Table 1. Baseline and clinical features of Turkish patients infected with HCV

	CH (n = 43)	LC (n = 12)	HCC (n = 12)	p
Gender, male (% age)	16 (37.2)	4 (33.3)	6 (50)	NS
Age	52±10.5	57.2±11.1	65±7.5	0.0002[b], 0.05[c]
Total protein, g/dl	7.8±0.6	6.5±0.9	6.9±0.9	NS
Albumin, g/dl	4.1±0.5	3.2±0.6	3.1±0.5	NS
Globulin, g/dl	3.7±0.7	3.3±0.6	3.8±0.9	NS
Platelet count, ($\times 10^{-3}$), mm^3	160.1±103.7	82.1±22.5	128.1±85.8	0.01[a]
INR	0.98±0.14	1.56±0.34	1.33±0.24	NS
ALT, IU/l	87±143	65±57	44±34	NS
AST, IU/l	74±126	74±40	75±57	NS
GGT, IU/l	62±71	87±100	91±90	NS
ALP, IU/l	163±105	112±60	133±82	NS
T-Bil, mg/dl	0.9±1.5	2.3±1	2.2±1.5	NS
D-Bil, mg/dl	0.4±1.2	0.9±0.6	1.1±0.8	NS
HCV RNA ($\times 10^{-6}$), IU/ml	3.4±7.6	1.1±0.1	7.9±10.2	0.01[c]

Data are expressed as mean ± SD. INR = International normalized ratio; AST = asparate aminotranseferase; GGT = γ-glutamyl transpeptidase; ALP = alkaline phosphatase. [a] CH vs. LC; [b] HCC vs. CH; [c] HCC vs. LC.

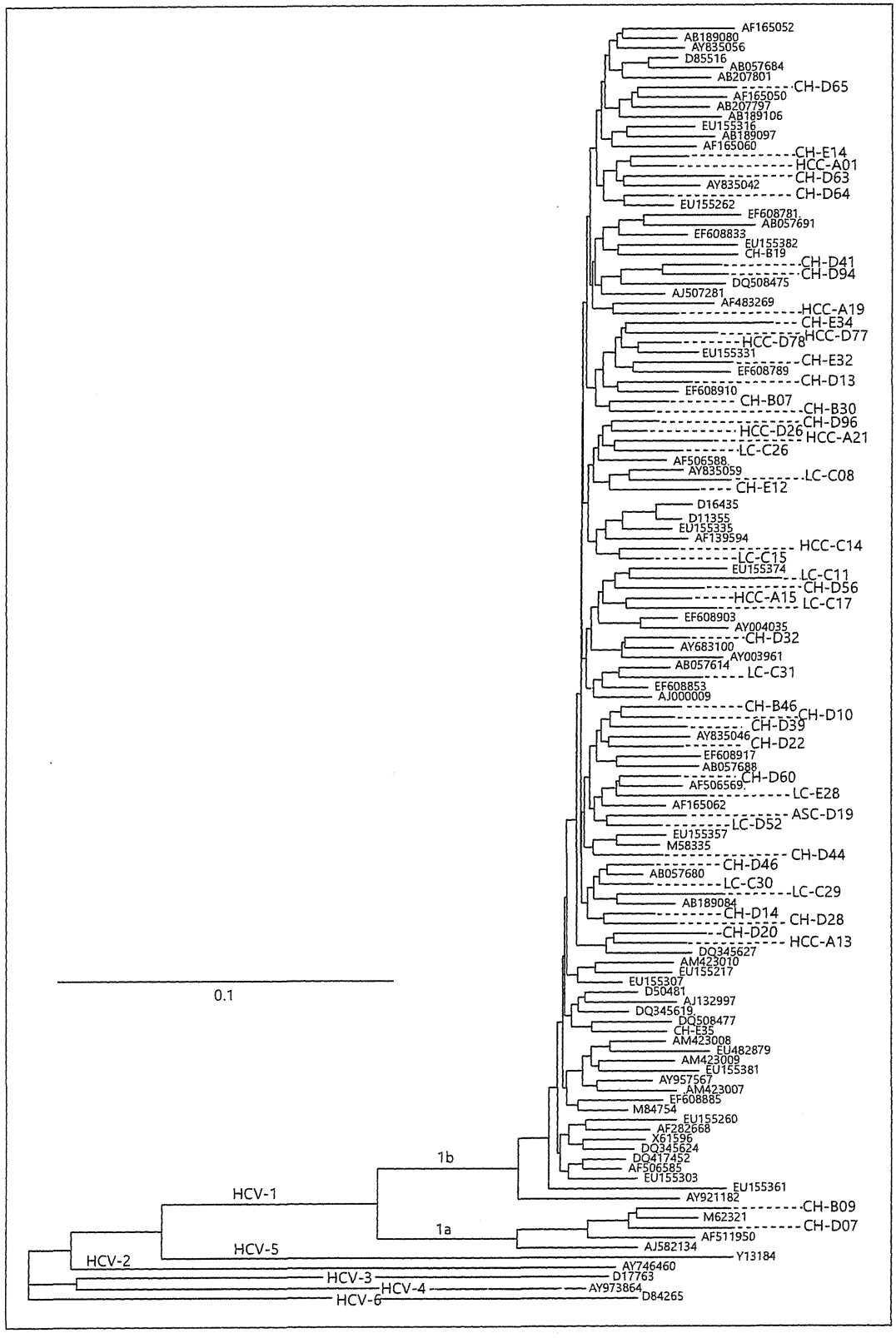
viral genotypes distributed worldwide [7]. Among those, genotypes 1, 2 and 3 and their subtypes have a global distribution, whereas other genotypes have more local distribution, such as genotype 4 that is found in the Middle East and Africa, genotype 5 in South Africa, and genotype 6 mainly in Asia [7]. Information on different genotypes can have epidemiological value by revealing specific features of local HCV epidemics [8, 9]. However, little is known about regional distribution and genetic features of HCV in Turkey. The aims of this study were to find out the most prevalent HCV genotypes in different regions of Turkey and trace out the HCV transmission patterns.

A total of 67 patients were enrolled in this study. These patients tested positive for anti-HCV and had a serum level of HCV RNA ≥50 IU/ml. The data was collected from six medical centers in four different Turkish cities. The patients were classified into three clinical groups: (1) patients with chronic liver disease with persistently elevated serum alanine aminotransferase (ALT) levels defined as chronic hepatitis (CH), (2) patients categorized under the liver cirrhosis (LC) group with clinical evidence of cirrhosis, and (3) patients diagnosed with hepatocellular carcinoma (HCC) on the basis of imaging results as well as on elevated serum fetoprotein (AFP) levels (≥400 ng ml^{-1}). The serological and biochemical tests were done at Ondokuz Mayis University. Samples were screened for HBsAg, anti-HBs, anti-HBc IgG, and anti-HCV by Architect (Abbott Diagnostics, USA). The molecular analysis was performed at the Department of Virology & Liver Unit, Nagoya City University, Graduate School of Medical Sciences, Japan. The study was approved by the Ethics Committee, School of Medicine, Ondokuz Mayis University.

Of the patients analyzed, 12 had diagnosed LC and 12 had diagnosed HCC. Comparative characteristics of these categories are summarized in table 1. There was also an unequal geographical distribution of the patients in each of the groups with most of the HCC patients enrolled from Ankara (8/12, 66.6%) and most of the LC patients (10/12, 83.3%) enrolled in Gaziantep.

Total RNA was extracted from the serum samples using the SepaGene RV-R Nucleic acid extraction kit (Sanko Junyaku Co., Ltd, Tokyo, Japan) in accordance with the manufacturer's protocol. Viral RNA were reverse transcribed into complementary DNA using SuperScript II RNase H Reverse Transcriptase (Invitrogen Corp. Carlsbad, Calif., USA) and random hexamer primer (Takara

Fig. 1. Phylogenetic analysis of HCV isolates from different regions in Turkey. Turkish isolates (in bold letters) were subject to bootstrap resampling with all available sequences in the HCV-NS5B region from the EMBL/DDBJ/GenBank database. The closest neighbors used for the phylogenetic tree are indicated under the corresponding accession numbers from DDBJ/EMBL/GeneBank.


AF165052
AB189080
AY835056
D85516
AB057684
AB207801
CH-D65
AF165050
AB207797
AB189106
EU155316
AB189097
AF165060
CH-E14
HCC-A01
CH-D63
AY835042
CH-D64
EU155262
EF608781
AB057691
EF608833
EU155382
CH-B19
CH-D41
CH-D94
DQ508475
AJ507281
AF483269
HCC-A19
CH-E34
HCC-D77
HCC-D78
EU155331
CH-E32
EF608789
CH-D13
EF608910
CH-B07
CH-B30
CH-D96
HCC-D26
HCC-A21
LC-C26
AF506588
AY835059
LC-C08
CH-E12
D16435
D11355
EU155335
AF139594
HCC-C14
LC-C15
EU155374
LC-C11
CH-D56
HCC-A15
LC-C17
EF608903
AY004035
CH-D32
AY683100
AY003961
AB057614
LC-C31
EF608853
AJ000009
CH-B46
CH-D10
CH-D39
AY835046
CH-D22
EF608917
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CH-D60
AF506569
LC-E28
AF165062
ASC-D19
LC-D52
EU155357
M58335
CH-D44
CH-D46
AB057680
LC-C30
LC-C29
AB189084
CH-D14
CH-D28
CH-D20
HCC-A13
DQ345627
AM423010
EU155217
EU155307
D50481
AJ132997
DQ345619
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CH-E35
AM423008
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EU155381
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AM423007
EF608885
M84754
EU155260
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X61596
DQ345624
DQ417452
AF506585
EU155303
EU155361
AY921182
CH-B09
M62321
CH-D07
AF511950
AJ582134
Y13184
AY746460
D17763
AY973864
D84265
0.1
1b
1a
HCV-1
HCV-2
HCV-3
HCV-4
HCV-5
HCV-6

1

Shuzo Co., Ltd, Tokyo, Japan) as described previously [10]. Confirmation of the presence of HCV-RNA in the samples was carried out by amplifying the highly conserved 5′-UTR region and HCV genotypes were determined in non-structural (NS5B) region by direct sequencing [11]. HCV RNA in all HCV RNA-positive samples was quantified by real-time PCR as described previously [12] with slight modifications in an ABI7500 FAST system. The detection limit of the assay was as few as 10 copies/ml.

Amplicons obtained in the NS5B region (nucleotides from 8,278 to 8,618) were directly sequenced with Prism Big Dye (Applied Biosystems, Foster City, Calif., USA) in an ABI 3100 DNA automated sequencer. The sequences for phylogenetic analysis were retrieved from DDBJ/EMBL/GeneBank. Alignments were performed using CLUSTALW [http://clustalw.ddbj.nig.ac.jp/top-e.html] and neighbor-joining tree was constructed [13]. Statistical differences were evaluated by Fisher's exact probability test and χ^2 test with Yates' correction where appropriate, using the STATA software version 8.0 (StataCorp. LP, College Station, Tex., USA). Differences were considered significant for p values <0.05.

