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Possible Recruitment of Peripheral Blood CXCR3$^+$ CD27$^+$ CD19$^+$ B Cells to the Liver of Chronic Hepatitis C Patients

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It has been suggested that hepatitis C virus (HCV) infects not only hepatocytes but also immune cells, including B cells. HCV infection of B cells is the likely cause of B-cell dysregulation disorders such as mixed cryoglobulinemia, rheumatoid factor production, and B-cell lymphoproliferative disorders that may evolve into non-Hodgkin's lymphoma. To clarify the effects of chronic HCV infection on B-cell dynamics, peripheral B cells from chronic hepatitis C patients (CHC) were characterized. We found that the frequency of CD27$^+$ B cells, that is memory phenotype, was significantly reduced in the peripheral blood of CHC. At the same time, the amount of IFN-γ-inducible protein-10 (IP-10), a CXCR3 ligand, was markedly elevated in the plasma of CHC. Furthermore, the CD27$^+$ B-cell population was found to highly express CXCR3 in CHC, thus suggesting that the CD27$^+$ B-cell population was recruited from peripheral blood to the inflammatory site of the liver of CHC, where IP-10 is produced. Immunohistochemical analyses of intrahepatic lymphocytes indicated that CXCR3$^+$ B cells were infiltrated in the liver of CHC. Our results thus offer new insight into the role of memory B cells in the HCV pathogenesis.

Introduction

NEARLY 200 MILLION PEOPLE worldwide are chronically infected with the hepatitis C virus (HCV; Lauer and Walker 2001). HCV infection persists for long periods and can lead to chronic liver damage that develops into chronic hepatitis, cirrhosis, or hepatocellular carcinoma. As there is no vaccine currently available to prevent HCV infection, uncovering the mechanisms that govern the pathogenesis of HCV infection is crucial in order to identify approaches to control disease progression.

HCV is a single-stranded, positive polarity RNA virus of the Flaviviridae family. CD81, a widely expressed tetraspanin molecule, has been shown to interact with the E2 region of HCV envelope protein (Pileri and others 1998), and is regarded as one of the key molecules for HCV infection. Although the liver is the major target organ for HCV, it also infects peripheral blood mononuclear cells (PBMCs), preferentially B cells that express CD81 (Dammacco and others 2000; Pal and others 2006). This B-tropism of HCV may be responsible, at least in part, for the extrahepatic manifestations of HCV infection, including mixed cryoglobulinemia (MC), Sjoegren-like syndrome, production of rheumatoid factor (RF), and B-lymphoproliferative disorder, which may evolve into non-Hodgkin's lymphoma (NHL; Galossi and others 2007; Zignego and others 2007). However, the mechanisms that underlie these immunological dysfunctions are poorly understood.

It has been demonstrated that CD81 molecule on human B cells forms a co-stimulatory complex with CD19 and CD21, and that cross-linking this complex lowers the threshold required for antigen-induced B-cell proliferation (Levy and others 1998). In the same way, binding of HCV E2 protein to

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the CD81/CD19/CD21 complex may affect the immune system by facilitating B-cell activation, and this may result in lymphoproliferative disorders.

The primary site of HCV infection and inflammatory response is the liver, in which various kinds of immunopathogenic events associated with the HCV infection take place. The infiltration of activated B cells recruited from draining lymph nodes increases during the course of disease progression, suggesting that infiltrating B cells play a role in the pathogenesis of chronic hepatitis (Vallat and others 2004), although the phenotype and characteristics of such B cells have not been well documented to date. It is plausible that infiltrating B cells in the liver are the malignant precursor population, as they are probably stimulated by inflammatory cytokines, and because HCV E2 protein and HCV have been known to induce DNA damage and mutation of cellular genes (Machida and others 2004, 2005, 2006).

In this study, we sought to analyze the effects of HCV infection on the fate of peripheral $CD27^+$ memory B cells that are thought to be vulnerable to antigenic stimulation. It was found that the frequencies of $CD27^+$ B cells that strongly express CXCR3 molecules, a receptor for IP-10, in the peripheral blood were significantly reduced compared with healthy subjects. Furthermore, the plasma level of IP-10 in CHC was markedly elevated. In accordance with these phenomena, $CXCR3^+$ B cells were detected in the CHC liver. The possible mechanisms controlling this memory B-cell recruitment in CHC are considered with regard to lymphocyte trafficking mediated by chemokines produced in the liver.

Materials and Methods

Patients and samples

Nineteen CHC were enrolled in this study. Demographic and clinical data were as follows: gender (M:F = 10:9); mean age (range) = 59.6 years (38–79 years); HCV genotype (No.) = 1b (18) and 2a (1); mean HCV RNA (KIU/mL) (SD) = 2,077 (1,016); mean alanine aminotransferase (ALT) (U/L) (SD) = 64.4 (23.5); and mean aspartate aminotransferase (AST) (U/L) (SD) = 61.3 (19.3). The details of this study were approved by the Review Board at the National Institute of Infectious Disease. All donors gave written informed consent before phlebotomy. Fourteen normal control subjects (CS) included anonymous volunteer blood donors visiting the Japanese Red Cross Blood Center.

Liver biopsy specimens were collected and stored until use at −80°C from 8 patients who gave written informed consent, including 5 patients with persistent HCV infection and 3 patients without HCV infection. Genotypes and plasma RNA titers of these patients with HCV infection are similar to those of the above 19 CHC.

Clinical tests

HCV genotype was determined by PCR of the core region with genotype-specific primers (Ohno and others 1997). HCV RNA was quantified by the Roche Amplicor assay (Roche Diagnostics, Branchberg, NJ), and results were standardized to international units (IU). Determination of the serum levels of ALT and AST was performed using standard methods.

Antibodies

The following directly conjugated Abs were used for flow cytometry: Allophycocyanin-anti-CD19 (MHCD1905; Invitrogen, Carlsbad, CA); FITC-anti-CD27 (555440; BD Biosciences, Franklin Lakes, NJ); and PE-anti-CXCR3 (FAB160P; R&D Systems, Minneapolis, MN). The following primary antibodies were used for immunohistochemical analyses: anti-CD19 (MCA1940; AbD serotec, Germany); anti-CXCR3 (SC-1395; Santa Cruz Biotechnology, Santa Cruz, CA); and anti-IP-10 (AF-266-NA; R&D Systems). The following Alexa-labeled secondary antibodies were purchased from Molecular Probes Inc., Invitrogen, Carlsbad, CA: Alexa635-Goat anti-mouse IgG (A31574); Alexa647-Rabbit anti-goat IgG (A21446); and Alexa488-Goat anti-mouse IgG (A11001).

Flow cytometry

Cells were washed twice with cold PBS containing 0.2% BSA, followed by incubation with an appropriate combination of directly conjugated Abs for 30 min on ice. Stained cells were analyzed by FACSCalibur (Becton Dickinson, San Jose, CA). Data were collected using CellQuest software (Becton Dickinson) and were analyzed using FlowJo software (Tree Star Inc., Ashland, OR).

Double fluorescence immunohistochemical analysis

Frozen sections (3 mm) of liver biopsy samples were fixed with ethanol for 10 min and with acetone for 1 min at −20°C, and then washed once with PBS. Primary antibodies were added, followed by incubation for 30 min at room temperature and washing with PBS (3×). Secondary antibodies were then added, followed by incubation for 30 min at room temperature and washing with PBS (3×). Additional primary antibodies were added, followed by incubation for 30 min at room temperature and washing with PBS (3×). Additional secondary antibodies were added, followed by incubation for 30 min at room temperature and washing with PBS (3×). Concentrations of primary antibodies used for double fluorescence staining were as follows: anti-CD19 (1:400); anti-CXCR3 (1:100); and anti-IP-10 (15 μL/mL). Concentrations of fluorescence-labeled secondary antibodies were as follows: Alexa635-Goat anti-mouse IgG (1:2,000); Alexa647-Rabbit anti-goat IgG (1:2,000); and Alexa488-Goat anti-mouse IgG (1:2,000). During the incubation period, light transmission was carefully avoided. Stained samples were then washed with distilled water for 1 min and mounted using Aqua poly/Mount (#18606, Polysciences, Inc., Warrington, PA). Stained samples were analyzed under a fluorescence microscope (Nikon C-SHG1, Nikon ECLIPSE E600; Nikon Co., Tokyo, Japan). BZ8000 fluorescence microscope (Keyence Co., Osaka, Japan) was used for deconvoluted fluorescence imaging in co-localization analyses.

Quantification of immunofluorescent pictures

Quantification of green (CXCR3) or red (CD19 and IP-10) fluorescing dots in the pictures was performed with the image analysis software, MetaMorph (Molecular Devices Co., Japan, Tokyo, Japan).

Plasma cytokine/chemokine/growth factor assay

Plasma samples were analyzed for IL-1β, IL-1RA, IL-2, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p40/p70, IL-13, IL-15, IL-17, TNF-α, IFN-α, IFN-γ, GM-CSF, MIP-1α, MIP-1β, IP-10, MIG, Eotaxin, RANTES, MCP-1, VEGF, G-CSF, EGF, FGF-basic and HGF using a Human Cytokine Thirty-plex antibody bead kit (BioSource Int. Inc., Camarillo, CA) according to the manufacturer's instructions. Assay results were obtained using a Luminex 100™ reader (Luminex Co., Austin, TX).

Statistics

Unpaired (two-tailed) Student's *t*-test was applied at 95% confidence level ($P < 0.05$) using Prism ver. 4 (GraphPad Software, Inc., San Diego, CA) in all cases.

Results and Discussion

Decreased frequency of peripheral CD27⁺ B cell in CHC

Two major B-cell subsets can be identified in human peripheral blood based on the expression of CD27, a member of the TNF receptor family. Functional differences between the two subsets have been extensively investigated and it is now generally accepted that CD27 is a memory B-cell marker (Agematsu and others 2000). As memory B cells are quickly activated upon antigenic stimulation and can rapidly produce high levels of immunoglobulins, we initially speculated that the frequency of CD27⁺ memory B cells is increased in the peripheral blood of patients with persistent HCV infection as a consequence of chronic antigenic stimulation by

