	T	6	0	(0.00)	0	(0.00)	0	(0.00)	6	(100.0)
5,087	G	2050	477	(23.27)	1	(0.05)	1572	(76.68)	0	(0.00)	A	5	3	(60.00)	0	(0.00)	2	(40.00)	0	(0.00)
5,114	G	1791	32	(1.79)	452	(25.24)	1306	(72.92)	1	(0.06)	C	3	0	(0.00)	2	(66.67)	1	(33.33)	0	(0.00)
5,117	A	1674	1262	(75.39)	1	(0.06)	411	(24.55)	0	(0.00)	G	3	1	(33.33)	0	(0.00)	2	(66.67)	0	(0.00)
5,156	G	1871	481	(25.71)	0	(0.00)	1389	(74.24)	0	(0.00)	A	3	2	(66.67)	0	(0.00)	1	(33.33)	0	(0.00)
5,462	C	2026	2	(0.10)	1452	(71.67)	3	(0.15)	569	(28.08)	T	11	0	(0.00)	3	(27.27)	0	(0.00)	8	(72.73)
5,535	T	2059	1	(0.05)	490	(23.80)	0	(0.00)	1568	(76.15)	C	9	0	(0.00)	5	(55.56)	0	(0.00)	4	(44.44)
5,799	G	2113	477	(22.57)	0	(0.00)	1634	(77.33)	1	(0.05)	A	3	2	(66.67)	0	(0.00)	1	(33.33)	0	(0.00)
5,804	C	2131	3	(0.14)	1613	(75.69)	3	(0.14)	511	(23.98)	T	3	0	(0.00)	1	(33.33)	0	(0.00)	2	(66.67)
5,807	T	2102	2	(0.10)	490	(23.31)	5	(0.24)	1604	(76.31)	C	3	0	(0.00)	2	(66.67)	0	(0.00)	1	(33.33)
5,831	T	2168	7	(0.32)	483	(22.28)	5	(0.23)	1672	(77.12)	C	3	0	(0.00)	2	(66.67)	0	(0.00)	1	(33.33)
5,834	T	2153	2	(0.09)	504	(23.41)	0	(0.00)	1647	(76.50)	C	3	0	(0.00)	3	(100.0)	0	(0.00)	0	(0.00)
5,837	C	2144	3	(0.14)	1674	(78.08)	2	(0.09)	465	(21.69)	T	3	0	(0.00)	1	(33.33)	0	(0.00)	2	(66.67)
5,882	T	2160	0	(0.00)	480	(22.22)	0	(0.00)	1680	(77.78)	C	3	0	(0.00)	2	(66.67)	0	(0.00)	1	(33.33)
5,883	C	2114	0	(0.00)	1502	(71.05)	1	(0.05)	611	(28.90)	T	3	0	(0.00)	1	(33.33)	0	(0.00)	2	(66.67)
5,969	T	2238	2	(0.09)	80	(3.57)	1	(0.04)	2155	(96.29)	A	1	1	(100.00)	0	(0.00)	0	(0.00)	0	(0.00)
5,978	C	2451	1	(0.04)	2013	(82.13)	1	(0.04)	436	(17.79)	T	1	0	(0.00)	0	(0.00)	0	(0.00)	1	(100.0)

Table 2. Cont.