Analysis of the HCV genotypes within a defined population is a useful epidemiological tool for the study of HCV infection evolution in different geographical regions and risk groups [8]. Direct sequencing is the most accurate method for HCV genotyping, but again the genotyping of the 5′-UTR is less informative, since sequence variation between genotype and/or subtypes is greatest in NS5, less in the envelope and the core, and least in the 5′-UTR [14, 15]. In this study, samples were collected from different Turkish cities to find the distribution of HCV genotypes in the partial NS5B region and trace the transmission routes by which HCV made inroads and spread in different geographical localities of the country.

This is the first study analyzing molecular evolutionary characteristics of HCV isolates collected from different regions of Turkey. As shown in figure 1, HCV-1b was found as the main subtype prevalent in Turkey. This is the most widespread variant of HCV, known as the most resistant strain to currently available treatment [16] and associated with HCV-related severe liver sequelae. HCC patients were significantly higher in age compared to the non-HCC, as evidenced in previous studies [17, 18]. Patients with LC had significantly low HCV RNA levels and platelet counts compared to patients with HCC and CH, respectively. However, due to the relatively small size of the cohort, concrete conclusions on clinical outcome may not be assumed. Regardless of the original region within Turkey, no regional bootstrap-significant phylogenetic cluster of HCV-1b was found. Phylogenetic analysis also did not reveal an indigenous phylogenetic cluster for Turkey and the closest matches were from both Asia (Japan) and Europe/USA. This indicates that the HCV spread in this country occurred from different sources possibly via different routes, which is in contrast to the single route exponential transmission described in Egypt or two independent waves as described in Japan [19, 20].

Turkey lies at the crossroads of two continents – Europe and Asia – and due to its geographic position, studying HCV epidemiology has its merits. The transmission of genotype 1b from Europe to Turkey is not exceptional. A fair role of HCV strains of genotypes 3, 4, and 6, predominant in neighboring Asian countries, in Turkey's transmission was obvious but not observed in this study, probably because of the small cohort of patients studied. Therefore, a comprehensive analysis is required across multiple loci of the HCV genome with a good number of samples taken from different regions of Turkey that may give more insight into the evolution of these viruses in Turkey.

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Human Blood Dendritic Cell Antigen 3 (BDCA3)$^+$ Dendritic Cells Are a Potent Producer of Interferon-λ in Response to Hepatitis C Virus

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The polymorphisms in the interleukin (*IL*)-*28B* (interferon-lambda [IFN]-λ3) gene are strongly associated with the efficacy of hepatitis C virus (HCV) clearance. Dendritic cells (DCs) sense HCV and produce IFNs, thereby playing some cooperative roles with HCV-infected hepatocytes in the induction of interferon-stimulated genes (ISGs). Blood dendritic cell antigen 3 (BDCA3)$^+$ DCs were discovered as a producer of IFN-λ upon Toll-like receptor 3 (TLR3) stimulation. We thus aimed to clarify the roles of BDCA3$^+$ DCs in anti-HCV innate immunity. Seventy healthy subjects and 20 patients with liver tumors were enrolled. BDCA3$^+$ DCs, in comparison with plasmacytoid DCs and myeloid DCs, were stimulated with TLR agonists, cell-cultured HCV (HCVcc), or Huh7.5.1 cells transfected with HCV/JFH-1. BDCA3$^+$ DCs were treated with anti-CD81 antibody, inhibitors of endosome acidification, TIR-domain-containing adapter-inducing interferon-β (TRIF)-specific inhibitor, or ultraviolet-irradiated HCVcc. The amounts of IL-29/IFN-λ1, IL-28A/IFN-λ2, and IL-28B were quantified by subtype-specific enzyme-linked immunosorbent assay (ELISA). The frequency of BDCA3$^+$ DCs in peripheral blood mononuclear cell (PBMC) was extremely low but higher in the liver. BDCA3$^+$ DCs recovered from PBMC or the liver released large amounts of IFN-λs, when stimulated with HCVcc or HCV-transfected Huh7.5.1. BDCA3$^+$ DCs were able to induce ISGs in the coexisting JFH-1-positive Huh7.5.1 cells. The treatments of BDCA3$^+$ DCs with anti-CD81 antibody, cloroquine, or bafilomycin A1 reduced HCVcc-induced IL-28B release, whereas BDCA3$^+$ DCs comparably produced IL-28B upon replication-defective HCVcc. The TRIF-specific inhibitor reduced IL-28B release from HCVcc-stimulated BDCA3$^+$ DCs. In response to HCVcc or JFH-1-Huh7.5.1, BDCA3$^+$ DCs in healthy subjects with IL-28B major (rs8099917, TT) released more IL-28B than those with IL-28B minor genotype (TG). *Conclusion*: Human BDCA3$^+$ DCs, having a tendency to accumulate in the liver, recognize HCV in a CD81-, endosome-, and TRIF-dependent manner and produce substantial amounts of IL-28B/IFN-λ3, the ability of which is superior in subjects with IL-28B major genotype. (HEPATOLOGY 2013;57:1705-1715)

Hepatitis C virus (HCV) infection is one of the most serious health problems in the world. More than 170 million people are chronically infected with HCV and are at high risk of developing liver cirrhosis and hepatocellular carcinoma. Genome-wide association studies have successfully identified the genetic polymorphisms (single nucleotide polymorphisms, SNPs) upstream of the promoter region of the

Abbreviations: Ab, antibody; HCV, hepatitis C virus; HCVcc, cell-cultured hepatitis C virus; HSV, herpes simplex virus; IHL, intrahepatic lymphocyte; INF-λ, interferon-lambda; IRF, interferon regulatory factor; ISGs, interferon-stimulated genes; JEV, Japanese encephalitis virus; Lin, lineage; mDC, myeloid DC; MOI, multiplicity of infection; PBMC, peripheral blood mononuclear cell; pDC, plasmacytoid DC; Poly IC, polyinosine-polycytidylic acid; RIG-I, retinoic acid-inducible gene-I; SNPs, single nucleotide polymorphisms; TLR, Toll-like receptor; TRIF, TIR-domain-containing adapter-inducing interferon-β.

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interleukin (IL)-28B / interferon-lambda 3 (IFN-λ3) gene, which are strongly associated with the efficacy of pegylated interferon-α (PEG-IFN-α) and ribavirin therapy or spontaneous HCV clearance.[1-4]

IFN-λs, or type III IFNs, comprise a family of highly homologous molecules consisting of IFN-λ1 (IL-29), IFN-λ2 (IL-28A), and IFN-λ3 (IL-28B). In clear contrast to type I IFNs, they are released from relatively restricted types of cells, such as hepatocytes, intestinal epithelial cells, or dendritic cells (DCs). Also, the cells that express heterodimeric IFN-λ receptors (IFN-λR1 and IL-10R2) are restricted to cells of epithelial origin, hepatocytes, or DCs.[5] Such limited profiles of cells expressing IFN-λs and their receptors define the biological uniqueness of IFN-λs. It has been shown that IFN-λs convey anti-HCV activity by inducing various interferon-stimulated genes (ISGs),[5] the profiles of which were overlapped but others were distinct from those induced by IFN-α/β. Some investigators showed that the expression of IL-28 in PBMC was higher in subjects with IL-28B major than those with minor; however, the levels of IL-28 transcripts in liver tissue were comparable regardless of IL-28B genotype.[2,6]

At the primary exposure to hosts, HCV maintains high replicative levels in the infected liver, resulting in the induction of IFNs and ISGs. In a case of successful HCV eradication, it is postulated that IFN-α/β and IFN-λ cooperatively induce antiviral ISGs in HCV-infected hepatocytes. It is of particular interest that, in primary human hepatocytes or chimpanzee liver, IFN-λs, but not type I IFNs, are primarily induced after HCV inoculation, the degree of which is closely correlated with the levels of ISGs.[7] These results suggest that hepatic IFN-λ could be a principal driver of ISG induction in response to HCV infection. Nevertheless, the possibility remains that DCs, as a prominent IFN producer in the liver, play significant roles in inducing hepatic ISGs and thereby suppressing HCV replication.

DCs, as immune sentinels, sense specific genomic and/or structural components of pathogens with various pattern recognition receptors and eventually release IFNs and inflammatory cytokines.[8] In general, DCs migrate to the organ where inflammation or cellular apoptosis occurs and alter their function in order to alleviate or exacerbate the disease conditions. Therefore, the phenotypes and/or capacity of liver DCs are deemed to be influenced in the inflamed liver. In humans, the existence of phenotypically and functionally distinct DC subsets has been reported: myeloid DC (mDC) and plasmacytoid DC (pDC).[9] Myeloid DCs predominantly produce IL-12 or tumor necrosis factor alpha (TNF-α) following proinflammatory stimuli, while pDCs release considerable amounts of type I IFNs upon virus infection.[9] The other type of mDCs, mDC2 or BDCA3$^+$(CD141) DCs, have been drawing much attention recently, since human BDCA3$^+$ DCs are reported to be a counterpart of murine CD8a$^+$ DCs.[10] Of particular interest is the report that BDCA3$^+$ DCs have a potent capacity of releasing IFN-λ in response to Toll-like receptor 3 (TLR3) agonist.[11] However, it is still largely unknown whether human BDCA3$^+$ DCs are able to respond to HCV.

Taking these reports into consideration, we hypothesized that human BDCA3$^+$ DCs, as a producer of IFN-λs, have crucial roles in anti-HCV innate immunity. We thus tried to clarify the potential of BDCA3$^+$ DCs in producing type III IFNs by using cell-cultured HCV (HCVcc) or hepatoma cells harboring HCV as stimuli. Our findings show that BDCA3$^+$ DCs are quite a unique DC subset, characterized by a potent and specialized ability to secrete IFN-λs in response to HCV. The ability of BDCA3$^+$ DCs to release IL-28B upon HCV is superior in subjects with IL-28B major (rs8099917, TT) to those with minor (TG or GG) genotype, suggesting that BDCA3$^+$ DCs are one of the key players in IFN-λ-mediated innate immunity.

Patients and Methods

Subjects. This study enrolled 70 healthy volunteers (male/female: 61/9) (age: mean ± standard deviation [SD], 37.3 ± 7.8 years) and 20 patients who underwent surgical resection of liver tumors at Osaka University Hospital (Supporting Table 1). The study was approved by the Ethical Committee of Osaka University Graduate School of Medicine. Written informed consent was obtained from all of them. All healthy volunteers were negative for HCV, hepatitis B virus (HBV), and human immunodeficiency virus (HIV) and had no apparent history of liver, autoimmune, or malignant diseases.

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Additional Supporting Information may be found in the online version of this article.

Reagents. The specifications of all antibodies used for FACS or cell sorting TLR-specific synthetic agonists, pharmacological reagents, and inhibitory peptides are listed in the Supporting Materials.

Separation of DCs from PBMC or Intrahepatic Lymphocytes. We collected 400 mL of blood from each healthy volunteer and processed them for PBMCs. Noncancerous liver tissues were obtained from patients who underwent resection of liver tumors (Supporting Table 1). For the collection of intrahepatic lymphocytes (IHLs), liver tissues were washed thoroughly with phosphate-buffered saline to remove the peripheral blood adhering to the tissue and ground gently. After Lin-negative ($CD3^-$, $CD14^-$, $CD19^-$, and $CD56^-$) cells were obtained by the MACS system, each DC subset with the defined phenotype was sorted separately under FACS Aria (BD). The purity was more than 98%, as assessed by FACS Canto II (BD). Sorted DCs were cultured at 2.5×10^4/well on 96-well culture plates.