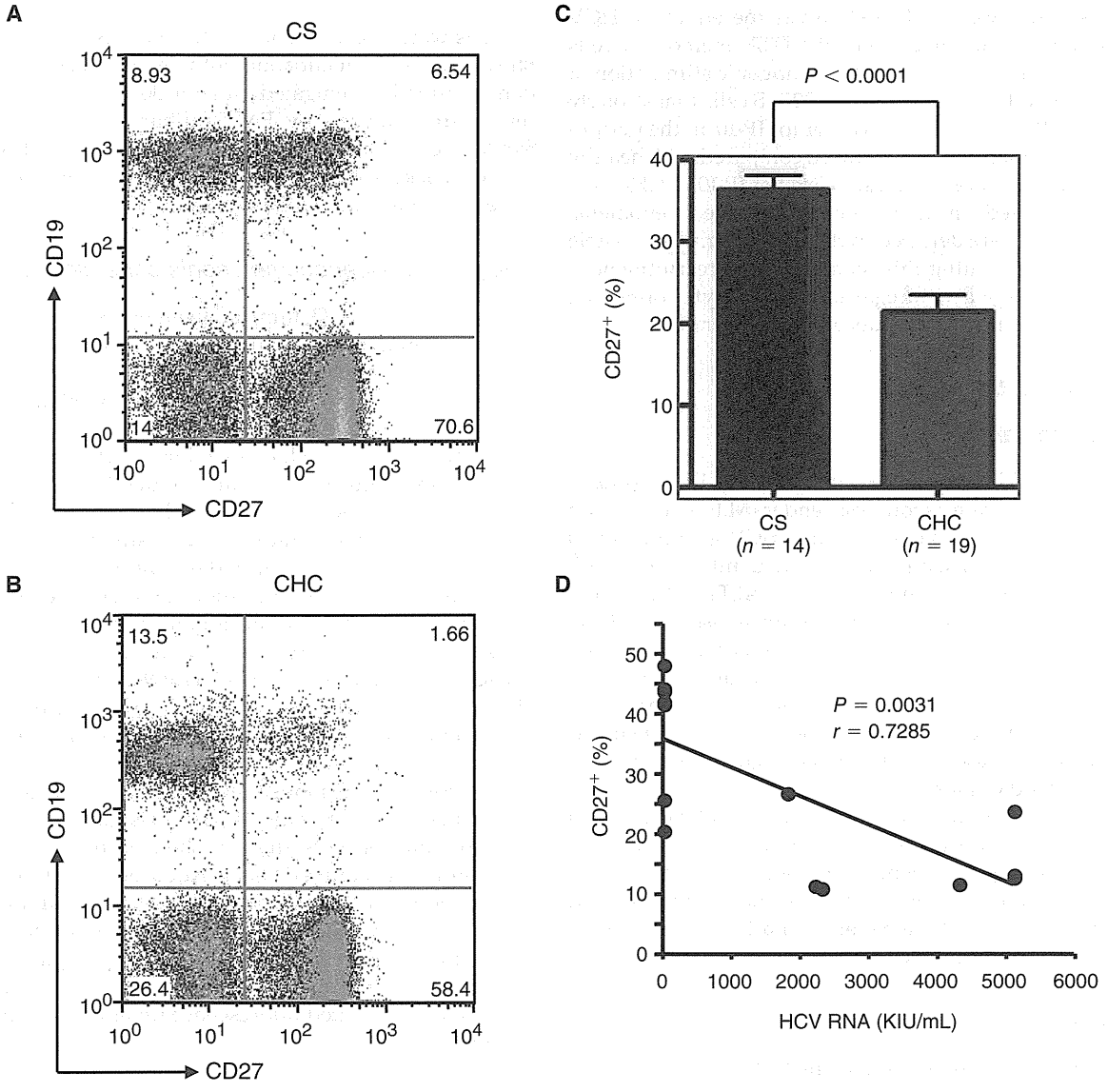

FIG. 1. Flow cytometry analysis of peripheral blood mononuclear cells (PBMCs) from control subjects (CS) and chronic hepatitis C patients (CHC). Lymphocytes were identified by forward and orthogonal light scatter characteristics. (**A**) Representative two-color (CD19 vs. CD27) analysis of lymphocytes in CS. (**B**) Representative two-color (CD19 vs. CD27) analysis of lymphocytes in CHC. (**C**) Percentage of CD27⁺ cells among gated CD19⁺ cells in CS ($n = 14$) and CHC ($n = 19$). (**D**) Correlation between plasma hepatitis C virus (HCV) RNA levels and percentages of CD27⁺ cells among gated CD19⁺ cells of 17 samples.

HCV antigens, such as the aforementioned E2 protein. We thus analyzed the frequencies of $CD27^+$ B cells in peripheral blood of control subjects (CS; $n = 14$) and chronic hepatitis C patients (CHC; $n = 19$) by flow cytometry.

Representative profiles (CD19 vs. CD27) of both CS and CHC are shown in Figure. 1A and 1B, respectively. It should be noted that the percentages of peripheral $CD19^+$ B cells in CHC were comparable to those of CS ($P = 0.7233$) (data not shown). In contrast to our expectations, the frequency of $CD27^+CD19^+$ B cells in CHC was lower than that in CS. As summarized in Figure 1C, it was concluded that the percentages of $CD27^+$ cells in $CD19^+$ B cells are significantly reduced ($P < 0.0001$) in CHC compared with CS.

Rosa et al. previously reported that HCV patients display a higher percentage of peripheral memory ($CD27^+$) B cells when compared with healthy controls (Rosa and others 2005). In contrast, Racanelli et al. found that the frequencies of $CD27^+$ B cells in persistently HCV-infected patients were significantly lower than those in healthy donors (Racanelli and others 2006). Ni et al. demonstrated that the frequencies of $CD27^+$ B cells in both normal subjects and HCV patients are comparable (Ni and others 2003). Among these controversial reports, our results are in good agreement with Racanelli and others (2006). In their report, patients with higher plasma viral loads had lower percentages of $CD27^+$ B cells, thus suggesting that high viral replication is associated with a reduction in $CD27^+$ B cells, although the negative correlation was not statistically significant in their study ($P = 0.0566$; $r = -0.4328$). In our study, the above negative correlation was statistically significant

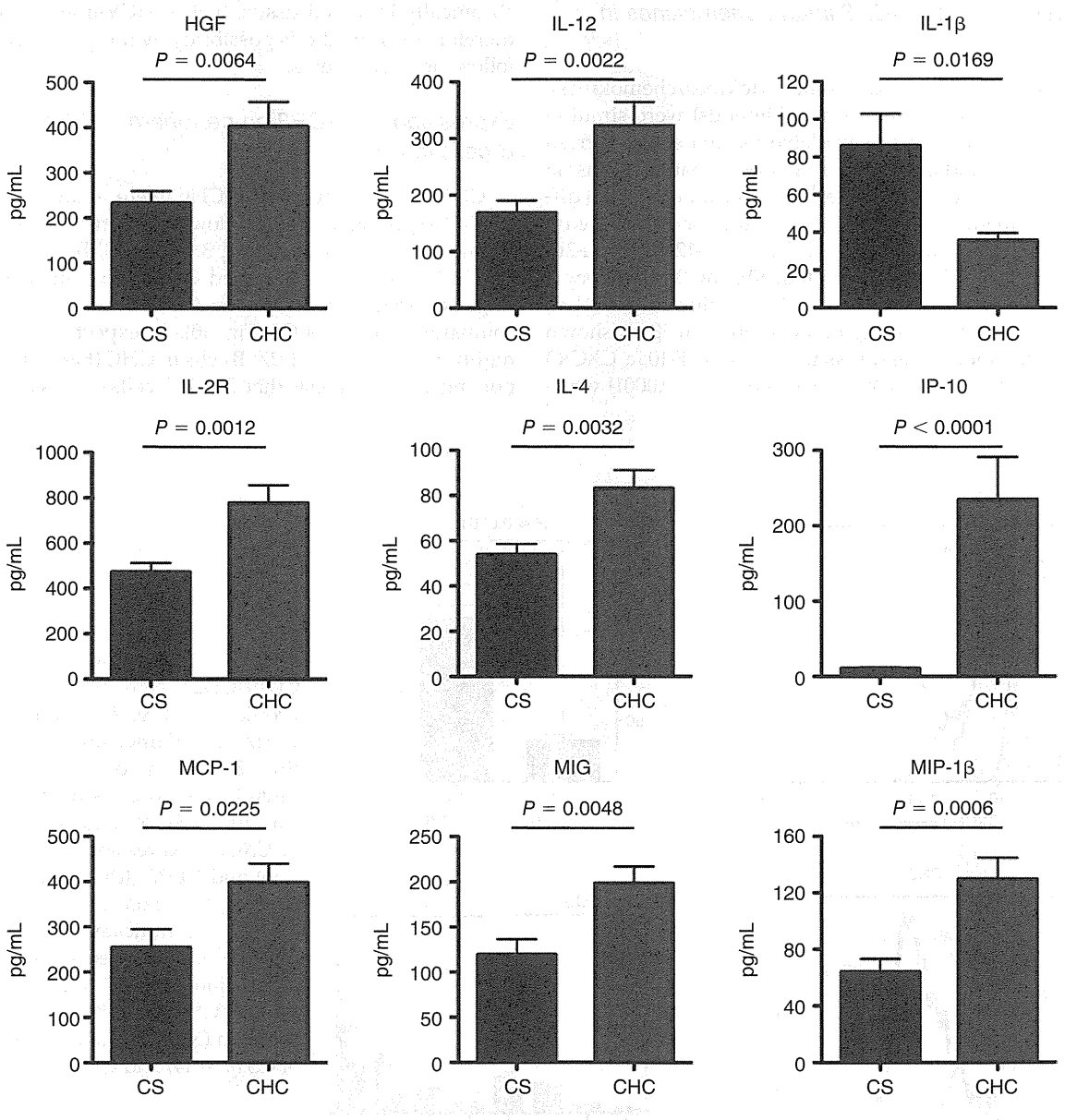

FIG. 2. Plasma levels of cytokine/chemokine/growth factors. The amounts of 30 different cytokines/chemokines/growth factors (see Materials and Methods) were simultaneously determined in the available plasma samples from both control subjects (CS; $n = 14$) and chronic hepatitis C patients (CHC; $n = 11$). Nine analytes that showed statistically significant ($P < 0.05$) differences between CS and CHC were indicated.

($P = 0.0031$; $r = -0.7285$; Fig. 1D) and thus agreeing with their suggestion.

Racanelli et al. hypothesized that under conditions of persisting HCV antigenemia, memory B cells not receiving specific B-cell receptor triggering before having T-cell help would be pushed to enhance Ig production and prone to apoptosis (Racanelli and others 2006), which may explain the reduction of $CD27^+$ memory B cells in HCV-infected patients. In subsequent experiments, we sought to examine an alternative possibility that the $CD27^+$ memory B cells are recruited from peripheral blood to the liver, where HCV infection triggers the production of IFN-γ and consequently induces attractants such as IFN-γ-inducible chemokines. We therefore examined plasma levels of various cytokines/chemokines/growth factors by a multiplex suspension bead array immunoassay.

Increased levels of CXCR3 ligand chemokines in plasma of CHC

The amounts of 30 different cytokines/chemokines/growth factors (see Materials and Methods) were simultaneously determined in the available plasma samples from both CS ($n = 14$) and CHC ($n = 11$; 11 out of 19 patients' plasma were available; Fig. 2). Statistically significant ($P < 0.05$) differences between the 2 groups were noted for the following analytes: HGF (hepatocyte growth factor), IL-12, IL-1β, IL-2R, IL-4, IP-10 (IFN-γ-inducible protein-10), MCP-1 (monocyte chemotactic protein-1), MIG (monokine induced by IFN-γ), and MIP (macrophage inflammatory protein)-1β as shown in Figure 2. Among these, plasma levels of IP-10, a CXCR3 ligand, in CHC were markedly elevated ($P < 0.0001$) when compared with CS. Plasma levels of another CXCR3 ligand, MIG, were also elevated ($P = 0.0048$).

These results prompted us to hypothesize that $CD27^+$ B cells express CXCR3 and that they migrate to inflammation sites in the liver of CHC, where the levels of IFN-γ are thought to be elevated and thus IP-10 is produced. It should be noted here that the enhanced expression of IP-10 mRNA has been demonstrated in the liver of CHC (Patzwahl and others 2001). The clinical significance of elevated serum levels of IP-10 in chronic HCV carriers has been already reported, and the levels of IP-10 in patient serum are regarded as a marker for therapeutic outcome (Narumi and others 1997; Itoh and others 2001; Patzwahl and others 2001). However, the physiological and immunological grounds for IP-10 production in HCV patients remain uncertain. If our predictions were correct, peripheral $CD27^+$ memory B cells in CHC would highly express CXCR3 and accumulate within chronically inflamed tissue, that is, HCV-infected liver. We therefore examined this possibility by flow cytometry in the following experiments.