Nucleo tide Position	LyHCVserumSR										LyHCVpbmcSR									
	Max base	No. of nucleotide	A (%)		C (%)		G (%)		T (%)		Max base	No. of nucleotide	A (%)		C (%)		G (%)		T (%)	
7,172	C	2409	2	(0.08)	1696	(70.40)	1	(0.04)	707	(29.35)	T	3	0	(0.00)	1	(33.33)	0	(0.00)	2	(66.67)
7,274	A	2654	1564	(58.93)	5	(0.19)	1074	(40.47)	11	(0.41)	G	7	3	(42.86)	0	(0.00)	4	(57.14)	0	(0.00)
7,349	G	2510	1019	(40.60)	5	(0.20)	1484	(59.12)	2	(0.08)	A	6	4	(66.67)	0	(0.00)	2	(33.33)	0	(0.00)
7,932	G	2810	1354	(48.19)	0	(0.00)	1453	(51.71)	3	(0.11)	A	9	7	(77.78)	0	(0.00)	2	(22.22)	0	(0.00)
8,093	G	2647	684	(25.84)	3	(0.11)	1956	(73.89)	4	(0.15)	A	7	4	(57.14)	0	(0.00)	3	(42.86)	0	(0.00)
8,168	G	1993	39	(1.96)	1	(0.05)	1952	(97.94)	1	(0.05)	A	2	1	(50.00)	0	(0.00)	1	(50.00)	0	(0.00)
8,237	C	2077	1	(0.05)	2033	(97.88)	0	(0.00)	42	(2.02)	T	3	0	(0.00)	1	(33.33)	0	(0.00)	2	(66.67)
8,672	G	2340	17	(0.73)	576	(24.62)	1734	(74.10)	12	(0.51)	C	3	0	(0.00)	2	(66.67)	1	(33.33)	0	(0.00)
8,693	T	2899	5	(0.17)	690	(23.80)	0	(0.00)	2204	(76.03)	C	3	0	(0.00)	2	(66.67)	0	(0.00)	1	(33.33)

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Table 3. Detection of St-Specific HCV-RNA in various kinds of lymphoid cell.

Immune cells	Raji				mir122Raji				Molt-4				naïve T (IL-2)				naïve T (IL6)				naïve T (TGF-β)				naïveT (IL6 and TGF-β)			
HCV-Strain	JFH	HCV	SB	Ly	JFH	HCV	SB	Ly	JFH	HCV	SB	Ly	JFH	HCV	SB	Ly	JFH	HCV	SB	Ly	JFH	HCV	SB	Ly	JFH	HCV	SB	Ly
Positive Strand																												
2 days	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
7 days	0	1	16	4	0	1	64	16	0	1	16	4	0	0	16	4	0	0	4	4	0	0	16	4	0	0	16	4
7 days-UV-irraidated	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Negative Strand																												
2 days	0	0	1	0	0	0	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
7days	0	0	4	1	0	0	16	4	0	0	4	1	0	0	4	1	0	0	1	1	0	0	4	1	0	0	4	1
7days-UV-irradiated	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

The titers of HCV-RNA were expressed as the highest dilution giving a visible band of the correct size.
Naïve T cells were incubated with IL-1β (10 ng/ml), IL23 (1 ng/ml), and CD3CD28 coated beads.
JFH-1 and HCV-1T are not lymphotropic HCV strains.
SB-HCV and Ly-HCV are lymphotropic HCV strains.
mir122Raji indicate miR122-transduced RIG-1/MDA-knockdown Raji.
doi:10.1371/journal.pone.0098521.t003