Immunofluorescence Staining of Human Liver Tissue. Tissue specimens were obtained from surgical resections of noncancerous liver from the patients as described above. Briefly, the 5-mm sections were incubated with the following antibodies: mouse biotinylated antihuman BDCA3 antibody (Miltenyi-Biotec), and mouse antihuman CLEC9A antibody (Biolegend) and subsequently with secondary goat antirabbit Alexa Fluor488 or goat antimouse Alexa Fluor594 (Invitrogen, Molecular Probes) antibodies. Cell nuclei were counterstained with Dapi-Fluoromount-GTM (Southern Biotech, Birmingham, AL). The stained tissues were analyzed by fluorescence microscopy (Model BZ-9000; Keyence, Osaka, Japan).

Cells and Viruses. The *in vitro* transcribed RNA of the JFH-1 strain of HCV was introduced into FT3-7 cells[12] or Huh7.5.1 cells. The stocks of HCVcc were generated by concentration of the medium from JFH-1-infected FT3-7 cells. The virus titers were determined by focus forming assay.[13] The control medium was generated by concentration of the medium from HCV-uninfected FT3-7 cells. Infectious JEVs were generated from the expression plasmid (pMWJEATG1) as reported.[14] HSV (KOS) was a generous gift from Dr. K. Ueda (Osaka University). Huh7.5.1 cells transduced with HCV JFH-1 strain was used for the coculture with DCs. The transcripts of ISGs in Huh7.5.1 were examined by reverse-transcription polymerase chain reaction (RT-PCR) methods using gene-specific primers and probes (Applied Biosystems, Foster City, CA).

Secretion Assays. IL-28B/IFN-λ3 was quantified by a newly developed chemiluminescence enzyme immunoassay (CLEIA) system.[15] IL-29/IFN-λ1, IL-28A/IFN-λ2, and IFN-β were assayed by commercially available enzyme-linked immunosorbent assay (ELISA) kits (eBioscience, R&D, and PBL, respectively). IFN-α was measured by cytometric beads array kits (BD) according to the manufacturer's instructions.

Statistical Analysis. The differences between two groups were assessed by the Mann-Whitney nonparametric *U* test. Multiple comparisons between more than two groups were analyzed by the Kruskal-Wallis nonparametric test. Paired *t* tests were used to compare differences in paired samples. All the analyses were performed using GraphPad Prism software (San Diego, CA).

Results

Human BDCA3$^+$ DCs Are Phenotypically Distinct from pDCs and mDCs. We defined BDCA3$^+$ DCs as $Lin^-HLA\text{-}DR^+BDCA3^{high+}$ cells (Fig. 1A, left, middle), and pDCs and mDCs by the patterns of CD11c and CD123 expressions (Fig. 1A, right). The level of CD86 on pDCs or mDCs is comparatively higher than those on BDCA3$^+$ DCs (Fig. 1B). The expression of CD81 is higher on BDCA3$^+$ DCs than on pDCs and mDCs (Fig. 1B, Supporting Fig. S1). CLEC9A, a member of C-type lectin, is expressed specifically on BDCA3$^+$ DCs as reported elsewhere,[16] but not on pDCs and mDCs (Fig. 1B).

Liver BDCA3$^+$ DCs Are More Mature than the Counterparts in the Periphery. BDCA3$^+$ DCs in infiltrated hepatic lymphocytes (IHLs) are all positive for CLEC9A, but liver pDCs or mDCs are not (data not shown). The levels of CD40, CD80, CD83, and CD86 on liver BDCA3$^+$ DCs are higher than those on the peripheral counterparts, suggesting that BDCA3$^+$ DCs are more mature in the liver compared to those in the periphery (Fig. 1C).

In order to confirm that BDCA3$^+$ DCs are localized in the liver, we stained the cells with immunofluorescence antibodies (Abs) in noncancerous liver tissues. Liver BDCA3$^+$ DCs were defined as $BDCA3^+CLEC9A^+$ cells (Fig. 1D). Most of the cells were found near the vascular compartment or in sinusoid or the space of Disse of the liver tissue.

BDCA3$^+$ DCs Are Scarce in PBMCs but More Abundant in the Liver. The percentages of BDCA3$^+$ DCs in PBMCs were much lower than those of the other DC subsets (BDCA3$^+$ DCs, pDCs and mDCs, mean $\pm$ SD [%], 0.054 ± 0.044, 0.27 ± 0.21 and 1.30 ± 0.65) (Fig. 2A). The percentages of BDCA3$^+$ DCs in IHLs were lower than those of the others (BDCA3$^+$ DCs, pDCs, and mDCs, mean $\pm$ SD [%],

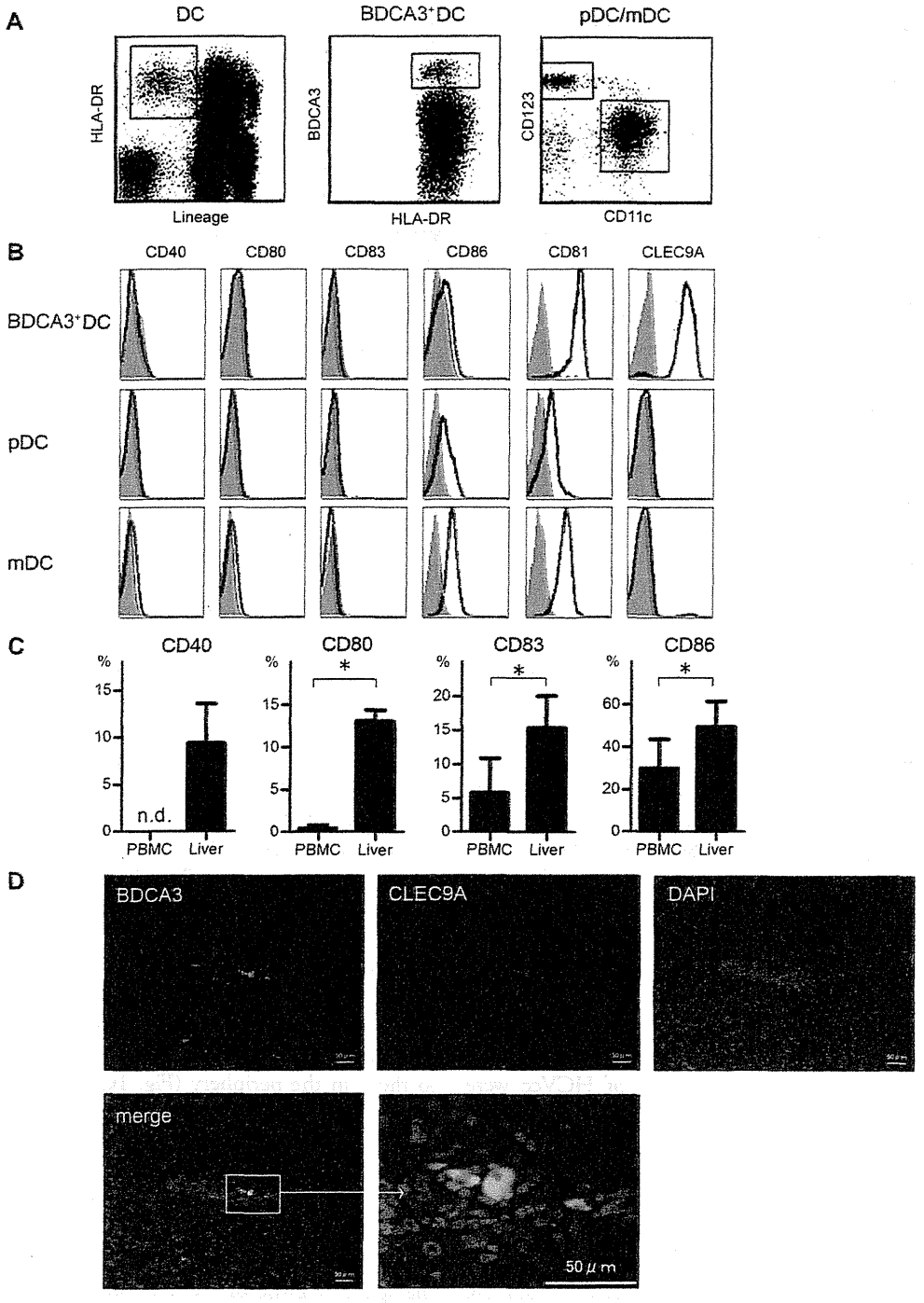

Fig. 1. Identification and phenotypic analyses of peripheral blood and intrahepatic BDCA3$^+$ DCs. (A) We defined BDCA3$^+$ DCs as Lineage$^-$HLA-DR$^+$BDCA3^{high+} cells (middle), pDCs as Lineage$^-$HLA-DR$^+$CD11c$^-$CD123^{high+} cells, and mDCs as Lineage$^-$HLA-DR$^+$CD11c$^+$CD123^{low+} cells (right). (B) The expressions of CD40, CD80, CD83, CD86, CD81, and CLEC9A on each DC subset in peripheral blood are shown. Representative results of five donors are shown in the histograms. Filled gray histograms depict data with isotype Abs, and open black ones are those with specific Abs. (C) The expressions of costimulatory molecules on BDCA3$^+$ DCs were compared between in PBMCs and in the liver. The results are shown as the percentage of positive cells. Results are the mean ± SEM from four independent experiments. *$P < 0.05$ by paired t test. (D) The staining for BDCA3 (green), CLEC9A (red) identifies BDCA3$^+$ DCs (merge, BDCA3$^+$CLEC9A$^+$) in human liver tissues. Representative results of the noncancerous liver samples are shown. BDCA, blood dendritic cell antigen; pDC, plasmacytoid DC; mDC, myeloid DC; CLEC9A, C-type lectin 9A.

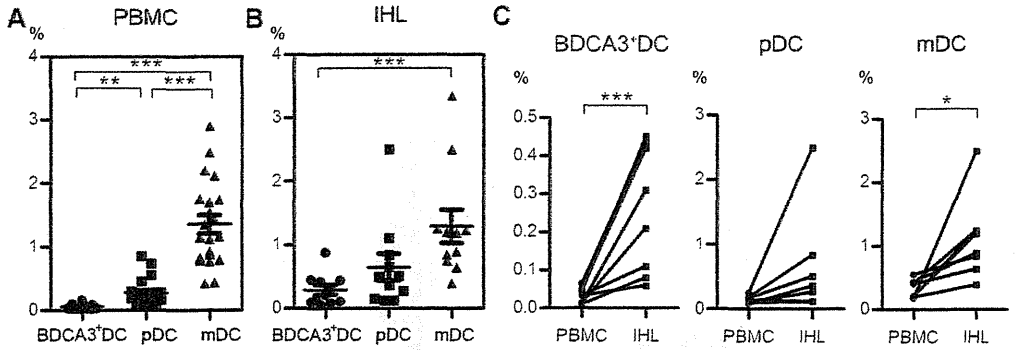

Fig. 2. Analysis of frequency of DC subsets in the peripheral blood and in the liver. Frequencies of BDCA3$^+$ DCs, pDCs, and mDCs in PBMCs (21 healthy subjects) (A) or in the intrahepatic lymphocytes (IHLs) (11 patients who had undergone surgical resection of tumors) (B) are shown. Horizontal bars depict the mean ± SD. $^{**}P < 0.005$; $^{***}P < 0.0005$ by Kruskal-Wallis test. (C) The paired comparisons of the frequencies of DC subsets between in PBMCs and in IHLs. the results of eight patients whose PBMCs and IHLs were obtained simultaneously are shown. $^{*}P < 0.05$; $^{***}P < 0.0005$ by paired *t* test. IHLs, intrahepatic lymphocytes; pDC and mDC, see Fig. 1.