Expression of CXCR3 on peripheral $CD27^+$ B cells in CHC

$CD19^+$ B cells in CS and CHC were analyzed for their CXCR3 expression levels by flow cytometry. Representative profiles are shown in Figure 3A and 3B. Both $CD27^-$ and $CD27^+$ B cells in CS expressed CXCR3 to some extent (Fig. 3A). Expression of CXCR3 on $CD27^-$ B cells in CHC was comparable to that of CS (Fig. 3B). As expected, CXCR3 was highly expressed on $CD27^+$ B cells in CHC (Fig. 3B), thus supporting our hypothesis that $CD27^+$ B cells expressing CXCR3

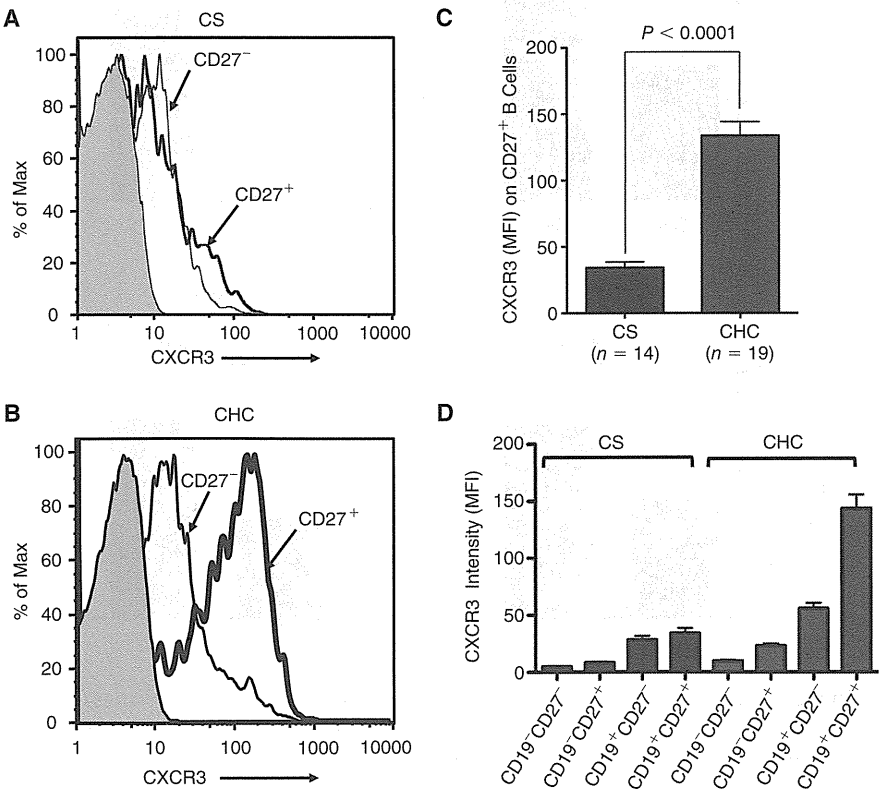

FIG. 3. Expression of CXCR3 on $CD19^+CD27^+$ cells. (**A**) Representative profile of CXCR3 expression on $CD27^-$ (thin line) and $CD27^+$ (thick line) B cells in control subjects (CS). Tinted line indicates negative control staining. (**B**) Representative profile of CXCR3 expression on $CD27^-$ (thin line) and $CD27^+$ (thick line) B cells in chronic hepatitis C patients (CHC). Tinted line indicates negative staining. (**C**) Summarized data of CXCR3 expression on $CD27^+$ B cells of CS ($n = 14$) and CHC ($n = 19$). (**D**) CXCR3 expression (MFI) of each cell population of CS ($n = 14$) and CHC ($n = 19$).

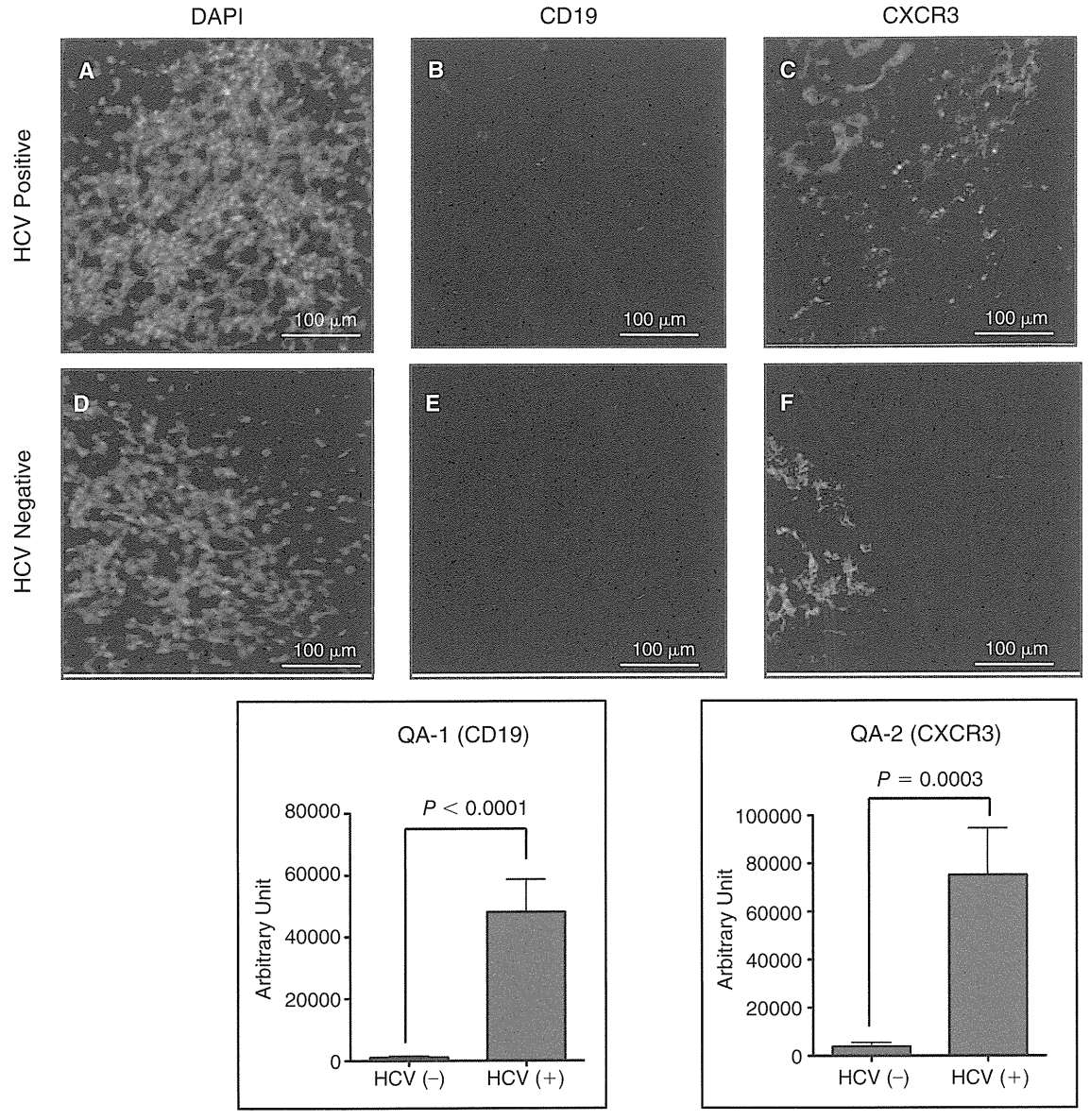

FIG. 4. Infiltration of B cells expressing CXCR3 to the chronic hepatitis C patient (CHC) liver. Panels **A–C**, **G**, and **H** are liver biopsy specimens of a patient who was chronically infected with hepatitis C virus (HCV). Panels **D–F**, **I**, and **J** are liver biopsy specimens of an alcohol-induced hepatitis patient who was HCV-negative. Specimens were stained with anti-CD19 (panels **B** and **E**), anti-CXCR3 (panels **C** and **F**), or anti-IP-10 (panels **H** and **J**) and analyzed as described in Materials and Methods. Panels **A**, **D**, **G**, and **I** are specimens stained with DAPI (4′,6-diamidino-2-phenylindole) to indicate cell nuclei. Panels QA-1 to QA-3 indicate the intensities (arbitrary unit) of red or green color dots quantified by the image analysis software, MetaMorph, as described in Materials and Methods. Data were collected from at least 10 fields of immunofluorescent pictures taken from 3 independent HCV-negative liver specimens and those of HCV-positive liver specimens. Error bars indicate SEM and *P* values were obtained by Student's *t*-test. Panels **K–N** are deconvoluted imagings of the merged (CXCR3 and IP-10) images taken from a liver specimen of a chronic hepatitis C patient. The white arrows indicate representative co-stained spots. Representative profiles from 5 independent analyses with similar results are shown.

are recruited to the liver, where levels of CXCR3 ligands, that is, IP-10 and MIG, are thought to be elevated. Figure 3C summarizes the expression levels of CXCR3 on $CD27^+$ B cells in both CS and CHC. The results shown in Figure 3D further confirmed that $CD27^+$ B cells of CHC express the highest amounts of CXCR3 among all the cell populations analyzed.

These results are compatible with a study by Muehlinghaus et al. in which CXCR3 was found to be expressed on a fraction of $CD27^+$ memory B cells but not on $CD27^-$ naïve B cells (Muehlinghaus and others 2005). Manz et al. proposed a possible mechanism for how the CXCR3-mediated recruitment of autoreactive memory B cells into inflamed tissues contributes to a break in self-tolerance (Manz and others 2006). As memory B cells have a lower threshold for antigenic activation and stably express CXCR3, which supports their accumulation within chronically inflamed sites, they may be resistant to the induction of peripheral tolerance.

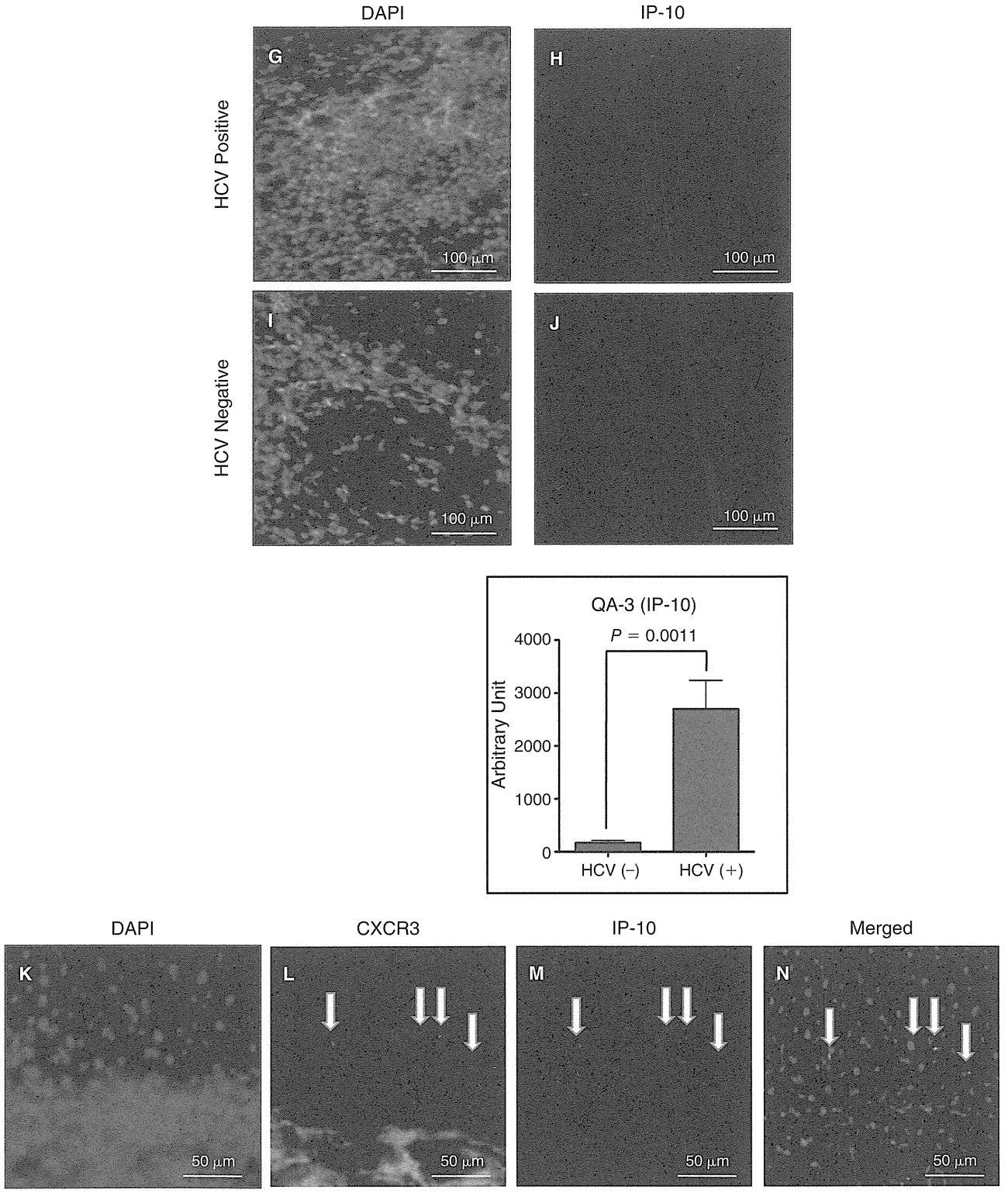

FIG. 4. (*Continued*)

Furthermore, in the inflamed tissue, inflammatory cytokines and autoantigens provide survival signals that promote plasma cell differentiation and survival, which may result in chronic inflammation and progressive tissue destruction. The present results are in line with this scenario when the HCV E2 protein is regarded as an autoantigen.