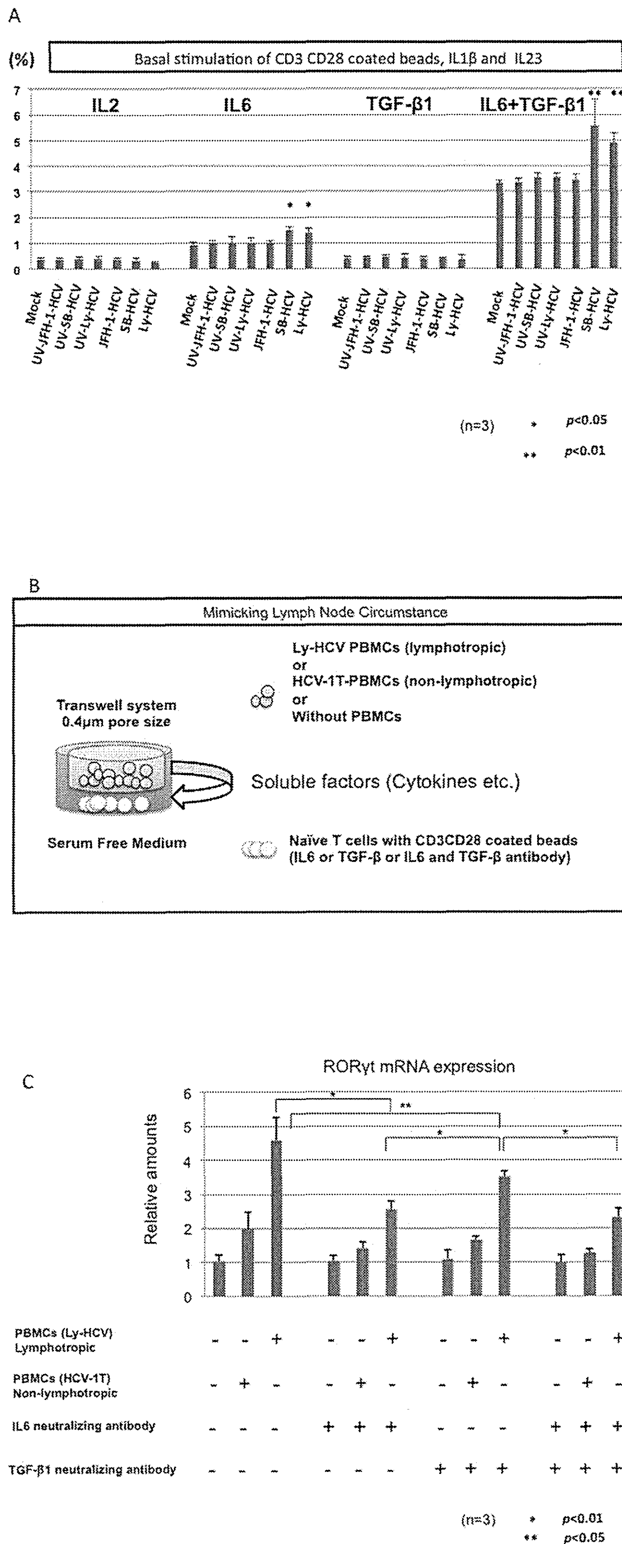


Figure 3. The effect of lymphotropic HCV on the Th17 development in various kinds of cytokines condition. Isolated naïve $CD4^{+}$ cells were exposed to SB-HCV, Ly-HCV, UV-irradiated-SB-HCV, Ly-HCV or Mock. Then, $CD3^{+}CD28^{+}$ coated beads and various kinds of cytokines were added to the culture medium to analyze the Th17 commitment and development (Suppl. Table 1). The cells were harvested at 7 days post-inoculation and IL17A-secreting cells were analyzed by MACS cytokine secretion assay. The frequencies of CD4+ IL17A+ cells among the CD4+ cells were shown in the bar graph (A). The bar graph indicates the frequencies of Th17 cells with or without lymphotropic HCV in the various kinds of cytokine conditions (A). The schema of the transwell system is shown (B). The expression of RORγt mRNA in naïve T lymphocytes with or without various kinds of stimulations is shown (C). The obtained data were analyzed by Mann-Whitney U test. Three independent experiments were carried out (A)(C).
doi:10.1371/journal.pone.0098521.g003

patients were significantly higher than in the other CH-C patients (*p*<*0.01*) (Fig. 2E). Moreover, these cytokines were significantly correlated with the amount of IL6 and TGF-β1 (data not shown). Then, we quantified the serum cytokines at the twelve months after the Peg-interferon/Ribavirin-treatment among double high patients. The serum amounts of IL-1β, IL17A, IL21 and IL23 were significantly decreased after the achievement of the sustained virological response (SVR) (Fig. 2F).

The relation between lymphotropic HCV and patients with high amounts of IL6 and TGF-β1 (Double-High)

Previously, Machida et al. described that HCV replication in B lymphocytes could induce their secretion of IL6. Therefore, we analyzed the relationship between lymphotropic HCV and patients with double-high by detecting strand-specific HCV-RNA in the $CD4^{+}$ T cells and $CD19^{+}$ B cells. The frequency of positive and negative-strand-specific-HCV-RNA in double-high CH-C patients was significantly higher than in the other CH-C patients (Table 1). These data indicated that the lymphotropism of HCV could be related to the IL6 and TGF-β1 double-high environment.