0.29 ± 0.25, 0.65 ± 0.69 and 1.2 ± 0.94) (Fig. 2B). The percentages of BDCA3$^+$ DCs in the IHLs were significantly higher than those in PBMCs from relevant donors (Fig. 2C). Such relative abundance of BDCA3$^+$ DCs in the liver over that in the periphery was observed regardless of the etiology of the liver disease (Supporting Table 1).

BDCA3$^+$ DCs Produce a Large Amount of IFN-λs upon Poly IC Stimulation. We compared DC subsets for their abilities to produce IL-29/IFN-λ1, IL-28A/IFN-λ2, IL-28B/IFN-λ3, IFN-β, and IFN-α in response to TLR agonists. Approximately 4.0×10^4 of BDCA3$^+$ DCs were recoverable from 400 mL of donated blood from healthy volunteers. We fixed the number of DCs at 2.5×10^4 cells/100 mL for comparison in the following experiments.

BDCA3$^+$ DCs have been reported to express mRNA for TLR1, 2, 3, 6, 8, and 10.[17] First, we quantified IL-28B/IFN-λ3 as a representative for IFN-λs after stimulation of BDCA3$^+$ DCs with relevant TLR agonists. We confirmed that BDCA3$^+$ DCs released IL-28B robustly in response to TLR3 agonist/poly IC but not to other TLR agonists (Fig. S2). In contrast, pDCs produced IL-28B in response to TLR9 agonist/CpG but much lesser to other agonists (Fig. S2). Next, we compared the capabilities of DCs inducing IFN-λs and IFN-β genes in response to relevant TLR agonists. BDCA3$^+$ DCs expressed extremely high levels of IL-29, IL-28A, and IL-28B transcripts compared to other DCs, whereas pDCs induced a higher level of IFN-β than other DCs (Fig. S3A).

Similar results were obtained with the protein levels of IFN-λs, IFN-β, and IFN-α released from DC subsets stimulated with TLR agonists. BDCA3$^+$ DCs produce significantly higher levels of IL-29, IL-28B, and IL-28A than the other DC subsets. In clear contrast, pDCs release a significantly larger amount of IFN-β and IFN-α than BDCA3$^+$ DCs or mDCs (Fig. 3A, Fig. S3B). As for the relationship among the quantity of IFN-λ subtypes from poly IC-stimulated BDCA3$^+$ DCs, the levels of IL-29/IFN-λ1 and IL-28B/IFN-λ3 were positively correlated ($R^2 = 0.76$, $P < 0.05$), and those of IL-28A/IFN-λ2 and IL-28B/IFN-λ3 were positively correlated as well ($R^2 = 0.84$, $P < 0.0005$), respectively (Fig. S3C). These results show that the transcription and translation machineries of IFN-λs may be overlapped among IFN-λ subtypes in BDCA3$^+$ DCs upon poly IC stimulation.

Liver BDCA3$^+$ DCs sorted from IHLs possess the ability to produce IL-28B in response to poly IC (Fig. 3B), showing that they are comparably functional. In response to poly IC, BDCA3$^+$ DCs were capable of producing inflammatory cytokines as well, such as TNF-α, IL-6, and IL-12p70 (Fig. S4A). By using Huh7 cells harboring HCV subgenomic replicons (HCV-N, genotype 1b), we confirmed that the supernatants from poly IC-stimulated BDCA3$^+$ DCs suppressed HCV replication in an IL-28B concentration-dependent manner (Fig. S4B). Therefore, poly IC-stimulated BDCA3$^+$ DCs are capable of producing biologically active substances suppressing HCV replication, some part of which may be mediated by IFN-λs.

BDCA3$^+$ DCs Produce IL-28B upon HCVcc or HCV/JFH-1-Transfected Huh7.5.1 Cells. We stimulated freshly isolated BDCA3$^+$ DCs, pDCs and mDCs with infectious viruses, such as HCVcc, Japanese encephalitis virus (JEV), and herpes simplex virus (HSV). In preliminary experiments, we confirmed that HCVcc stimulated BDCA3$^+$ DCs to release IL-28B in a dose-dependent manner (Fig. S5). BDCA3$^+$ DCs

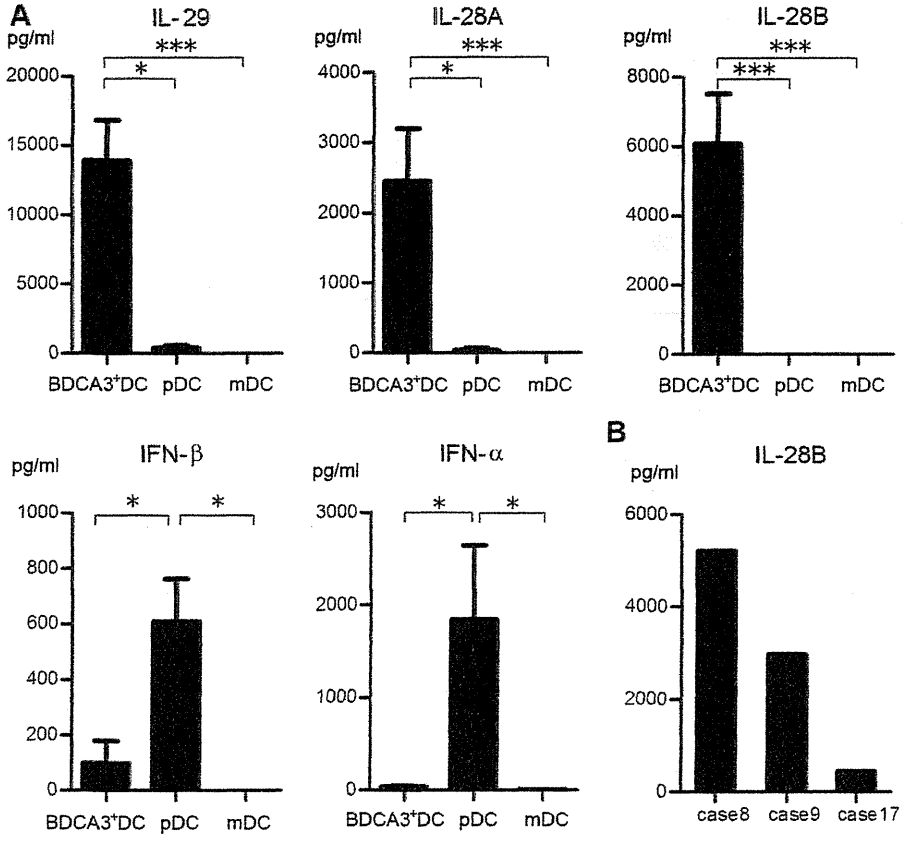

Fig. 3. BDCA3$^+$ DCs recovered from peripheral blood or intrahepatic lymphocytes produce large amounts of IL-29/IFN-λ1, IL-28A/IFN-λ2, and IL-28B/IFN-λ3 in response to poly IC. (A) BDCA3$^+$ DCs and mDCs were cultured at 2.5×10^4 cells with 25 mg/mL poly IC, and pDCs were with 5 mM CPG for 24 hours. The supernatants were examined for IL-29, IL-28A, IL-28B, IFN-β and IFN-α. Results are shown as mean $\pm$ SEM from 15 experiments. $^*P < 0.05$; $^{***}P < 0.0005$ by Kruskal-Wallis test. (B) For the IL-28B production, BDCA3$^+$ DCs in intrahepatic lymphocytes were cultured at 2.5×10^4 cells with 25 mg/mL poly IC for 24 hours. The samples of cases 8 and 9 were obtained from patients with non-B, non-C liver disease and that of case 17 was from an HCV-infected patient (Supporting Table 1).

produced a large amount of IL-28B upon exposure to HCVcc and released a lower amount of IFN-α upon HCVcc or HSV (Fig. 4A). In contrast, pDCs produced a large amount of IFN-α in response to HCVcc and HSV and a much lower level of IL-28B upon HCVcc (Fig. S6). In mDCs, IL-28B and IFN-α were not detectable with any of these viruses (data not shown).

BDCA3$^+$ DCs produced significantly higher levels of IL-28B than the other DCs upon HCVcc stimulation (Fig. 4B). By contrast, HCVcc-stimulated pDCs released significantly larger amounts of IFN-β and IFN-α than the other subsets (Fig. 4B). Liver BDCA3$^+$ DCs were capable of producing IL-28B in response to HCVcc (Fig. 4C). These results show that, upon HCVcc stimulation, BDCA3$^+$ DCs produce more IFN-λs and pDCs release more IFN-β and IFN-α than the other DC subsets, respectively. Taking a clinical impact of IL-28B genotypes on HCV eradication into consideration, we focused on IL-28B/IFN-λ3 as a representative for IFN-λs in the following experiments.

In a coculture with JFH-1-infected Huh7.5.1 cells, BDCA3$^+$ DCs profoundly released IL-29, IL-28A, and IL-28B (Fig. 4D, the results of IL-29 and IL-28A, not shown), whereas BDCA3$^+$ DCs failed to respond to Huh7.5.1 cells lacking HCV/JFH-1, showing that IL-28B production from BDCA3$^+$ DCs is dependent on HCV genome (Fig. 4D). In the absence of BDCA3$^+$ DCs, IL-28B is undetectable in the supernatant from JFH-1-infected Huh7.5.1 cells, demonstrating that BDCA3$^+$ DCs, not HCV-replicating Huh7.5.1 cells, produce detectable amount of IL-28B (Fig. 4D). In the coculture, BDCA3$^+$ DCs comparably released IL-28B either in the presence or the absence of transwells, suggesting that cell-to-cell contact between DCs and Huh7.5.1 cells is dispensable for IL-28B response (Fig. 4E). In parallel with the quantity of IL-28B in the coculture, ISG15 was significantly induced only in JFH-1-infected Huh7.5.1 cells cocultured with BDCA3$^+$ DCs (Fig. 4F). A strong induction was observed with other ISGs in JFH-1-infected Huh7.5.1 in the presence of BDCA3$^+$ DCs, such as IFIT1, MxA, RSD2, IP-10, and USP18 (Fig. S7). The results clearly show that BDCA3$^+$ DCs are capable of producing large amounts of IFN-λs in response to cellular or cell-free HCV, thereby inducing various ISGs in bystander liver cells.