MC, a chronic autoimmune disorder, is usually associated with chronic liver inflammation by HCV infection (Dore and others 2007; Galossi and others 2007; Zignego and others 2007). Excessive production of antibodies known as RF is the main cause of MC. The fact that the majority of circulating B cells of patients with HCV-associated MC are

of the CD27+ phenotype (Carbonari and others 2005) further supports the contribution of memory B cells to extrahepatic autoimmune disorders.

Migration of B cells expressing CXCR3 into the CHC liver

In order to directly assess the migration of B cells expressing CXCR3 to the liver, immunohistochemical analyses were carried out by staining liver biopsy specimens. Unfortunately, as we could not obtain the biopsy specimens from the CHC analyzed in the above experiments, they were collected from other chronic hepatitis C patients. Liver biopsy specimens of alcohol-induced HCV-negative hepatitis patients instead of normal subjects were used as controls, since we could not obtain normal liver biopsy specimens.

As shown in Figure 4, the infiltration of CD19+ B cells were recognized in the liver of a HCV-positive hepatitis C patient, and the distribution of the CD19+ cells in the liver was almost identical to that of CXCR3+ cells (panels B vs. C). These CXCR3+ B-cell infiltrations were not observed in an HCV-negative alcohol-induced hepatitis patient specimen (panels E and F). Five HCV-positive and three HCV-negative independent liver biopsy specimens were analyzed, and similar staining profiles as shown in the figure were obtained. As expected, IP-10 was expressed in the liver of a CHC (panel H) but not in an HCV-negative specimen (panel J). Figure 4K–4N indicated that CXCR3+ cells are localized at the sites where IP-10 is expressed positive. Together, these results are concordant with our hypothesis that CD27+ B cells expressing CXCR3 migrate into the liver of PIP.

In this study, it was demonstrated that significant reductions in peripheral CD27+ B cells are seen in CHC, while plasma levels of IP-10, a CXCR3 ligand, are markedly increased in CHC. Furthermore, it was found that the CD27+ B cells highly express the CXCR3. In accordance with these phenomena, immunohistochemical analyses revealed that CXCR3+ B cells migrate in the liver of CHC where IP-10 is produced.

Finally, it is worth mentioning here that the recruitment of peripheral memory B cells into inflamed tissue may not be unique to CHC but rather is a general phenomenon. A recent report by Hansen et al. showed accumulation of memory B cells in the inflamed salivary glands of Sjoegren's syndrome patients (Hansen and others 2002), and Malbran et al. found that peripheral CD27+ memory B cells are reduced in patients with X-linked lymphoproliferative disease (Malbran and others 2004). Further investigation into the dynamics of memory B cells in chronically HCV-infected patients will be necessary, not only to better understand the HCV pathogenesis in particular, but also to gain insight into immunological disorders such as tolerance breakdown in general.

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Author Disclosure Statement

The authors have no conflicting financial interests.

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Functional phenotypes and gene expression profiles of peripheral blood mononuclear cells in chronic hepatitis C patients who developed non-Hodgkin's B-cell lymphoma

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ABSTRACT

Epidemiological data have indicated a close relationship between chronic HCV infection and non-Hodgkin's B-cell lymphoma (B-NHL). In this study, functional phenotypes and gene expression profiles of PBMCs were analyzed in chronic hepatitis C (CHC) patients who developed B-NHL. The frequencies of effector $CD8^+$ T cells and cytotoxic natural killer cells increased in CHC patients with B-NHL compared to those in CHC patients without B-NHL. These phenotypic changes may reflect the host's immune response to neoplasia. The mRNA expression levels of several oncogenes increased in CHC patients without B-NHL, but were much higher in CHC patients with B-NHL, while mRNA levels of type I IFNs were decreased in CHC patients without B-NHL and were nearly negligible in CHC patients with B-NHL. Interestingly, the mRNA expression levels of activation-induced cytidine deaminase and caspase recruitment domain-containing proteins markedly increased in CHC patients without B-NHL but decreased in CHC patients with B-NHL. These results are discussed in view of the possible involvement of HCV infection in B-cell lymphomagenesis.

Introduction

Hepatitis C virus (HCV) is an enveloped positive-stranded RNA virus belonging to the *Flaviviridae* family. It has infected nearly 200 million people worldwide and is therefore an important public health problem [1]. The liver is considered to be the primary target of HCV infection, which is also associated with a number of extrahepatic manifestations, such as mixed cryoglobulinemia, rheumatoid factor production, Sjogren-like syndrome, and B-cell lymphoproliferative disorders that may develop into overt non-Hodgkin's B-cell lymphoma (B-NHL) [2]. Recent epidemiological data have suggested a close relationship between chronic HCV infection and B-NHL [3]; however, experimental data concerning these conditions remain elusive. The aim of the present study was to compare the functional phenotypes and gene expression profiles of PBMCs between CHC patients without B-NHL and those with B-NHL. Results of this study suggest the possible involvement of HCV infection in the progression of B-NHL.

Materials and methods

Patients and PBMCs. Thirteen healthy subjects, 19 CHC patients without B-NHL, and 13 CHC patients with B-NHL were enrolled in this study. Demographic and clinical data were as follows: (1) CHC patients without B-NHL ($n = 19$): gender (M:F = 10:9); mean age (range) = 59.6 years (38–79 years); mean alanine aminotransferase (ALT) (IU/L) (SD) = 64.4 (23.5); mean aspartate aminotransferase (AST) (IU/L) (SD) = 61.3 (19.3); HCV genotype (No.) = 1b (18) and 2b (1); and mean HCV RNA titer = 2077 KIU/mL. (2) CHC patients with B-NHL ($n = 13$): gender (M:F = 7:6); mean age (range) = 73.1 years (52–85 years); ALT (IU/L) (SD) = 28.5 (12.4); AST (IU/L) (SD) = 32.4 (11.2); HCV genotype (No.) = 1b (13); and mean HCV RNA titer = 1288 KIU/mL. All CHC patients were confirmed to be negative for other viral infections, including hepatitis B virus and human immunodeficiency virus infections. Study protocols were approved by the review board at the National Institute of Infectious Diseases and Jikei University School of Medicine. All donors gave written informed consent. PBMCs were isolated by Fi-

Abbreviations: AID, activation-induced cytidine deaminase; CARD, caspase recruitment domain; CHC, chronic hepatitis C; HCV, hepatitis C virus; B-NHL, B-cell non-Hodgkin's lymphoma; NK, natural killer; PBMCs, peripheral blood mononuclear cells.

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coll-HyPaque (Pharmacia Biotech, Quebec, Canada) gradient centrifugation.

Flow cytometry. The following fluorescence-conjugated Abs were used for flow cytometry: Peridinin–chlorophyll–protein complex-anti-CD3 (Cat. 347344, BD Biosciences, San Jose, CA), APC-anti-CD8 (Cat. IM2469, Beckman Coulter, Fullerton, CA), PE-anti-CCR7 (Cat. FAB197P, R&D Systems, Minneapolis, MN), FITC-anti-CD45R (Cat. A07786, Beckman Coulter), and APC-anti-CD56 (Cat. IM2474, Beckman Coulter). Cells were washed twice with cold PBS containing 0.2% BSA and incubated with an appropriate combination of conjugated Abs for 30 min on ice. Stained cells were analyzed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). Data were collected using CellQuest software (Becton Dickinson, San Jose, CA) and analyzed using FlowJo software (Tree Star, Inc., Ashland, OR).

Semiquantitative real-time PCR. Total RNA was extracted from lymphoid cells using Isogen (Nippon Gene Co., Ltd., Tokyo, Japan). cDNA was synthesized using SuperScript™ III reverse transcriptase (Invitrogen, Carlsbad, CA) with oligo(dT) 12–18 primers (Invitrogen). PCR amplification was performed using SYBR Premix Ex Taq™ II (TAKARA Shuzo, Kyoto, Japan) with gene-specific primers (Bex Co., Ltd., Tokyo, Japan) available in the public database RTPrimerDB under the following entry codes (in parentheses): CCND2 (803), AID (2285), IFNA (3541), IFNB (3542), FN1 (3523), LMO2 (693), and GAPDH (3539); real-time PCR primer sets (http://www.realtimeprimers.org/): CCND1 (JS-209); and the Universal ProbeLibrary Assay Design Center (http://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp; Roche Applied Science): MYC (#34, 04687671001), TP53 (#58, 04688554001), MLLT3 (#25, 04686993001), serine/threonine kinase 15 (STK15; #79, 04689020001), FHIT (#33, 04687663001), CASP1 (#17, 04686900001), and CASP4 (#29, 04687612001). The sequences of primers for RIG-I were 5′-GTG CAA AGC CTT GGC ATG T-3′ (forward) and 5′-TGG CTT GGG ATG TGG TCT ACT C-3′ (reverse).