Detection of a new lymphotropic HCV from a patient with lympho-proliferative disease

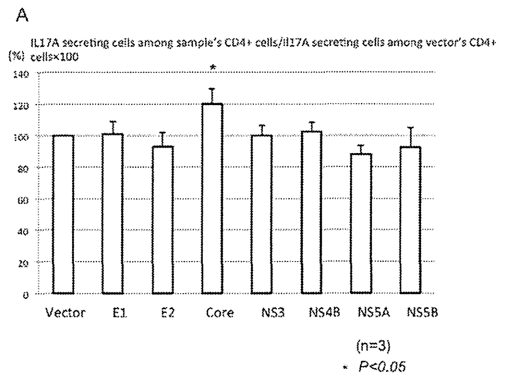
Previously, we used a lymphotropic SB-HCV that was reported by Sung et al[29]. In this study, we found a patient who had higher amounts of HCV RNA in the lymphocytes in comparison to other CH-C patients. This lymphotropic HCV (named Ly-HCV) is genotype 1b. The full-length sequence of this strain was analyzed by deep sequencing of both serum and PBMC samples. Phylogenetic tree analysis was then carried out (Fig. S1A). To characterize the metagenomics of HCV infection in human serum, LyHCVserumSR (registered in DDBJ; the accession number, AB779562) and PBMC, LyHCVpbmcSR (registered in DDBJ; the accession number, AB779679), we analyzed the samples by paired end deep sequencing. The coverage was 100.0% and the average depth was 2092.1× (Fig. S1B).

The LyHCVserumSR and LyHCVpbmcSR isolates were 99.5% identical to each other within the overlapping region. In 42 nucleotide bases, the major nucleotide bases showed differences. However, only the proportions of nucleotide sequences were different (Table 2). The sequences of HCV-RNA obtained from serum and PBMCs were almost the same (Table 2). Therefore, we used the diluted Ly-HCV-serum for the *in vitro* infection study.

Analysis of Infectivity of Ly-HCV and SB-HCV under the various kinds of cytokines

We examined the infectivity of Ly-HCV and SB-HCV into several lymphoid-cell lines (Raji, miR122-transduced RIG-1/MDA-knockdown Raji, and Molt-4) and primary naïve $CD4^{+}$ T cells. Semi-quantitative strand-specific nested PCR was carried out as in our previous reports (Table 3). The infectivity of HCV in the IL-6 and TGF-β cytokine combination conditions with low dose IL1β, IL23 and CD3CD28 coated beads was no better than that

A



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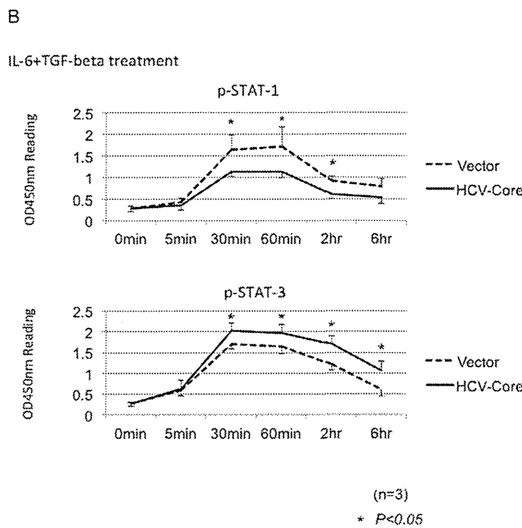