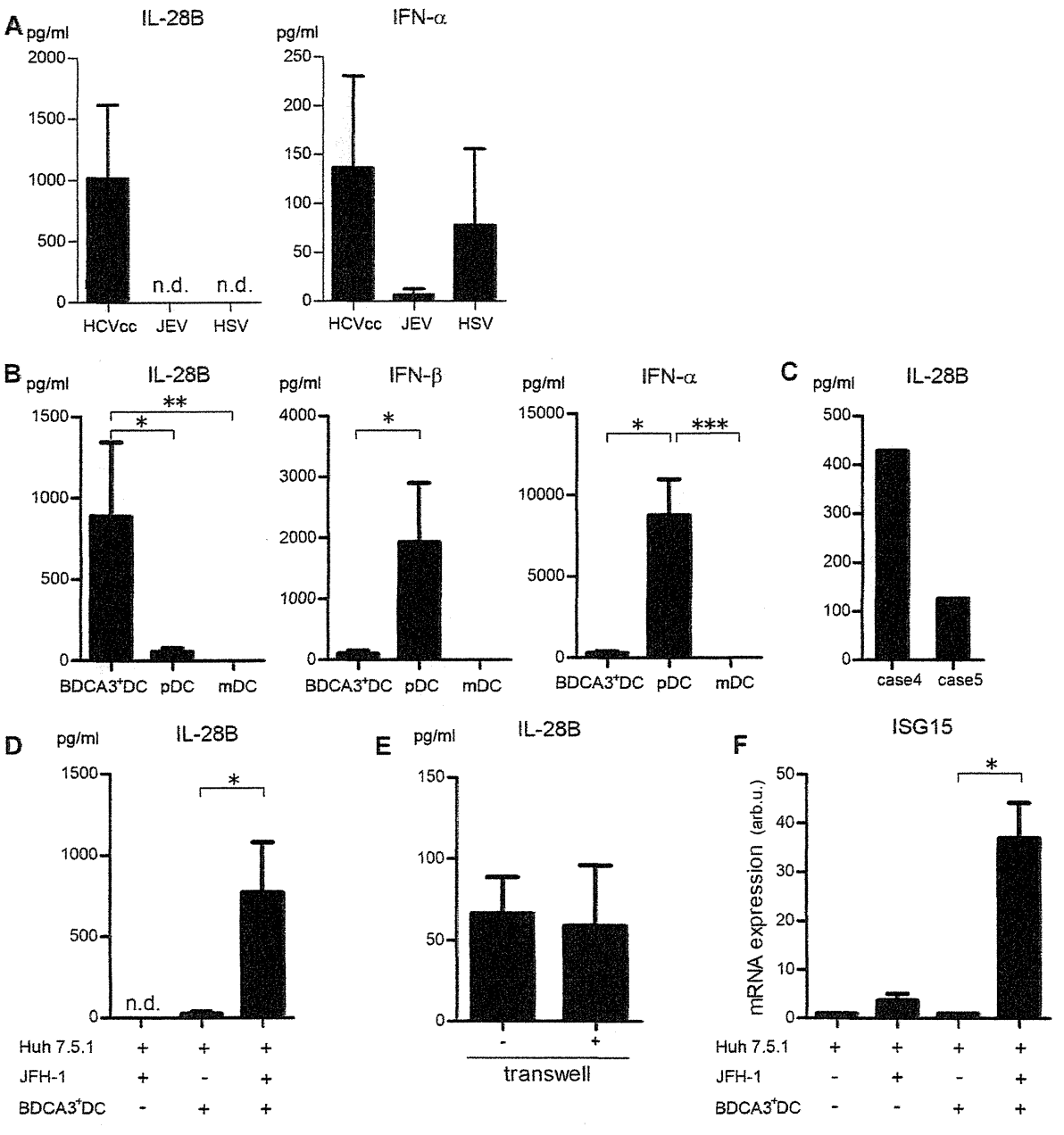

Fig. 4. BDCA3$^+$ DCs produce IL-29, IL-28A, and IL-28B upon cell-cultured HCV or HCV/JFH-1-transfected Huh7.5.1 cells, thereby inducing ISG. (A) BDCA3$^+$ DCs were cultured at 2.5 $\times$ 10^4 cells for 24 hours with HCVcc, JEV, or HSV at a multiplicity of infection (MOI) of 10. Results are shown as mean $\pm$ SEM from six experiments. n.d.; not detected. (B) BDCA3$^+$ DCs, pDCs, and mDCs were cultured at 2.5 $\times$ 10^4 cells for 24 hours with HCVcc at an MOI of 10. The results are shown as mean $\pm$ SEM from 11 experiments. *$P < 0.05$; **$P < 0.0005$; ***$P < 0.0005$ by Kruskal-Wallis test. (C) BDCA3$^+$ DCs recovered from intrahepatic lymphocytes were cultured at 2.5 $\times$ 10^4 cells for 24 hours with HCVcc at an MOI of 10. Both of the samples (cases 4 and 5) were obtained from patients with non-B, non-C liver disease. (D,E) BDCA3$^+$ DCs were cocultured at 2.5 $\times$ 10^4 cells with JFH-1-transfected (MOI = 2) or -untransfected Huh7.5.1 cells for 24 hours. The supernatants of JFH-1-transfected Huh7.5.1.cells without BDCA3$^+$ DCs were also examined. In some experiments of the coculture with JFH-1-transfected Huh7.5.1 cells and BDCA3$^+$ DCs, transwells were inserted into the wells (E). Results are shown as mean $\pm$ SEM from five experiments. *$P < 0.05$ by paired t test. (F) BDCA3$^+$ DCs were cocultured at 2.5 $\times$ 10^4 cells with JFH-1-transfected Huh7.5.1 cells (MOI = 2) or -untransfected Huh7.5.1 cells for 24 hours. The Huh7.5.1.cells were harvested and subjected to real-time RT-PCR analyses for ISG15 expression. The results are shown as mean $\pm$ SEM from five experiments. *$P < 0.05$ by paired t test. HCVcc, cell-cultured HCV; JEV, Japanese encephalitis virus; HSV, herpes simplex virus.

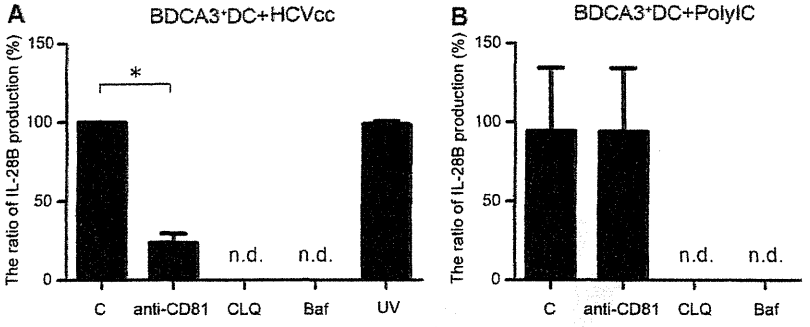

Fig. 5. The CD81 and endosome acidification is involved in the production of IL-28B from HCV-stimulated BDCA3$^+$ DCs, but HCV replication is not necessary. (A,B) BDCA3$^+$ DCs were cultured at 2.5×10^4 cells with HCVcc at an MOI of 10 (A) or poly IC (25 μg/mL) (B). In some experiments, UV-irradiated HCVcc was used at the same MOI, and BDCA3$^+$ DCs were treated with anti-CD81Ab (5 mg/mL), chloroquine (10 mM), or bafilomycin A1 (25 nM). The results are expressed as ratios of IL-28B quantity with or without the treatments. They are shown as mean $\pm$ SEM from five experiments. *$P < 0.05$ by paired t test. C, control; CLQ, treatment with chloroquine; Baf, treatment with bafilomycin A1; UV, ultraviolet-irradiated HCVcc; n.d., not detected.

CD81 and Endosome Acidification Are Involved in IL-28B Production from HCV-Stimulated BDCA3$^+$ DCs, but HCV Replication Is Not Involved. It is not known whether HCV entry and subsequent replication in DCs is involved or not in IFN response.[18,19] To test this, BDCA3$^+$ DCs were inoculated with UV-irradiated, replication-defective HCVcc. We confirmed that UV exposure under the current conditions is sufficient to negate HCVcc replication in Huh7.5.1 cells, as demonstrated by the lack of expression of NS5A after inoculation (data not shown). BDCA3$^+$ DCs produced comparable levels of IL-28B with UV-treated HCVcc, indicating that active HCV replication is not necessary for IL-28B production (Fig. 5A).

We next examined whether or not the association of HCVcc with BDCA3$^+$ DCs by CD81 is required for IL-28B production. It has been reported that the E2 region of HCV structural protein is associated with CD81 on cells when HCV enters susceptible cells.[13,20] We confirmed that all DC subsets express CD81, the degree of which was most significant on BDCA3$^+$ DCs (Fig. 1B, Fig. S1). Masking of CD81 with Ab significantly impaired IL-28B production from HCVcc-stimulated BDCA3$^+$ DCs in a dose-dependent manner (Fig. 5A, Fig. S8), suggesting that HCV-E2 and CD81 interaction is involved in the induction. The treatment of poly IC-stimulated BDCA3$^+$ DCs with anti-CD81 Ab failed to suppress IL-28B production (Fig. 5B).

HCV enters the target cells, which is followed by fusion steps within acidic endosome compartments. Chloroquine and bafilomycin A1 are well-known and broadly used inhibitors of endosome TLRs, which are reported to be capable of blocking TLR3 response in human monocyte-derived DC.[21,22] In our study, the treatment of BDCA3$^+$ DCs with chloroquine, bafilomycin A1, or NH4Cl significantly suppressed their IL-28B production either in response to HCVcc or poly IC (Fig. 5A,B, NH4Cl, data not shown). These results suggest that the endosome acidification is involved in HCVcc- or poly IC-stimulated BDCA3$^+$ DCs to produce IL-28B. The similar results were obtained with HCVcc-stimulated pDCs for the production of IL-28B (Fig. S9). We validated that such concentration of chloroquine (10 mM) and bafilomycin A1 (25 nM) did not reduce the viability of BDCA3$^+$ DCs (Fig. S10).

BDCA3$^+$ DCs Produce IL-28B in Response to HCVcc by a TIR-Domain-Containing Adapter-Inducing Interferon-β (TRIF)-Dependent Mechanism. TRIF/TICAM-1, a TIR domain-containing adaptor, is known to be essential for the TLR3-mediated pathway.[23] In order to elucidate whether TLR3-dependent pathway is involved or not in IL-28B response of BDCA3$^+$ DCs, we added the cell-permeable TRIF-specific inhibitory peptide (Invivogen) or the control peptide to poly IC- or HCVcc-stimulated BDCA3$^+$ DCs. Of particular interest, the TRIF-specific inhibitor peptide, but not the control one, significantly suppressed IL-28B production from poly IC- or HCVcc-stimulated BDCA3$^+$ DCs (Fig. 6A,B). In clear contrast, the TRIF-specific inhibitor failed to suppress IL-28B from HCVcc-stimulated pDCs (Fig. 6C), suggesting that pDCs recognize HCVcc in an endosome-dependent but TRIF-independent pathway. These results show that BDCA3$^+$ DCs may recognize HCVcc by way of the TRIF-dependent pathway to produce IL-28B.