Real-time PCR was performed for 45 cycles at 94 °C for 1 min and 60 °C for 25 s (two-step) using a Light Cycler (Roche Diagnostics, Basel, Switzerland). Amplification of predicted fragments was confirmed by melt curve analysis and gel electrophoresis. Standard curves were obtained with 10-fold serial dilutions of the amplified products. Measured amounts of mRNA were normalized against

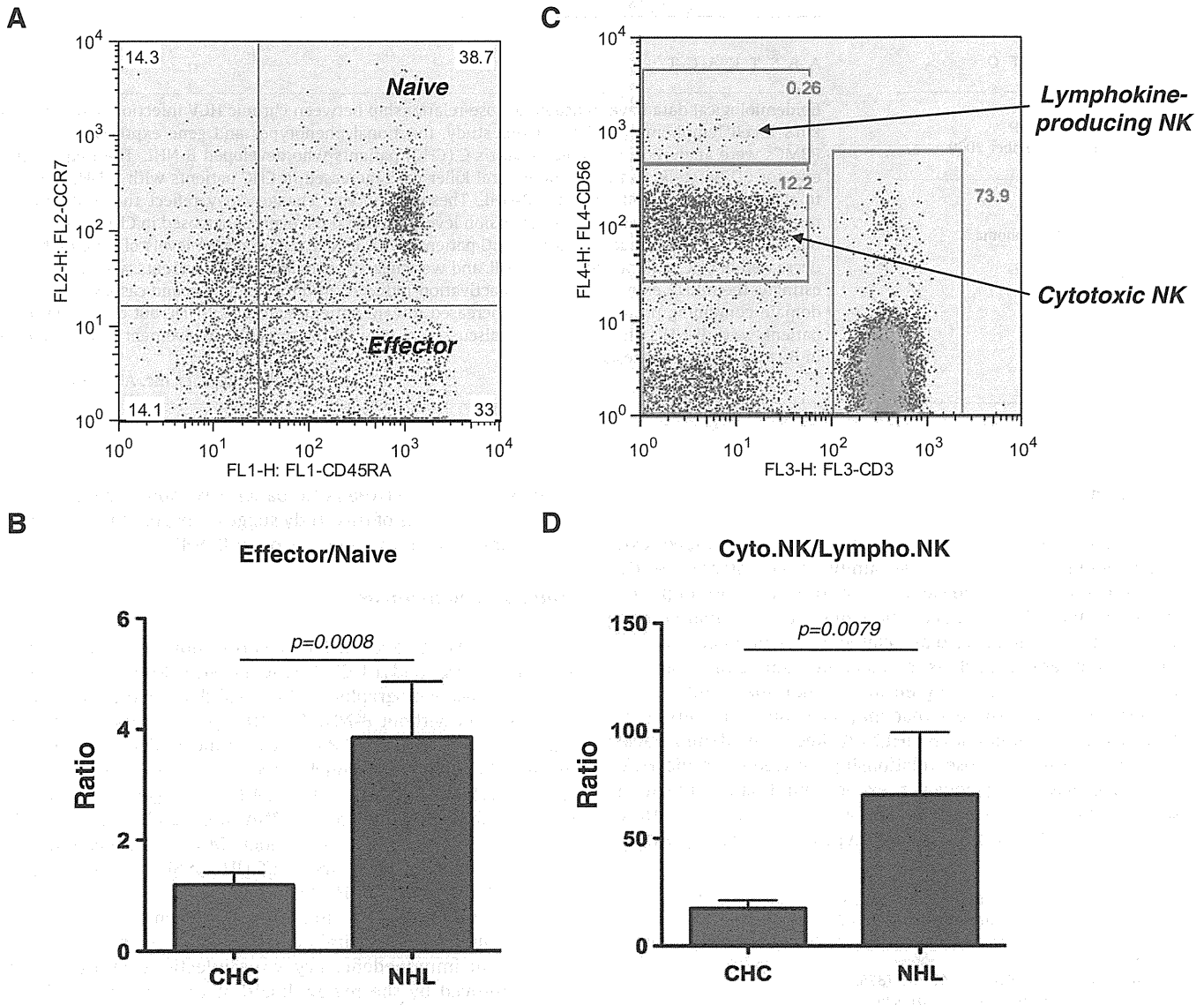

Fig. 1. Phenotypic analysis of PBMCs. (A) $CD3^+CD8^+$cells ($CD8^+$ T cells) were analyzed by 2-color flow cytometry (CD45RA vs. CCR7). Fractions of naïve $CD8^+$ T cells ($CD45RA^+CCR7^+$) and effector $CD8^+$ T cells ($CD45RA^+CCR7^-$) are shown. (B) Ratios of effector $CD8^+$ T cells to naïve $CD8^+$ T cells in CHC patients without B-NHL ($n = 19$) and CHC patients with B-NHL (referred to as NHL; $n = 7$) are shown. Error bars indicate SEM. (C) Lymphocytes were analyzed by 2-color flow cytometry (CD3 vs. CD56). The $CD56^{dim}$ fraction (cytotoxic NK cells) and $CD56^{bright}$ fraction (lymphokine-producing NK cells) are shown. (D) Ratios of cytotoxic NK cells ($CD56^{dim}$) to lymphokine-producing NK cells ($CD56^{bright}$) in CHC patients without B-NHL ($n = 19$) and CHC patients with B-NHL (referred to as NHL; $n = 7$) are shown. Error bars indicate SEM.

the amounts of GAPDH mRNA. The mRNA expression levels of normal PBMCs were arbitrarily defined as 1.0.

Statistics. Unpaired two-tailed Student's *t* tests at the 95% confidence level ($p < 0.05$) were applied in all cases using Prism ver.4 (GraphPad Software, Inc., San Diego, CA).

Results and discussion

Functional phenotypes of PBMCs in CHC patients with or without B-NHL

Based on CD45RA and CCR7 expressions, $CD8^+$ T cells are classified into four subsets: naïve cells ($CD45RA^+CCR7^+$), central memory cells ($CD45RA^-CCR7^+$), effector memory cells ($CD45RA^-CCR7^-$), and effector cells ($CD45RA^+CCR7^-$), as shown in Fig. 1A [4]. The ratio of effector $CD8^+$ T cells to naïve $CD8^+$ T cells markedly increased in CHC patients with B-NHL compared to that in CHC patients without B-NHL, as shown in Fig. 1B. These results suggest that phenotypic changes, i.e., naïve to effector, occurred in PBMCs during the course of B-NHL development.

Human natural killer (NK) cells constitute approximately 15% of PBMCs and are divided into two subsets based on the cell surface density of CD56, i.e., $CD56^{bright}$ and $CD56^{dim}$ [5], as shown in Fig. 1C. There is ample evidence to suggest that these NK cell subsets have unique functional attributes and distinct roles in human immune responses [6]. The $CD56^{dim}$ NK subset has higher cytotoxicity than the $CD56^{bright}$ subset, which is capable of producing abundant lymphokines [5]. The ratio of cytotoxic NK cells ($CD56^{dim}$) to lymphokine-producing NK cells ($CD56^{bright}$) markedly increased in CHC patients with B-NHL compared to that in CHC patients without B-NHL, as shown in Fig. 1D.

Taken together, these phenotypic changes in PBMCs of CHC patients with or without B-NHL may be attributed to the development of B-NHL and may thus reflect the host's immune response to neoplasia.

Gene expression profiles of PBMCs in CHC patients with or without B-NHL

Our preliminary analyses of the dynamics of innate immune-related gene expressions in PBMCs demonstrated that the mRNA expression levels of type I IFN, i.e., IFN-α and IFN-β, decreased significantly, while those of caspase recruitment domain (CARD)-containing proteins, such as RIG-I, caspase 1, and caspase 4, markedly increased in CHC patients without B-NHL compared to normal subjects. The mRNA expression levels of various lymphomagenesis-related genes were also analyzed, and the significant increase in the mRNA expression levels of AID in CHC patients was of particular interest. These levels were thought to be closely associated with the occurrence of B-NHL [7]. In this study, the mRNA expression levels of interest were those of the above-mentioned genes in PBMCs of CHC patients with or without B-NHL. Relative mRNA expression levels of 15 genes in PBMCs were analyzed (Fig. 2), and significant differences were observed between the three groups: normal, CHC patients without B-NHL, and CHC patients with B-NHL. Interestingly, there seemed to be three distinct categories in terms of mRNA expression level dynamics: (A) upregulated in CHC patients without B-NHL and further more in CHC

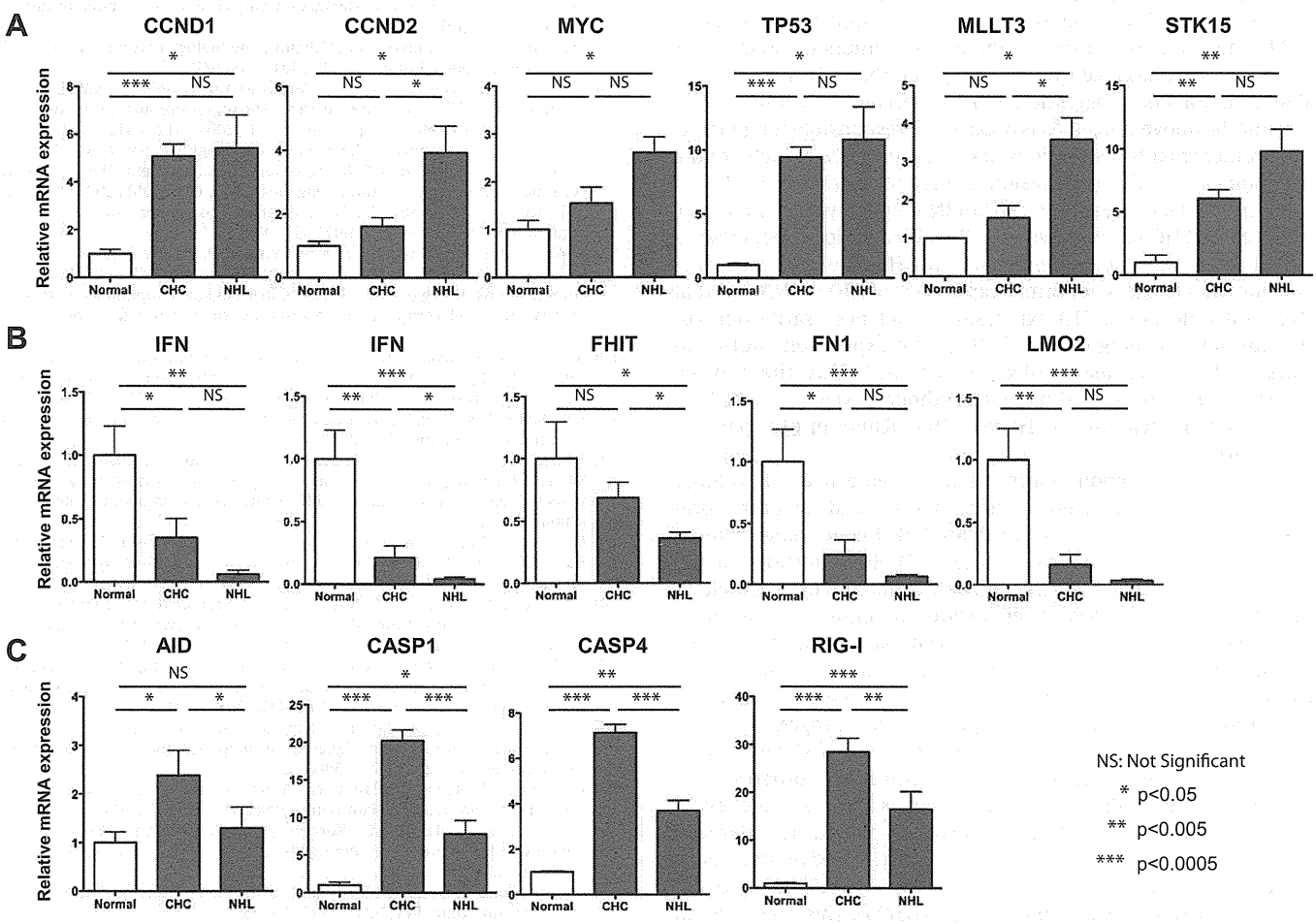

Fig. 2. Gene expression profiles of PBMCs. mRNA expression levels of 15 genes in normal subjects ($n = 7$), CHC patients without B-NHL ($n = 7$), and CHC patients with B-NHL (referred to as NHL; $n = 7$) are shown. mRNA expression dynamics are categorized into A, B, and C (see text). Error bars indicate SEM.

patients with B-NHL, (B) downregulated in CHC patients without B-NHL and further more in CHC patients with B-NHL, and (C) upregulated in CHC patients without B-NHL and downregulated to normal levels in CHC patients with B-NHL.

Oncogenes and proto-oncogenes are included in category A in which gene expression levels are highly upregulated in CHC patients with B-NHL. Overexpression of CCND1, which alters cell cycle progression, is frequently observed in various tumors and may contribute to tumorigenesis [8]. Furthermore, CCND2 is known to be expressed at constitutively high levels in B-NHL [9] and may thus be correlated with B-cell lymphomagenesis. It is reported that STK15 is strongly expressed in histologically aggressive NHL [10]. Taken together, these results suggest that upregulation of these genes is responsible for the development of B-NHL.