C

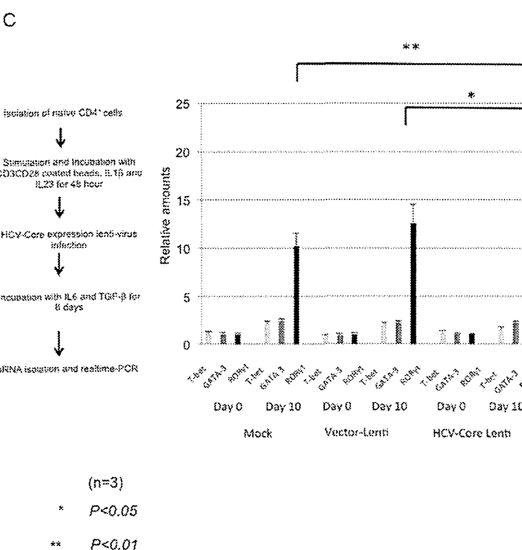

Figure 4. The identification of proteins responsible for enhancing the Th17 development (A). The transfection of various kinds of plasmids expressing HCV-individual proteins (E1, E2, Core, NS3, NS4B, NS5A, NS5B and vector) was carried out by nucleofector. The cells were analyzed at 72 hours post-transfection. The bar graph indicates the IL17A-secreting cells among the sample's $CD4^{+}$ cells/IL17A secreting cells and the vector's $CD4^{+}$ cells × 100. The obtained data were analyzed by Mann-Whitney U test. Three independent experiments were carried out. **The analysis of STAT-1 and STAT-3 signaling (B).** We used a pathscan to quantify sequentially the phosphor-STAT-1 and STAT-3. The dotted lines indicate data of the vector control. Three independent experiments were carried out. **Long-term culture affected the commitment of naïve T lymphocytes with HCV-core expressing Lenti-virus (C).** The gene expressions of T-bet, GATA-3 and ROR-γt were analyzed by real-time PCR. The relative amounts of mRNA were calculated by ΔΔCT methods. The target gene expressions were analyzed at pre-inoculation of Lent-virus and 10 days after the inoculation of lenti-virus. Three independent experiments were carried out.

doi:10.1371/journal.pone.0098521.g004

in IL2, IL6, or TGF-β cytokine only conditions with low dose IL1β, IL23 and CD3CD28 coated beads.

The effect of lymphotropic HCV on the Th17 development

The addition of both IL6 and TGF-β1 could significantly induce IL17-secreting T cells (Th17) in comparison to IL6 or TGF-β1 alone (Fig 3A). Both lymphotropic HCV strains (SB-HCV and Ly-HCV) could significantly up-regulate the Th17 development in comparison to Mock and these strains that had been UV-irradiated. Then, we used a co-culture system to analyze the blocking of IL6 and TGF-β1 effects since the expressions of IL6 and TGF-β1 mRNA in PBMCs of double high patients were significantly higher than those in other CH-C patients (Fig. 2D and Fig. 3B). The IL-6 was produced from B lymphocytes. Moreover, the major TGF-β1 producing cells were monocytes in double high patients (Data not shown). The soluble factors produced from PBMCs of Ly-HCV-patient could significantly induce Th17 master gene RORγt in comparison to mock and PBMCs of HCV-1T patient (Fig. 3C). The addition of IL6 and TGF-β1 neutralizing antibody significantly reduced the expression of RORγt, especially IL6 neutralizing antibody (Fig. 3C).

The identification of HCV proteins and signal transduction responsible for the production of IL17A

We used E1, E2, Core, NS3, NS4B, NS5A and NS5B expressing plasmids to transiently express these proteins in naïve T cells. The transfection efficiencies were 45.4±4.96% (average±standard deviation). Among these proteins, only HCV-Core protein could significantly enhance the production of IL17A cells (Fig. 4A)($p<0.05$). In addition to in vitro circumstance, we used NOD/scid/γc^{null} (NOG) mice that are super-immunodeficiency mice[33]. The transfusions of HCV-core expressing human primary lymphoid cells were carried out (*ongoing Kondo Y et al.*). The higher amount of IL17A and RORγt mRNA were detected in the HCV-Core expressing $CD4^{+}$ cells in comparison to the control groups (data not shown). Then, we sequentially analyzed the STAT-1 and STAT-3 activation by IL6 and TGF-β1 stimulation in the HCV-Core expressing T cells. The results indicated that STAT-3 signaling was significantly enhanced in comparison to mock-transfected T cells (Fig. 4B)($p<0.05$). These data indicated that HCV-Core protein enhanced the STAT-3 signaling following the induction of the Th17 master gene-RORγt.

Long-term culture of primary naïve T lymphocytes with HCV-core expressing lenti-virus

We constructed the HCV-core expressing lenti-virus to analyze the long-term culture of primary naïve T lymphocytes with the expression of HCV core protein. The efficiency of lenti-virus infection was 27.7±3% (average±standard deviation). In addition to IL1β and IL23, the IL6 and TGF-β1 cytokine conditions could remarkably induce the RORγt mRNA (Fig.4C). Moreover, significantly higher amounts of RORγt mRNA were detected in the HCV-core expressing T lymphocytes in comparison with the control groups (Fig. 4C)($p<0.05$).