BDCA3$^+$ DCs in Subjects with IL-28B Major Genotype Produce More IL-28B in Response to HCV than Those with IL-28B Minor Type. In order to compare the ability of BDCA3$^+$ DCs to release IL-28B in healthy subjects between IL28B major (rs8099917, TT)

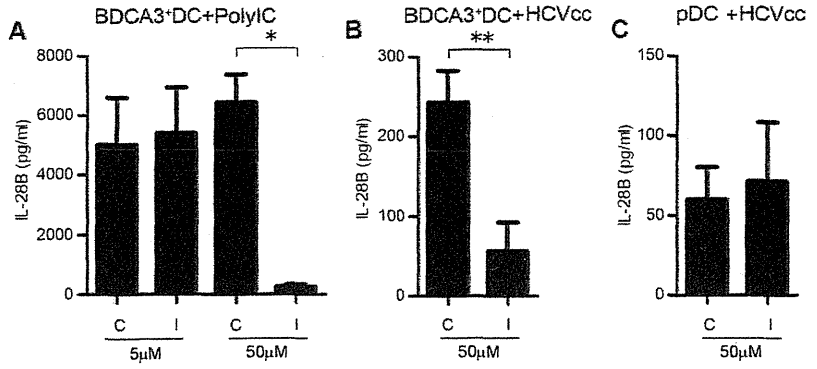

Fig. 6. BDCA3$^+$ DCs produce IL-28B upon HCVcc stimulation in a TRIF-dependent mechanism. BDCA3$^+$ DCs or pDCs had been treated with 5 or 50 mM TRIF inhibitory peptide or control peptide for 2 hours. Subsequently, BDCA3$^+$ DCs were stimulated with Poly IC (25 μg/mL) or HCVcc (MOI = 10), and pDCs were stimulated with HCVcc (MOI = 10), respectively. IL-28B was quantified by ELISA. They are shown as mean ± SEM from five experiments. *$P < 0.05$ by paired t test. C, TRIF control peptide; I, TRIF inhibitory peptide.

and minor hetero (TG) genotypes, we stimulated BDCA3$^+$ DCs of the identical subjects with poly IC (25 mg/mL, 2.5 mg/mL, 0.25 mg/mL), HCVcc or JFH-1-infected Huh 7.5.1, and subjected them to ELISA. The levels of IL-28B production by poly IC-stimulated BDCA3$^+$ DCs were comparable between subjects with IL-28B major and minor type (Fig. 7A). Similar results were obtained with the lesser concentrations of poly IC (Fig. S11). Of particular interest, in response to HCVcc or JFH-1 Huh7.5.1 cells, the levels of IL-28B from BDCA3$^+$ DCs were significantly higher in subjects with IL-28B major than those with minor type (Fig. 7B,C, S12).

Discussion

In this study we demonstrated that human BDCA3$^+$ DCs (1) are present at an extremely low frequency in PBMC but are accumulated in the liver; (2) are capable of producing IL-29/IFN-λ1, IL-28A/IFN-λ2, and IL-28B/IFN-λ3 robustly in response to HCV; (3) recognize HCV by a CD81-, endosome acidification and TRIF-dependent mechanism; and (4) produce larger amounts of IFN-λs upon HCV stimulation in subjects with IL-28B major genotype (rs8099917, TT). These characteristics of BDCA3$^+$ DCs are quite unique in comparison with other DC repertoires in the settings of HCV infection.

At the steady state, the frequency of DCs in the periphery is relatively lower than that of the other immune cells. However, under disease conditions or physiological stress, activated DCs dynamically migrate to the site where they are required to be functional. However, it remains obscure whether functional BDCA3$^+$ DCs exist or not in the liver. We identified BDCA3$^+$CLEC9A$^+$ cells in the liver tissue (Fig. 1D). In a paired frequency analysis of BDCA3$^+$ DCs between in PBMCs and in IHLs, the cells are more abundant in the liver. The phenotypes of liver BDCA3$^+$ DCs were more mature than the PBMC counterparts. In support of our observations, a recent publication showed that CD141$^+$ (BDCA3$^+$) DCs are accumulated and more mature in the liver, the trend of which is more in HCV-infected liver.[24] We confirmed that liver BDCA3$^+$ DCs are functional, capable of releasing IFN-λs in response to poly IC or HCVcc.

BDCA3$^+$ DCs were able to produce large amounts of IFN-λs but much less IFN-β or IFN-α upon TLR3 stimulation. In contrast, in response to TLR9 agonist,

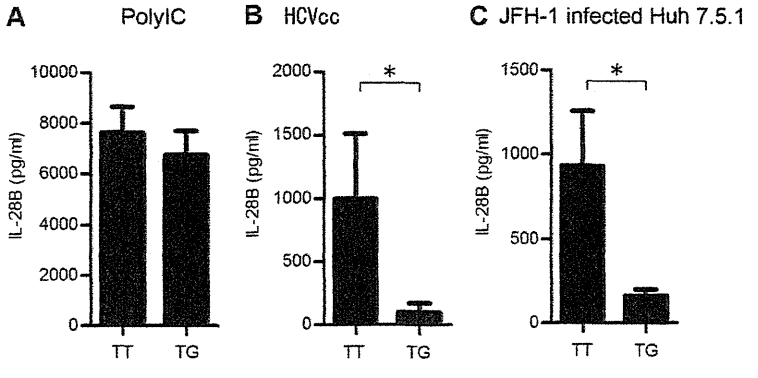

Fig. 7. In response to HCVcc, BDCA3$^+$ DCs of healthy donors with IL-28B major genotype (rs8099917, TT) produced more IL-28B than those with minor type (TG). BDCA3$^+$ DCs of healthy donors with IL-28B TT (rs8099917) or TG genotype were cultured at 2.5 × 10^4 cells with 25 mg/mL poly IC (A), with HCVcc at an MOI of 10 (B), or with JFH-1-infected Huh 7.5.1 cells (C) for 24 hours. The supernatants were subjected to IL-28B ELISA. The same healthy donors were examined for distinct stimuli. The results are the mean ± SEM from 15 donors with TT and 8 with TG, respectively. *$P < 0.05$ by Mann-Whitney U test.

pDCs released large amounts of IFN-β and IFN-α but much less IFN-λs. Such distinctive patterns of IFN response between BDCA3$^+$ DCs and pDCs are of particular interest. It has been reported that interferon regulatory factor (IRF)-3, IRF-7, or nuclear factor kappa B (NF-κB) are involved in IFN-β and IFN-λ1, while IRF-7 and NF-κB are involved in IFN-α and IFN-λ2/λ3.[5] Presumably, the stimuli with TLR3/retinoic acid-inducible gene-I (RIG-I) (poly IC) or TLR9 agonist (CpG-DNA) in DCs are destined to activate these transcription factors, resulting in the induction of both types of IFN at comparable levels. However, the results of the present study did not agree with such overlapping transcription factors for IFN-λs, IFN-β, and IFN-α. Two possible explanations exist for different levels of IFN-λs and IFN-α production by BDCA3$^+$ DCs and pDCs. First, the transcription factors required for full activation of IFN genes may differ according to the difference of DC subsets. The second possibility is that since type III IFN genes have multiple exons, they are potentially regulated by post-transcriptional mechanisms. Thus, it is possible that such genetic and/or posttranscriptional regulation is distinctively executed between BDCA3$^+$ DCs and pDCs. Comprehensive analysis of gene profiles downstream of TLRs or RIG-I in BDCA3$^+$ DCs should offer some information on this important issue.

BDCA3$^+$ DCs were found to be more sensitive to HCVcc than JEV or HSV in IL-28B/IFN-λ3 production. Such different strengths of IL-28B in BDCA3$^+$ DCs depending on the virus suggest that different receptors are involved in virus recognition. Again, the question arises of why BDCA3$^+$ DCs produce large amounts of IFN-λs compared to the amounts produced by pDCs in response to HCVcc. Considering that IRF-7 and NF-κB are involved in the transcription of the IL-28B gene, it is possible that BDCA3$^+$ DCs successfully activate both transcription factors upon HCVcc for maximizing IL-28B, whereas pDCs fail to do so. In support for this possibility, in pDCs it is reported that NF-κB is not properly activated upon HCVcc or hepatoma cell-derived HCV stimulations.[25]

In the present study we demonstrated that HCV entry into BDCA3$^+$ DCs through CD81 and subsequent endosome acidification are critically involved in IL-28B responses. Involvement of TRIF-dependent pathways in IL-28B production was shown by the significant inhibition of IL-28B with TRIF inhibitor. Nevertheless, active HCV replication in the cells is not required. Based on our data, we considered that BDCA3$^+$ DCs recognize HCV genome mainly by an endosome and TRIF-dependent mechanism. Although the results with UV-irradiated HCVcc, anti-CD81 blocking Ab, and chloroquine were quite similar, the TRIF-specific inhibitor failed to suppress IL-28B from pDCs (Fig. 6, Fig. S9).

In the coculture with JFH-transfected Huh7.5.1 cells, BDCA3$^+$ DCs presumably receive some signals for IL-28B production by way of cell-to-cell dependent and independent mechanisms. In the present study, most of the stimuli to BDCA3$^+$ DCs for IL-28B production may be the released HCVcc from Huh7.5.1 cells, judging from the inability of suppression with transwells. However, a contribution of contact-dependent mechanisms cannot be excluded in the coculture experiments. HCV genome is transmissible from infected hepatocytes to uninfected ones through tight junction molecules, such as claudin-1 and occuludin. Further investigation is needed to clarify whether such cell-to-cell transmission of viral genome is operated or not in BDCA3$^+$ DCs.

The relationship between IL-28B expression and the induction of ISGs has been drawing much research attention. In primary human hepatocytes, it is reported that HCV primarily induces IFN-λ, instead of type-I IFNs, subsequently enhancing ISG expression.[7] Of particular interest is that the level of hepatic IFN-λs is closely correlated with the strength of ISG response.[26] These reports strongly suggest that hepatic IFN-λs are a crucial driver of ISG induction and subsequent HCV eradication. Besides, it is likely that BDCA3$^+$ DCs, as a bystander IFN-λ producer in the liver, have a significant impact on hepatic ISG induction. In support of this possibility, we demonstrated in this study that BDCA3$^+$ DCs are capable of producing large amounts of IFN-λs in response to HCV, thereby inducing ISGs in the coexisting liver cells.