In contrast, the mRNA expression levels of type I IFN, i.e., IFN-α and IFN-β, markedly decreased in CHC patients without B-NHL and were almost negligible in CHC patients with B-NHL (category B). This downregulated type I IFNs gene expression may be due to strategies employed by HCV to evade antiviral innate immune responses in the host [11]. Expression of fragile histidine triad (FHIT), fibronectin 1 (FN1) and LIM domain only 2 (LMO2) mRNAs was also downregulated in CHC without B-NHL and further downregulated in CHC with B-NHL. Decreased expression of FHIT is correlated with worse prognosis in CHC patients with B-NHL [12]. Lossos et al. reported that augmented expression of FN1 and LMO2 is correlated with prolonged survival in patients with DLBCL [13]. Thus, downregulation of these genes in CHC patients may be correlated with B-NHL malignancy.

Up- and downregulation of mRNA expression was noted in AID and CARD-containing proteins, including CASP1, CASP4, and RIG-I (category C). AID is essential for both somatic hypermutation (SHM) and class switch recombination of immunoglobulin genes in B cells [14]. It has recently been proposed that AID is instrumental in initiation and progression of B-NHL because a malfunction in either of the above processes is apparently responsible for generating chromosomal translocations and aberrant SHM, which are the two main causes of genetic lesions associated with B-NHL [15].

Interestingly, expression of AID mRNA, which was significantly enhanced in CHC patients without B-NHL, was downregulated to normal levels in CHC patients with B-NHL. We speculate that HCV infection triggers abnormal expression of AID mRNA and that after B-NHL develops, AID expression is not necessarily upregulated further. Downregulation of AID mRNA expression in CHC patients with B-NHL may reflect lower HCV RNA titers when compared to those of CHC patients without B-NHL (2077 KIU/mL in CHC patients without B-NHL vs. 1288 KIU/mL in CHC patients with B-NHL).

CARDs are interaction motifs found in a wide array of proteins, particularly those involved in inflammatory and apoptotic processes [16]. Caspases 1 and 4, which are CARD-containing proteins, are classified as inflammatory caspases [17]. HCV infection is a major cause of liver disease characterized by inflammation, cell damage, and fibrotic reactions of hepatocytes; apoptosis has also been implicated in the pathogenesis. Enhanced expression of both caspase mRNA may be a consequence of HCV infection in CHC patients without B-NHL. On the other hand, both caspases are also involved in apoptosis and thought to suppress tumor progression [18]. Therefore, reduced mRNA expression of CASP1 and CASP4 in CHC patients with B-NHL may be correlated with B-NHL progression.

RIG-I, another CARD-containing protein, is a cytoplasmic sensor molecule for dsRNA, including HCV RNA, and plays a critical role in antiviral innate immune responses [19]. RIG-I mRNA expression increased in CHC patients without B-NHL ($p < 0.0005$) and decreased in CHC patients with B-NHL ($p < 0.005$). This may account for the fact that enhanced RIG-I expression results in augmentation of antitumor activity by producing inflammatory cytokines and inducing apoptosis [20].

In conclusion, the present study indicates noticeable alterations in both functional phenotypes and gene expression profiles between PBMCs of CHC patients without B-NHL and those of CHC patients with B-NHL. Our results support the notion that HCV infection is at least partly responsible for the development of B-NHL in CHC patients.

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Differential susceptibility of peripheral blood CD5$^+$ and CD5$^-$ B cells to apoptosis in chronic hepatitis C patients

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ABSTRACT

A body of evidence has suggested a close link between chronic hepatitis C virus (HCV) infection and B cell abnormalities, including mixed cryoglobulinemia, rheumatoid factor (RF) production, and lymphoproliferative disorders that may develop into non-Hodgkin's lymphoma. Recent studies have demonstrated the expansion of CD5$^+$ B cells in the peripheral blood of chronic hepatitis C patients (CHC). As CD5$^+$ B cells, which are capable of producing autoantibodies and RF, are apparently crucial for the development of HCV-associated pathogenesis, the fate of both the CD5$^+$ and CD5$^-$ B cell subsets upon chronic HCV infection is of interest. In this study, the degree to which chronic HCV infection induces apoptosis in each B cell subset was investigated. Our results demonstrated that peripheral CD5$^-$ B cells were more susceptible to apoptosis than CD5$^+$ B cells in CHC. Furthermore, plasma levels of IL-4, IL-10, and IL-12 were significantly elevated in CHC, thus suggesting that these interleukins protect CD5$^+$ B cells from apoptosis. The rationale for the differential susceptibility of distinct B cell subsets in CHC is also discussed with regard to extrahepatic manifestations associated with chronic HCV infection.

Introduction

Hepatitis C virus (HCV) infection is one of the major causes of chronic liver disease, affecting nearly 200 million people worldwide [1]. Although the liver is considered to be the primary target, HCV infection is also associated with extrahepatic manifestations such as mixed cryoglobulinemia (MC), rheumatoid factor (RF) production and B cell lymphoproliferative disorders that may develop into overt non-Hodgkin's B-lymphoma (B-NHL) [2]. However, the pathogenic relationship between HCV infection and these immunological disorders remains uncertain.

The CD5$^+$ B cell subset identified in early 1980s [3] has attracted considerable interest because of its association with autoimmune pathology [4]. CD5$^+$ B cells are the predominant B cell population in the fetus but are rare in adults, and seem to constitute a primitive but effective first line of defense against foreign pathogens [5]. The expansion of CD5$^+$ B cells in rheumatoid arthritis [6], Sjoegren syndrome [7] and MC [8] has also implicated them in the development of autoimmune disorders. Interestingly, these disorders seem to be closely correlated with HCV infection [9]. Curry et al. recently demonstrated the expansion of CD5$^+$ B cells in peripheral blood from chronic hepatitis C patients (CHC) [10] and within the liver of non-cirrhotic CHC [11]. These results strongly suggest a correlation between HCV pathogenesis and the expansion of peripheral CD5$^+$ cells; however, the mechanisms underlying this CD5$^+$ B cell subset-restricted expansion upon HCV infection remain unknown.

In this study, we compared the susceptibility of the peripheral CD5$^+$ and CD5$^-$ B cell subsets to apoptosis in both normal subjects and CHC. Our results demonstrated the differential susceptibility to apoptosis between the two B cell subsets in CHC but not in normal subjects. Enhanced levels of anti-apoptotic cytokines in CHC plasma may be responsible for the apoptosis resistance of CD5$^+$ B cell in CHC.

Materials and methods

Patients and samples. A total of 25 CHC were enrolled in this study. Demographic and clinical data were as follows: gender (M:F) = 15:10; mean age (range) = 60.8 years (44–80 years); HCV genotype (No.) = 1b (15) and 2a (10); mean HCV RNA (KIU/mL) (SD) = 1836 (1506); mean alanine aminotransferase (ALT) (U/L) (SD) = 57.3 (18.1); and mean aspartate aminotransferase (AST) (U/L) (SD) = 47.3 (17.7). The study protocols were approved by the Review Board at the National Institute of Infectious Disease. All donors gave written informed consent before phlebotomy. A

Abbreviations: B-NHL, non-Hodgkin's B-lymphoma; CHC, chronic hepatitis C patients; HCV, hepatitis C virus; MC, mixed cryoglobulinemia; RF, rheumatoid factor.

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total of 15 normal control subjects included anonymous volunteer blood donors visiting the Japanese Red Cross Blood Center (Tokyo, Japan), who were confirmed to be negative for HCV, HBV, and HIV.

Clinical tests. HCV genotype was determined by PCR of the core region with genotype-specific primers [12]. HCV RNA was quantified by the Roche Amplicor assay (Roche Diagnostics, Branchberg, NJ), and results were standardized to international units (IU). Determination of serum levels of ALT and AST was performed using standard methods.

Flow cytometry. The following fluorescence-conjugated antibodies (Abs) were used for flow cytometry: allophycocyanin-anti-CD19 (MHCD1905; Invitrogen, Carlsbad, CA); and PerCP-Cy5.5-anti-CD5 (Cat. 341089; BD Biosciences, San Jose, CA). Cells were washed twice with cold PBS containing 0.2% BSA, followed by incubation with an appropriate combination of directly conjugated Abs for 30 min on ice. Stained cells were analyzed by FACSCallibur (Becton Dickinson, San Jose, CA). Data were collected using CellQuest software (Becton Dickinson, San Jose, CA) and were analyzed using FlowJo software (Tree Star Inc., Ashland, OR).

Assessment of apoptotic cell death. Levels of Annexin V binding to both $CD5^+$ and $CD5^-$ B cells were assessed with a commercially available Annexin V apoptosis detection kit Annexin V-FITC (PN IM3546, Beckman Coulter, Fullerton, CA) according to the manufacturer's instructions.

Plasma cytokine assay. Plasma samples were analyzed for IL-2, IL-4, IL-5, IL-10, IL-12p40/p70, IL-13, IFN-γ, and TNFα using a Human Cytokine Thirty-plex antibody bead kit (BioSource Int. Inc., Camarillo, CA) according to the manufacturer's instructions. Assay results were obtained using a Luminex 100™ reader (Luminex Co., Austin, TX).

Statistics. Unpaired (two-tailed) Student's *t*-test was applied at the 95% confidence level ($p < 0.05$) using Prism ver.4 (GraphPad Software, Inc., San Diego, CA) in all cases.

Results and discussion

Increased frequency of peripheral blood $CD5^+$ B cells in CHC

The frequencies of peripheral blood $CD19^+$ cells (B cells) and $CD5^+$ cells (mostly T cells) were comparable between normal subjects and CHC, as shown in Fig. 1A and B, respectively. Although CD5 is primarily a pan-T cell marker, a particular B cell subset, termed B-1 or B-1a cells, expresses the CD5 molecule [13,14]. When the percentages of peripheral $CD5^+$ cells in $CD19^+$ cells was analyzed, a significant increase was noticed in CHC when compared to normal subjects (Fig. 1C). It was also verified that the frequencies of peripheral $CD5^+CD19^+$ cells were significantly elevated in CHC (Fig. 1D). These results were concordant with a previous study by Curry et al., in which they concluded that immune complex formation by expanded $CD5^+$ B cells may limit the development of progressive liver disease [10].

$CD5^+$ B cells, which are characterized by the production of low-affinity IgM with RF activity [5], have been shown to expand in patients with MC [8] and in those with Sjoegren's syndrome [7]. Interestingly, the correlation between chronic HCV infection and the above-mentioned manifestations has been widely appreciated [15]. Therefore, we aimed to investigate the fate of both the $CD5^+$ and $CD5^-$ B cell subsets upon chronic HCV infection.

Differential susceptibility of peripheral blood $CD5^+$ and $CD5^-$ B cells to apoptosis in CHC

The levels of spontaneous apoptosis among peripheral blood $CD5^+$ and $CD5^-$ B cells in both normal subjects and CHC were analyzed using three-color flow cytometry by staining with allophycocyanin-anti-CD19, PerCP-Cy5.5-anti-CD5 and Annexin V-FITC. Representative staining data from experiments analyzing eight normal subjects and ten CHC with similar results are shown in Fig. 2A and B, respectively. The patterns of Annexin V binding were almost identical between $CD5^+$ and $CD5^-$ B cells in normal subjects (Fig. 2A). In contrast, as shown in Fig. 2B, $CD5^-$ B cells bound to much larger amounts of Annexin V than $CD5^+$ B cells in CHC. The percentages of each cell subset bound to large amounts of Annexin V are shown in Fig. 2C (the cut-off point was tentatively set at a fluorescence intensity of 2000). It was concluded that, $CD5^-$ B cells were more vulnerable to apoptosis than $CD5^+$ B cells upon HCV infection; in other words, $CD5^+$ B cells were apparently resistant to apoptosis.