Discussion

Autoimmune thyroiditis, systemic lupus erythematosus, idiopathic thrombocytopenic purpura, autoimmune hepatitis and rheumatoid arthritis etc. could be classified not only as HCV-related diseases but also as Th17-related autoimmune diseases[16–19,34–36]. In this study, we clearly demonstrated the relevance of lymphotropic HCV to autoimmune-related diseases including an important role of Th17 cells in CH-C patients. This study revealed two important mechanisms by which Th17 development is enhanced. In the first, the existence of lymphotropic HCV can result in the IL6 and TGF-β1 double high condition that can enhance Th17 development. Previously, Machida et al. described that HCV replication in B cells induced IL6 production from B cells[24]. In the second, the existence of HCV in naïve T cells can enhance Th17 development in the IL6 and TGF-β1 double high condition by enhancing STAT-3/RORγt signaling. Previously, we showed that lymphotropic HCV could suppress IFN-γ/STAT-1/T-bet signaling, which could contribute to the persistence of hepatitis C virus infection[1,4,13]. STAT-3 signaling could be enhanced by the suppression of STAT-1 signaling. However, the finding of our research was surprising. Therefore, we examined this phenomenon studiously and carefully. First, we found a novel genotype, 1b lymphotropic HCV strain (Ly-HCV), that could infect Raji and human primary lymphocytes. Although the infectivity of this strain was lower than that of SB-HCV[29], we could detect negative and positive strand RNA in naïve T lymphocytes with stimulation.

Therefore, we used two lymphotropic HCV strains to analyze the effect on Th17 development. Moreover, two kinds of expression experiments showed that HCV-Core could enhance the STAT-3/RORγt signaling, since the method of gene expression and the period of incubation might affect the result of T cell development. However, the results of our two kinds of experiments (plasmids and lenti-virus) consistently showed that the expression of HCV-core protein in T lymphocytes could enhance the Th17 development. Other groups previously reported that HCV-core protein could affect anergy-related genes and T cell responses by inducing spontaneous and alternating T-cell receptor-triggered Ca2+ oscillations[37,38]. Therefore, the expression of HCV-core protein in T lymphocyte might be important for the functional changes in T lymphocytes. Although our study confirmed that the replication of HCV in lymphocytes is important, there was a bystander effect by exosome produced from HCV-infected lymphocytes. Previously, our group reported that exosome could transport miRNA and proteins in the microenviroment[39,40]. This phenomenon could explain the significant effects of a low level of HCV infection in lymphocytes. Moreover, the naïve T lymphocyte is located upstream of Th17 development. Therefore, we should not underestimate the effect of a low-level of HCV replication in lymphocytes.

In conclusion, we report the detailed mechanism of Th17 development and HCV infection, which might be involved in the pathogenesis of autoimmune-related disease in CH-C patients (Fig S2). Recently, a novel therapy targeting STAT-3 signaling was reported[23,41,42]. We should consider the clinical use of such treatments for autoimmune-related diseases in CH-C patients.

Supporting Information

Figure S1 Phylogenetic trees constructed based on the nearly entire nucleotide sequence of HCV by using the unweighted pair group method with the arithmetic mean (Michener 1957). The tree includes the three genotype 1a isolates, forty 1b, one 1c, three 2a, two 2b, one 2c and one 3a, 3b, 6b, 7b, 9b, 10a, whose nucleotide sequence data were retrievable from the GenBank/EMBL/DDBJ database (A). Mapping to the consensus HCV genome sequence. For Ly-HCV 0183-4, 197,414 reads were mapped (Fig S1B). The coverage was 100.0%, and the average depth was 2092.1x. For Ly-HCV 0186-1, 410 reads were aligned. The coverage was 95.9%, and the average depth was 4.3× (B).
(TIFF)

Figure S2 The schema of Th17 induction in perihepatic lymph node of CH-C patient with lymphotropic HCV are shown.
(TIFF)

Table S1 Various kinds of cytokines conditions for in vitro analysis are shown in Table S1.
(DOC)

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Author Contributions

Conceived and designed the experiments: YK MN OK TK ML TS. Performed the experiments: YK MN OK KM RF TN KK EK KN. Analyzed the data: YK MN OK TN KN ML TS. Contributed reagents/materials/analysis tools: KM. Wrote the paper: YK MN TN ML TS.

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