Controversial results have been reported regarding the relationship between IL28B genotypes and the levels of IL-28 expression. Nevertheless, in chronic hepatitis C patients with IL-28B major genotype, the IL-28 transcripts in PBMCs are reported to be higher than those with minor genotype.[2] In this study, by focusing on a prominent IFN-λ producer (BDCA3$^+$ DCs) and using the assay specific for IL-28B, we showed that the subjects with IL-28B major genotype could respond to HCV by releasing more IL-28B. Of interest, such a superior capacity of BDCA3$^+$ DCs was observed only in response to HCV but not to poly IC. Since the pathways downstream of TLR3-TRIF leading to IL-28B in BDCA3$^+$ DCs should be the same, either HCV or poly IC stimulation, two plausible explanations exist for such a distinct IL-28B response. First, it is possible that distinct epigenetic regulation may be

involved in IL-28B gene according to the IL-28B genotypes. Recently, in influenza virus infection, it is reported that micro-RNA29 and DNA methyltransferase are involved in the cyclooxygenase-2-mediated enhancement of IL-29/IFN-λ1 production.[27] This report supports the possibility that similar epigenetic machineries could be operated as well in HCV-induced IFN-λs production. Second, it is plausible that the efficiency of the stimulation of TLR3-TRIF may be different between the IL-28B genotypes. Since HCV reaches endosome in BDCA3$^+$ DCs by way of the CD81-mediated entry and subsequent endocytosis pathways, the efficiencies of HCV handling and enzyme reactions in endosome may be influential in the subsequent TLR3-TRIF-dependent responses. Certain unknown factors regulating such process may be linked to the IL-28B genotypes. For a comprehensive understanding of the biological importance of IL-28B in HCV infection, such confounding factors, if they exist, need to be explored.

In conclusion, human BDCA3$^+$ DCs, having a tendency to accumulate in the liver, recognize HCV and produce large amounts of IFN-λs. An enhanced IL-28B/IFN-λ3 response of BDCA3$^+$ DCs to HCV in subjects with IL-28B major genotype suggests that BDCA3$^+$ DCs are one of the key players in anti-HCV innate immunity. An exploration of the molecular mechanisms of potent and specialized capacity of BDCA3$^+$ DCs as IFN-λ producer could provide useful information on the development of a natural adjuvant against HCV infection.

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Mechanism of the dependence of hepatitis B virus genotype G on co-infection with other genotypes for viral replication

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SUMMARY. Hepatitis B virus (HBV) is classified into several genotypes. Genotype G (HBV/G) is characterised by worldwide dispersion, low intragenotypic diversity and a peculiar sequence of the precore and core region (stop codon and 36-nucleotide insertion). As a rule, HBV/G is detected in co-infection with another genotype, most frequently HBV/A2. In a previous *in vivo* study, viral replication of HBV/G was significantly enhanced by co-infection with HBV/A2. However, the mechanism by which co-infection with HBV/A2 enhances HBV/G replication is not fully understood. In this study, we employed 1.24-fold HBV/A2 clones that selectively expressed each viral protein and revealed that the core protein expressing construct significantly enhanced the replication of HBV/G in Huh7 cells. The introduction of the HBV/A2 core promoter or core protein or both genomic regions into the HBV/G genome showed that both the core promoter and core protein are required for efficient HBV/G replication. The effect of genotype on the interaction between foreign core protein and HBV/G showed that HBV/A2 was the strongest enhancer of HBV/G replication. Furthermore, Western blot analysis of Dane particles isolated from cultures of Huh7 cells co-transfected by HBV/G and a cytomegalovirus (CMV) promoter–driven HBV/A2 core protein expression construct indicated that HBV/G employed HBV/A2 core protein during particle assembly. In conclusion, HBV/G could take advantage of core proteins from other genotypes during co-infection to replicate efficiently and to effectively package HBV DNA into virions.

Keywords: co-transfection, core protein, genotype A, genotype G, hepatitis B virus, replication.

INTRODUCTION

Hepatitis B virus (HBV) infection affects more than 350 million people and is one of the major causes of acute and chronic liver disease. Acute HBV infection in adults is usually self-limiting, while chronic HBV infection can cause chronic hepatitis, liver cirrhosis or hepatocellular carcinoma [1]. As the clinical course in infected individuals depends on a complex interplay among various factors including viral, host and environmental factors, molecular characteristics of HBV including the genotype could become increasingly important in our understanding of HBV clinical implications [2].

Abbreviations: CMV, cytomegalovirus; CP, core promoter; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; SEAP, secreted alkaline phosphatase.

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Eight major HBV genotypes (A–H) have been identified by a sequence divergence >8% in the entire HBV genome [3,4] and have a relatively distinct geographical distribution, which may be associated with anthropological history [5]. Hepatitis B virus genotype G (HBV/G) was first described in 2000 by studies carried out in France [6]. It is usually detected during co-infection with other genotypes, most frequently with HBV/A2 [7,8]. Co-infection with HBV/C and H has also been reported [9–11]. One of the features distinguishing HBV/G from other genotypes is the 36-nucleotide (nt) insertion in its core gene [6,12]. Recent studies indicated that the 36-nt insertion increased core protein translation without enhancing mRNA abundance [13], and insertion of the 36-nt in the core region of genotypes A and D impaired genome replication, despite upregulation of core protein expression, indicating that the 36-nt insertion could alter core protein expression without altering the mRNA expression [14]. The other feature of the HBV/G genome that is unique is the possession of two stop codons in the precore region that prevents the expression of hepatitis B e antigen (HBeAg) [6,12]. Nevertheless, some HBV/G carriers are

HBeAg positive, which is explained by co-infection with an HBeAg-expressing HBV/A strain [7].

As previously reported, HBV/G monoinfection in uPA/SCID mice that had been transplanted with human hepatocytes (hereafter referred to as chimeric mice) resulted in very low level viral replication, but HBV replication increased markedly when the animals were co-infected with HBV/A2, C or H [11,15]. Furthermore, the co-infection induced more pronounced fibrosis, which concurs with findings from studies of immunosuppressed patients [16]. However, as it is still unclear how the interaction between HBV/G and other genotypes enhances the replication of HBV/G and affects the virological and clinical manifestation within an individual, we conducted *in vitro* studies using 1.24-fold HBV clones to elucidate the mechanism of HBV/G replication during co-infection.

MATERIALS AND METHODS

Plasmid constructs of HBV DNA and sequencing

Hepatitis B virus DNA was extracted from 100 μL of serum using the QIAamp DNA blood kit (Qiagen GmbH, Hilden, Germany). Four primer sets were designed to amplify two fragments (A and B) covering the entire HBV/G genome. PCR with nested primers was performed using TaKaRa LA Taq polymerase (Takara Biochemicals, Kyoto, Japan) for 35 cycles (30 s at 95° C, 30 s at 60° C and 2 min at 72° C). The primer pairs and protocols for plasmid construction are outlined in the Supporting Information. As reported previously [17], these fragments were added to the pUC19 vector, which had been deprived of promoters (Invitrogen Corp., Carlsbad, CA, USA), by digestion with *Hin*dIII and *Eco*RI, resulting in the 1.24-fold HBV genome – required to transcribe the oversized pregenome and precore messenger RNA. Cloned HBV DNA sequences were confirmed with Prism BigDye (Applied Biosystems, Foster City, CA, USA) using the ABI 3100 automated sequencer.

HBV DNA mutagenesis and construct design

HBV/A2 and HBV/G clones containing the 1.24-fold HBV genome were constructed using isolates obtained from a co-infected Caucasian patient from the San Francisco cohort described in our previous study (patient #1) [7]. The study design conformed to the 1975 Declaration of Helsinki and was approved by our institutional ethics committee. Written informed consent was obtained from the patient. The HBV/A2 clones isolated from the patient's blood specimen did not possess any precore or core promoter mutations that are known to affect HBeAg expression. To study the interaction between the different genotype isolates, the following viral protein expression constructs were prepared (outlined in Fig. 1) in HBV/A2 recombinant plasmids: HBV/A2-S, HBV/A2-core, HBV/A2-pol and HBV/A2-X and were each able to selectively translate one of the four viral proteins (the large surface, precore/core, polymerase and X proteins, respectively), whereas translation of the other three was prevented by the introduction of point mutations that produced corresponding stop codons (Fig. 1a). The following stop codons were used: (i) for surface protein: change from TTA to TAG in the 15th codon of the S gene (T198A) [18], (ii) for core protein: change from AAG to TAG in the 96th codon of the core gene (A2186T), (iii) for polymerase: change from CAACAA to TAATAA in the 283rd and 284th codons of the pol gene (C2558T/C2592T) and (iv) for X protein: change from CAA to TAA in the 7th codon of the HBx gene (C1395T) [19]. All of the above HBV/A2 recombinant plasmids possessed a TCTG motif after nucleotide position 1876, which abolished genome replication by altering the ε loop (CTGT to TCTG, nt 1877–1880) [20]. The 'HBV/A2-N' clone contained all six mutations and was used as an experimental negative control. All of the mutations in this study (substitutions, insertions and deletions) were created by overlapping PCR extension followed by the exchange of endonuclease enzyme-restricted fragments, as described previously [13,21].

Three cytomegalovirus (CMV) promoter-driven expression clones were constructed containing the whole core genes (not including the precore section) of HBV/G (nt 1901–2488), HBV/A2 (nt 1901–2458) and HBV/C (nt 1901–2452): CMV-HBV/G/core, CMV-HBV/A2/core and CMV-HBV/C/core, respectively (Fig. 1b).

Three replicating recombinant constructs were created by recombination of different genomic sections of HBV/G and HBV/A2 (Fig. 1c). The 'HBV-G/A2-CP' clone was a HBV/G-based construct in which the leading fragment containing the core promoter (CP) region (nt 1413–1806) was replaced with that of HBV/A2. The 'HBV-G/A2-CP +core' clone was also an HBV/G-based construct, in which the leading fragment containing the core promoter (CP), precore and core region (nt 1413–2821) of HBV/G was replaced with those of HBV/A2. The 'HBV-G/A2-core' clone was an HBV/G-based construct in which the fragment of the precore and core region (nt 1806–2821) was replaced with those of HBV/A2.

Cell culture and transfection

After 16 h of culture, Huh7 cells were transfected with 5 μg of DNA construct per 10-cm diameter dish using the Fugene 6 transfection reagent (Promega, Madison, WI, USA) according to the manufacturer's protocol and harvested 3 days later. Transfection efficiency was measured by co-transfection with 0.5 μg of a reporter plasmid expressing secreted alkaline phosphatase (SEAP) and normalised with subsequent SEAP measurement from culture supernatant using a SEAP reporter assay kit (TOYOBO, Osaka, Japan) [17]. Three experiments were conducted for each clone.