Elevation of anti-apoptotic cytokine levels in CHC plasma

A number of cytokines, including IL-2, IL-4, IL-10, IL-12p40/p70, IL-13, IFN-γ, and TNFα, are known to suppress apoptosis of leukemic $CD5^+$ B cells, and may be closely involved in the pathogenesis

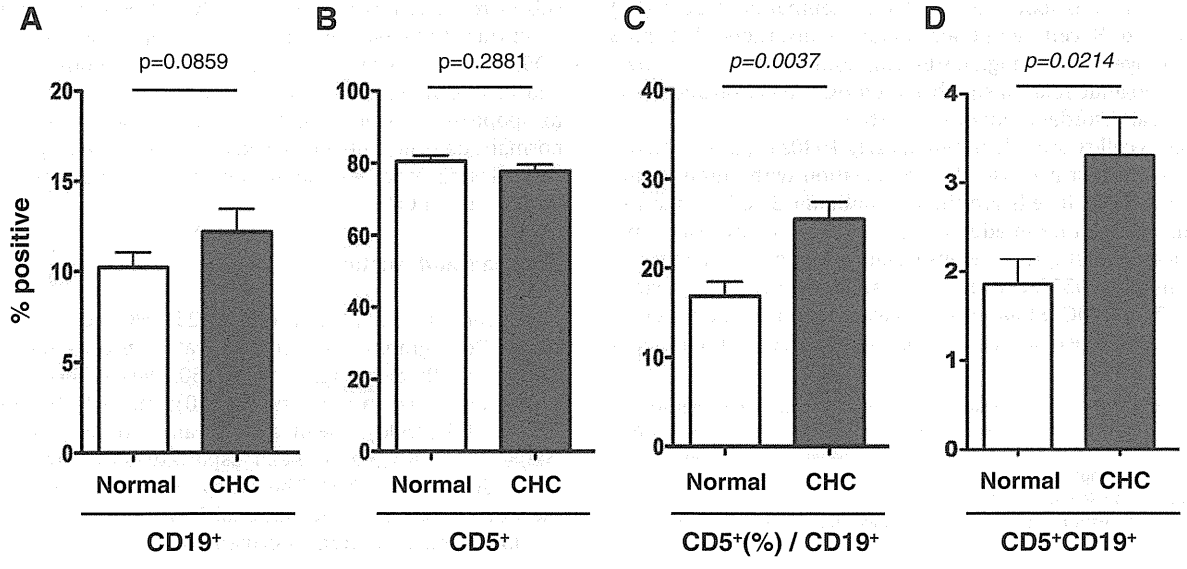

Fig. 1. Flow cytometric analysis of PBMC from normal subjects and CHC. Lymphocytes were identified by forward and orthogonal light scatter characteristics. Percentages of $CD19^+$ cells (A), $CD5^+$ cells (B), $CD5^+$ (%) in $CD19^+$ cells (C), and $CD5^+CD19^+$ cells (D) in normal ($n = 10$) and CHC ($n = 25$) are shown with SEM bars and *p*-values.

of chronic lymphocytic leukemia (B-CLL) [16,17]. Some anti-apoptotic cytokines, i.e., IL-4 and IL-10, have been shown to protect cord blood $CD5^+$ B cells from apoptosis [18,19]. These findings prompted us to examine the cytokine levels in CHC plasma. Our assumption was that anti-apoptotic cytokine levels are elevated in CHC, thereby preventing apoptosis of $CD5^+$ B cells. As shown in Fig. 3A, B, and C, plasma levels of IL-4, IL-10, and IL-12 were significantly elevated in CHC when compared with normal subjects, which supported our prediction. In addition, plasma levels of other anti-apoptotic cytokines for leukemic $CD5^+$ B cells, i.e., IL-2, IL-13, IFN-γ, and TNFα,

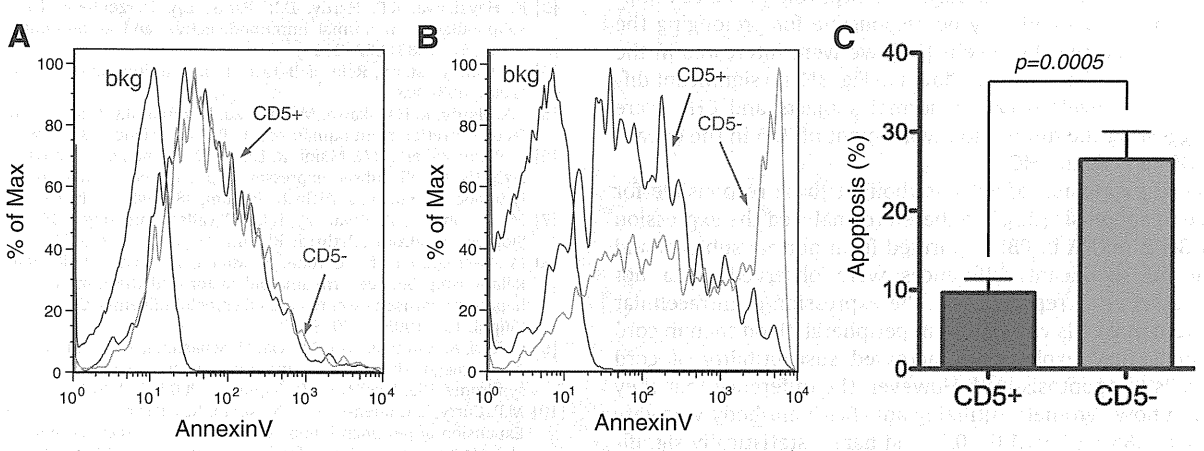

Fig. 2. Annexin V binding to $CD5^+$ and $CD5^-$ B cells. Representative staining patterns for Annexin V binding to $CD5^+$ (red line) and $CD5^-$ (green line) B cells are shown in normal subjects (A) and in CHC (B). Blue lines indicate background (bkg) staining in negative controls. (C) Summary of data on Annexin V binding to $CD5^+$ (red bar) and $CD5^-$ (green bar) B cells in CHC ($n = 10$) are shown with SEM and p-values. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

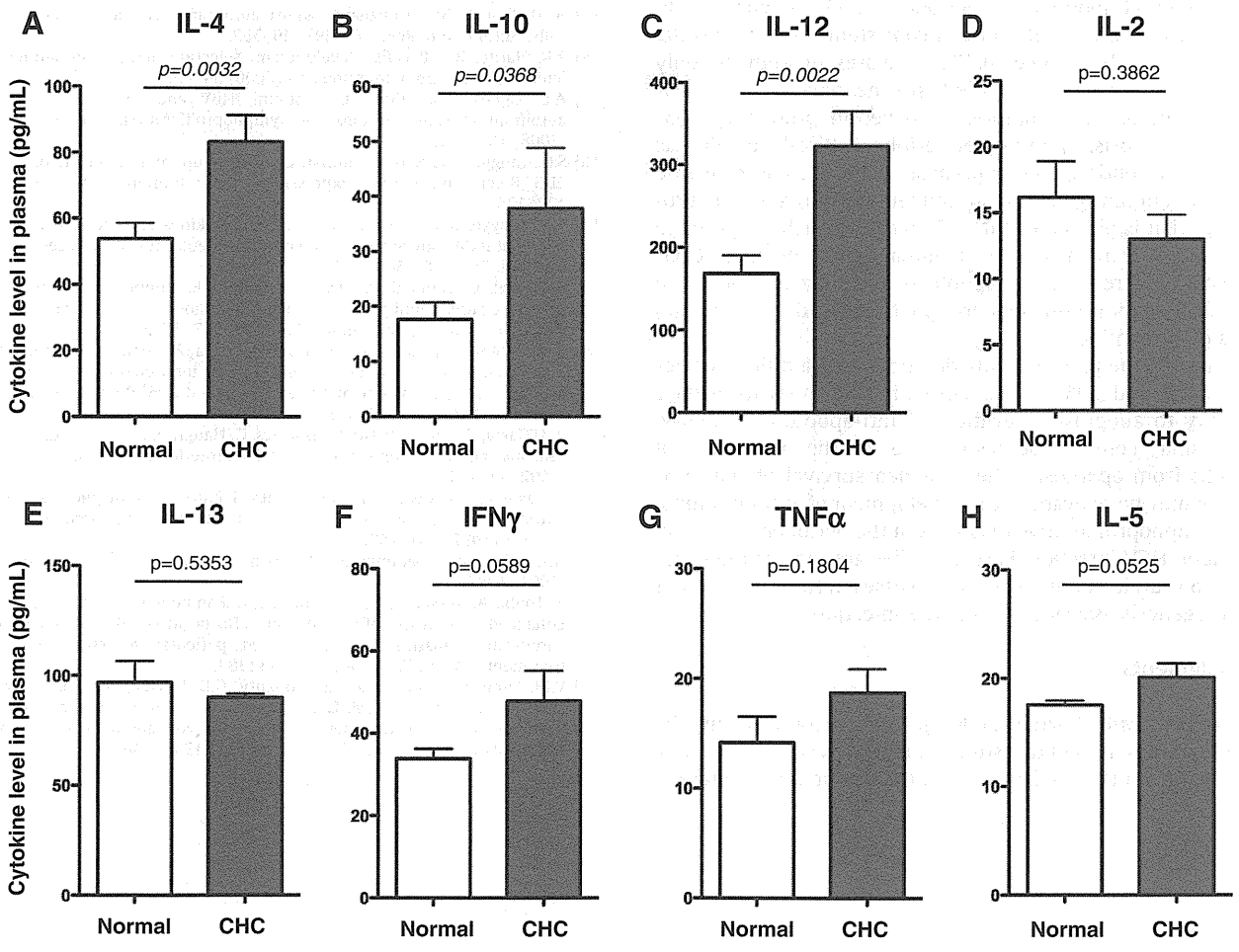

Fig. 3. Plasma levels of cytokines. Amounts of IL-4 (A), IL-10 (B), IL-12 (C), IL-2 (D), IL-13 (E), IFN-γ (F), TNFα (G), and IL-5 (H) were simultaneously determined in plasma samples from both normal ($n = 14$) and CHC ($n = 11$), and are shown with SEM and p-values.

were not significantly elevated (Fig. 3D–G). These results reflect the differences between leukemic $CD5^+$ B cells and CHC $CD5^+$ B cells in terms of the functional expression of cytokine receptors.

The elevation of IL-10 levels in CHC plasma is of particular interest because $CD5^+$ B cells themselves are known to produce IL-10 [20], and this autocrine loop may result in further expansion of the $CD5^+$ B cell subset. As IL-5 plays a critical role in the development of $CD5^+$ B cells and may be responsible for prolonging the lifespan of immature $CD5^+$ B cells [21], we were interested in the plasma levels of IL-5 in CHC. As shown in Fig. 3H, no significant differences in IL-5 levels between normal subjects and CHC were found, suggesting the minimum involvement of IL-5 in the expansion of $CD5^+$ B cells in CHC.

Enhanced expression of Bcl-2 is thought to be responsible for resistance to apoptosis [22]. We therefore analyzed the expression levels of Bcl-2 mRNA in PBMC purified from normal subjects and CHC, but no significant differences were observed (data not shown). Kessel et al. reported that the expression of intracellular Bcl-2 protein in B cells was higher in peripheral blood than in cord blood, which may explain the increased susceptibility of cord blood B cells to apoptosis [18]. However, the difference that they observed in flow cytometry utilizing anti-Bcl-2 antibody was very subtle (MFI: 2.85 ± 1.3 vs 1.6 ± 0.9) and barely statistically significant ($p = 0.05$). Toubi et al. analyzed the Bcl-2 expression levels in B cells from healthy individuals and HCV-positive patients, but did not detect any differences between them [23]. Thus, the underlying mechanism, other than augmented expression of Bcl-2, responsible for the resistance to apoptosis recognized in the $CD5^+$ B cell subset remains uncertain and needs to be clarified.

The preferential apoptosis of peripheral $CD5^-$ B cells in CHC demonstrated in the present study is consistent with the results previously reported by Toubi et al. [23]. The present study not only confirmed their findings but, for the first time, revealed the possible involvement of anti-apoptotic cytokines in protecting $CD5^+$ B cells from apoptosis. Apoptosis of peripheral B cells in CHC has received some attention, chiefly because of the increased release of cell nuclear autoantigens and subsequent development of autoimmunity [24]. It is plausible that $CD5^-$ B cells, which are prone to apoptosis, are the main source of autoantigens, whereas $CD5^+$ B cells, which are resistant to apoptosis and may expand in an autocrine fashion by producing anti-apoptotic IL-10, are the main producers of autoantibodies.

In conclusion, the present study demonstrated a difference between the $CD5^+$ and $CD5^-$ B cell subsets in CHC in terms of their susceptibility to apoptosis. Elevation of anti-apoptotic cytokines in CHC plasma seems to be responsible for the prevention of $CD5^+$ B cells from apoptosis. The extended survival of the $CD5^+$ B cell subset may be relevant to the development of autoimmunity, as well as lymphoproliferative disorders in the periphery or within the liver upon HCV infection. Future studies are required in order to enhance our understanding of the processes in the development of the pathogenesis associated with HCV infection.

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Molecular mechanism of hepatitis C virus-induced glucose metabolic disorders

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Hepatitis C virus (HCV) infection causes not only intrahepatic diseases but also extrahepatic manifestations, including metabolic disorders. Chronic HCV infection is often associated with type 2 diabetes. However, the precise mechanism underlying this association is still unclear. Glucose is transported into hepatocytes via glucose transporter 2 (GLUT2). Hepatocytes play a crucial role in maintaining plasma glucose homeostasis via the gluconeogenic and glycolytic pathways. We have been investigating the molecular mechanism of HCV-related type 2 diabetes using HCV RNA replicon cells and HCV J6/JFH1 system. We found that HCV replication down-regulates cell surface expression of GLUT2 at the transcriptional level. We also found that HCV infection promotes hepatic gluconeogenesis in HCV J6/JFH1-infected Huh-7.5 cells. HCV infection transcriptionally up-regulated the genes for phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6-phosphatase (G6Pase), the rate-limiting enzymes for hepatic gluconeogenesis. Gene expression of PEPCK and G6Pase was regulated by the transcription factor forkhead box O1 (FoxO1) in HCV-infected cells. Phosphorylation of FoxO1 at Ser319 was markedly diminished in HCV-infected cells, resulting in increased nuclear accumulation of FoxO1. HCV NS5A protein was directly linked with the FoxO1-dependent increased gluconeogenesis. This paper will discuss the current model of HCV-induced glucose metabolic disorders.

Keywords: HCV, diabetes, gluconeogenesis, GLUT2, FoxO1, JNK, NS5A

INTRODUCTION

Hepatitis C virus (HCV) is a positive-sense, single stranded RNA virus that belongs to the genus *Hepacivirus* of the family *Flaviviridae*. The approximately 9.6-kb HCV genome encodes a unique open reading frame that is translated into a polyprotein of about 3,000 amino acids, which is cleaved by cellular signalases and viral proteases to generate at least 10 viral proteins, such as core, envelope 1 (E1) and E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B (Choo et al., 1991; Lemon et al., 2007).

Hepatitis C virus is the main cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. More than 170 million people worldwide are chronically infected with HCV (Poynard et al., 2003). Persistent HCV infection causes not only liver diseases but also extrahepatic manifestations. It is well established that HCV perturbs the glucose metabolism, leading to insulin resistance and type 2 diabetes in predisposed individuals. Several epidemiological, clinical, and experimental data suggested that HCV infection serves as an additional risk factor for the development of diabetes (Mason et al., 1999; Negro and Alaei, 2009; Negro, 2011). HCV-related glucose metabolic changes and insulin resistance and diabetes have significant clinical consequences, such as accelerated fibrogenesis, increased incidence of hepatocellular carcinoma, and reduced virological response to interferon (IFN)-α-based therapy (Negro, 2011). Therefore, it is very important to clarify the molecular mechanism of HCV-related diabetes. However, the precise mechanisms are poorly understood.

Experimental data suggest a direct interference of HCV with the insulin signaling pathway. Transgenic mice expressing HCV core gene exhibit insulin resistance (Shintani et al., 2004; Koike, 2007). In this transgenic mice model, both tyrosine phosphorylation of the insulin receptor substrate (IRS)-1 and IRS-2 are decreased. These decreases are recovered when the proteasome activator PA28γ is deleted, suggesting that the HCV core protein suppresses insulin signaling through a PA28γ-dependent pathway (Miyamoto et al., 2007). Several other reports also showed a link of the HCV core protein with insulin resistance (Kawaguchi et al., 2004; Pazienza et al., 2007).

Hepatocytes play a crucial role in maintaining plasma glucose homeostasis by adjusting the balance between hepatic glucose production and utilization via the gluconeogenic and glycolytic pathways, respectively. Gluconeogenesis is mainly regulated at the transcriptional level of the glucose 6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) genes, whereas glycolysis is mainly regulated by glucokinase (GK). Gluconeogenesis and glycolysis are coordinated so that one pathway is highly active within a cell while the other is relatively inactive. It is well known that increased hepatic glucose production via gluconeogenesis is a major feature of type 2 diabetes (Clore et al., 2000).

To identify a novel mechanism of HCV-related diabetes, we have been investigating the effects of HCV on glucose production in hepatocytes using HCV RNA replicon cells (Lohmann et al., 1999) and HCV J6/JFH1 cell culture system (Lindenbach et al., 2005; Wakita et al., 2005; Bungyoku et al., 2009). We previously reported that HCV replication suppresses cellular glucose uptake through down-regulation of cell surface expression of glucose transporter 2 (GLUT2; Kasai et al., 2009). Furthermore, we

recently reported that HCV promotes hepatic gluconeogenesis via an NS5A-mediated, forkhead box O1 (FoxO1)-dependent pathway, resulting in increased cellular glucose production in hepatocytes (Deng et al., 2011). This paper discusses our current model for HCV-induced glucose metabolic disorders.

HCV REPLICATION DOWN-REGULATES CELL SURFACE EXPRESSION OF GLUT2

The uptake of glucose into cells is conducted by the facilitative glucose carrier, glucose transporters (GLUTs). GLUTs are integral membrane proteins that contain 12 membrane-spanning helices. To date, a total of 14 isoforms have been identified in the GLUT family (Wu and Freeze, 2002; Macheda et al., 2005; Godoy et al., 2006). Glucose is transported into hepatocytes by GLUT2. We previously reported that HCV J6/JFH1 infection suppresses hepatocytic glucose uptake through down-regulation of surface expression of GLUT2 in human hepatoma cell line, Huh-7.5 cells (Kasai et al., 2009). We also demonstrated that GLUT2 expression in hepatocytes of the liver tissues from HCV-infected patients was significantly lower than in those from patients without HCV infection. Our data suggest that HCV infection down-regulates GLUT2 expression at transcriptional level. We are currently analyzing transcriptional control of human GLUT2 promoter in HCV replicon cells as well as in HCV J6/JFH1-infected cells.

HCV INFECTION PROMOTES HEPATIC GLUCONEOGENESIS

Then we analyzed hepatic glucose production and expression of transcription factors using HCV replicon cells and HCVcc system in order to clarify a role of HCV infection in glucose metabolic changes. Hepatic glucose production is usually regulated by gluconeogenesis and glycolysis. Therefore, we examined whether HCV infection induces gluconeogenesis or glycolysis. We found that the PEPCK and G6Pase genes were transcriptionally up-regulated in J6/JFH1-infected cells (**Figure 1**). On the other hand, the GK gene was transcriptionally down-regulated in HCV-infected cells. We obtained similar data in HCV replicon cells (both in subgenomic replicon cells and full-genomic replicon cells). When HCV replication was suppressed by IFN treatment, the up-regulation of PEPCK and G6Pase gene expression as well as the down-regulation of GK gene expression were canceled. From these results, HCV infection selectively up-regulates PEPCK and G6Pase genes, whereas HCV infection down-regulates GK gene (Deng et al., 2011).

Both HCV replicon cells and HCV-infected cells produced greater amounts of glucose than the control cells. IFN treatment canceled the enhanced glucose production in HCV replicon cells as well as in HCV-infected cells. G6P is an important precursor molecule that is converted to glucose in the gluconeogenesis pathway (**Figure 1**). Our metabolite analysis showed that a significantly higher level of G6P was accumulated in HCV-infected cells than in the control cells, suggesting that HCV indeed promotes hepatic gluconeogenesis to cause hyperglycemia. There is a trend toward an increase in gluconeogenesis in HCV-infected cells (**Figure 1**).

HCV SUPPRESSES FoxO1 PHOSPHORYLATION AT Ser319, LEADING TO THE NUCLEAR ACCUMULATION OF FoxO1

It has been reported that G6Pase, PEPCK, and GK are regulated by certain transcription factors, including FoxO1 (Hirota et al., 2008), hepatic nuclear factor 4α (HNF-4α; Hirota et al.,

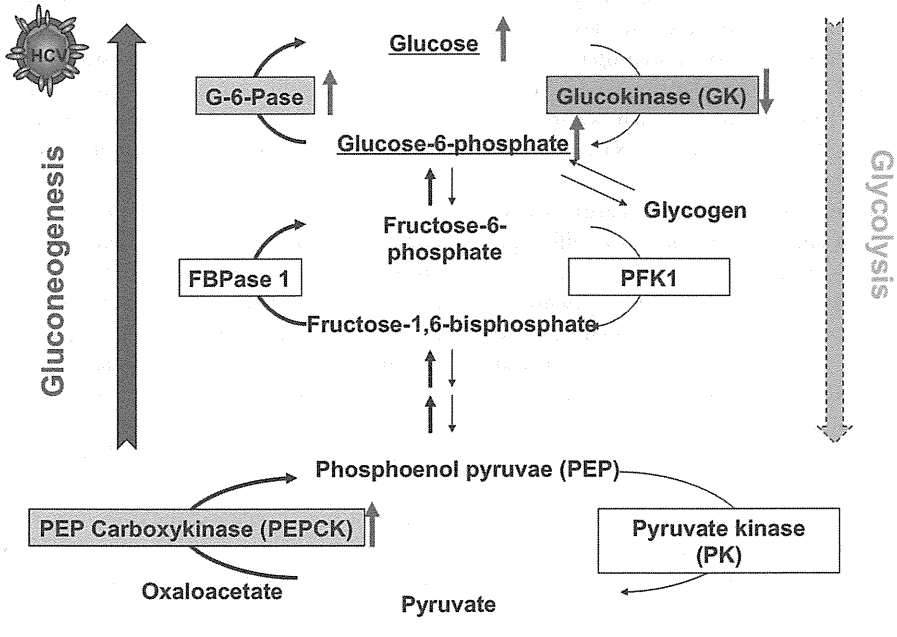

FIGURE 1 | Regulation of Gluconeogenesis and Glycolysis in the HCV-infected cells. HCV infection promotes gluconeogenesis via transcriptional up-regulation of the genes for PEPCK and G6Pase, the rate-limiting enzymes for hepatic gluconeogenesis, and transcriptional down-regulation of the gene for GK, the rate-limiting enzyme for hepatic glycolysis.