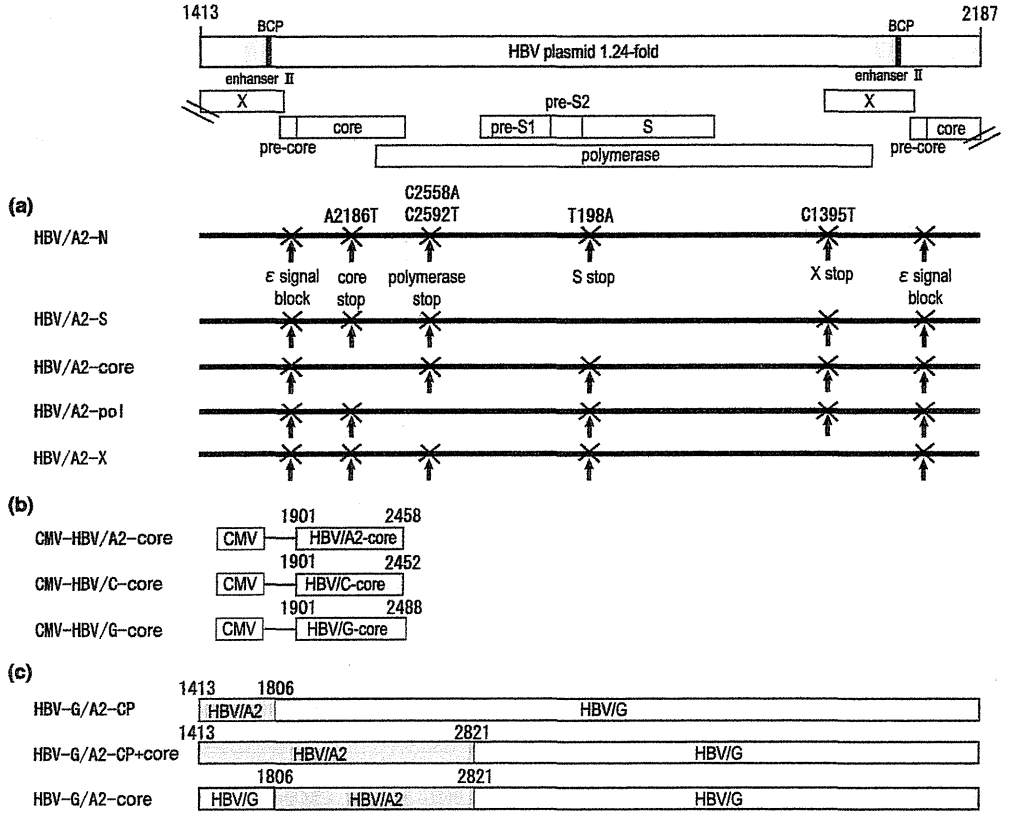

Fig. 1 HBV constructs (1.24-fold) and CMV-driven HBV core protein expression constructs used for the present study. CP, core promoter; BCP, basal core promoter; CMV, cytomegalovirus promoter. Stop codons for the corresponding HBV protein are indicated by crosses and arrows. All HBV/A2 recombinant plasmids consisted of the packaging-negative mutation (ε signal block). In three recombinant constructs between HBV/A2 and HBV/G, the corresponding recombinant genomic parts are shown by the grey bar. CMV-core constructs produce core protein without generating HBeAg in the absence of the preceding ε signal.

Determination of HBV markers

The expression levels of hepatitis B surface antigen (HBsAg) and HBeAg were determined by chemiluminescent enzyme immunoassay using commercial assay kits (Fujirebio Inc., Tokyo, Japan). The detection limit of the HBsAg assay is 0.05 IU/mL. HBV core-related antigen (HBcrAg) was measured in serum using a previously described chemiluminescent enzyme immunoassay [22]. The detection limit of the HBcrAg assay is 1.0 kU/mL.

Southern blot hybridisation

Southern blot hybridisation was performed with full-length probes for each genotype/subgenotype according to previously described methods [23]. In brief, cells were harvested and lysed in 1.5 mL of lysis buffer containing 50 mM Tris–HCl (pH 7.4), 1 mM EDTA and 1% NP-40. Half of the cell lysate was treated with 100 μg/mL of RNase A and 200 μg/mL of DNase I for 2 h at 37° C, in the presence of 6 mM Mg acetate. Then, HBV DNA was released by proteinase K digestion, extracted with phenol and precipitated with ethanol after the addition of 20 μg of glycogen. DNA was separated on a 1.2% agarose gel, transferred to a positively charged nylon membrane (Roche Diagnostics GmbH, Mannheim, Germany) and hybridised with an alkaline phosphatase-labelled full-length HBV/G or HBV/A2 fragment generated with a Gene Images AlkPhos direct labelling module (GE Healthcare, Hertfordshire, UK). The detection was performed with CDP-Star, ready-to use (Roche Diagnostics GmbH). The signals were analysed by using a LAS-3000 image analyzer (Fuji Photo Film, Tokyo, Japan).

Western blot analysis

Serum or culture medium samples were subjected to SDS-PAGE under 15–25% polyacrylamide gel electrophoresis conditions. The proteins in the gel were electroblotted onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Billerica, MA, USA) at 15 V for 45 min. The

membrane was then blocked and probed using alkaline phosphatase-conjugated HB50 (for HBcAg) or HB91 (for HBcrAg) monoclonal antibody [22] at room temperature for 1 h, before being washed and incubated with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate solution (KPL, Gaithersburg, MD, USA) for 15 min (for HBcrAg) or 90 min (for HBcAg).

Sucrose density gradient ultracentrifugation

Aliquots (1.7 mL) of 10%, 20%, 30%, 40%, 50% or 60% (w/w) sucrose in 10 mM Tris–HCl, 150 mM NaCl and 1 mM EDTA (pH 7.5) were carefully layered in a 12–mL ultracentrifuge tube and left at room temperature for 6 h. The culture supernatant of Huh7 cells that had been co-transfected with the 1.24-fold HBV genome construct (HBV/G or HBV/A) and/or the CMV-HBV/A2-core plasmid was layered onto this sucrose gradient, and ultracentrifugation was performed at 200 000 × *g* for 15 h at 4° C in a Beckman Sw40Ti rotor (Beckman Coulter, Brea, CA, USA). Fractions were collected from the top to the bottom of the gradient. The density of each fraction was calculated from its weight and volume. Each fraction was diluted 10-fold and tested for HBcrAg, HBsAg, HBeAg and HBV DNA.

Immunoprecipitation

Immunoprecipitation was carried out using magnetic beads coated with monoclonal anti-HBs from the 'Magnosphere™ MS300/Caboxyl' kit (JSR Corp., Tokyo, Japan) [24]. A 100-μL aliquot of sample was mixed with 100 μL of a magnetic bead suspension. The mixture was then incubated for 1 h at room temperature under gentle agitation and then magnetically separated. The core protein in the precipitate was analysed by Western blotting.

RESULTS

The replication of HBV/G is enhanced by HBV/A2 in co-transfection experiments

In this study, HBV/G and HBV/A2 genome clones (1.24-fold) were constructed from the serum of a HBV carrier that had been co-infected with HBV/G and HBV/A2. The HBV/G-d36 clone is a HBV/G genome-based construct in which the genotype-specific 36-nt insertion was deleted. We performed co-transfection with HBV/A2 and HBV/G clones and assessed virological features. Because of an over 12% sequence divergence between genotype A and G at the nucleotide level [12], the blot was hybridised successively with genotype-specific probes to DNA of each genotype. However, due to the unbiased binding of each probe at lower efficiency in Southern blot analysis [although the replication of HBV/A2 was higher than that of HBV/G, relative value of HBV/A2 with probe G became lower (0.63), as well as the detection of HBV/G with probe A was very weak (0.24)], each probe of genotype G or A was used for hybridisation with the HBV/A2 and HBV/G clones (Fig. 2a). The density of single-strand HBV DNA detected by the genotype-specific probes in Southern blot analysis revealed that co-transfection with HBV/A2 resulted in increased replication of the wild-type HBV/G

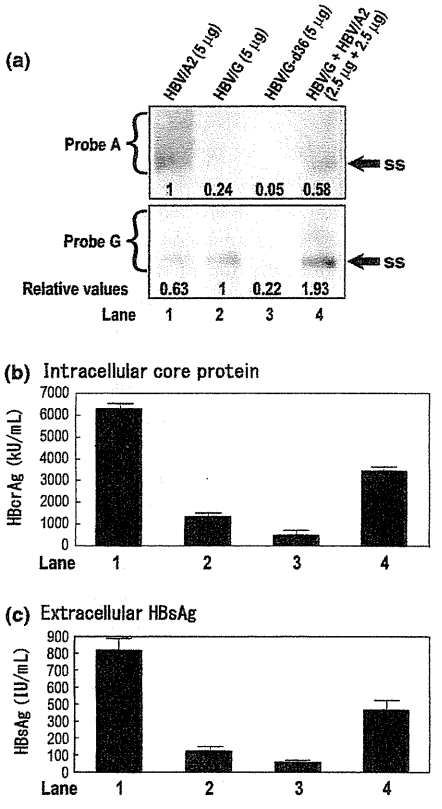

Fig. 2 (a) Southern blot analysis for replicative activity among HBV/G monotransfection, HBV/G-d36 monotransfection, HBV/A2 monotransfection and co-transfection with HBV/A2 and HBV/G (3 days after transfection). HBV/G-d36 clone was a deletion mutant lacking the 36-nt unique insertion in the core gene of the wild-type HBV/G clone. Hybridisation of the blot with genotype-specific probes of genotype A2 (upper) and G (lower). The density values shown at the bottom were measured to the probe-specific DNA sample. Single-stranded (SS) DNA is indicated by arrows. (b) Intracellular expression of core protein was estimated by detecting HBV-core-related antigen (HBcrAg) [22] as measured by a commercial chemiluminescent enzyme immunoassay (mean and standard deviation, $n = 3$). (c) HBsAg levels in the supernatant as detected by a commercial chemiluminescent enzyme immunoassay (mean and standard deviation, $n = 3$). All experiments were tested at least three times.

clone, compared with monotransfection of the wild-type HBV/G (Fig. 2a). The intracellular expression of core protein in the cell lysates and the expression of HBsAg in the culture supernatant were also enhanced by the co-transfection with both HBV/A2 and HBV/G clones (Fig. 2b and 2c). Removing the 36-nt insertion from the wild-type HBV/G genome resulted in a significant reduction in viral replication and core protein expression compared with the wild-type HBV/G clone. These results are in agreement with the observations of a previous study [13].

The core protein of HBV/A2 is essential for efficient replication of HBV/G

To determine how HBV/A2 rescues HBV/G replication during co-transfection, we constructed four HBV/A2 recombinant plasmids that selectively expressed one of the four viral proteins, whereas translation of the other three was prevented by the introduction of stop codons (Fig. 1a). All of these plasmids were prevented from coding for the viral pregenomic RNA containing the 'packaging-negative' mutation in the ε signal loop to abrogate encapsidation (see Materials and methods). Huh7 cells were co-transfected with the wild-type HBV/G clone and one of the four plasmids expressing a single viral protein. According to Southern blot analysis, the expression of intracellular HBV DNA was greatly increased when HBV/G was co-transfected with HBV/A2-core compared with the other three expression plasmids or the experimental control (pUC19 or HBV/A2-N) (Fig. 3a). The intracellular expression of core protein in the cell lysates was also the highest when HBV/G was co-transfected with HBV/A2-core (Fig. 3b). The expression of HBsAg in the culture supernatant was only increased when HBV/G was co-transfected with the HBV/A2-S plasmid (Fig. 3c). These results indicated that the core protein translated from the HBV/A2 recombinant plasmid can enhance HBV/G replication.

The core protein of HBV/A2 is more effective than those of HBV/C and HBV/G at promoting HBV/G replication

To compare the effects of genotype on the ability of the core protein to increase HBV/G replication in co-transfection experiments, we generated three genotype-specific core protein expression constructs (HBV/G, HBV/A2 and HBV/C) driven by the CMV promoter, which produced core protein in the absence of a preceding ε signal (Fig. 1c). Huh7 cells were co-transfected with HBV/G and one of the three core protein expression vectors. Southern blot analysis showed that the level of intracellular HBV DNA was highest during co-transfection with CMV-HBV/A2/core, followed by CMV-HBV/G/core, and was lowest for CMV-HBV/C/core (Fig. 4a), although the expression of core protein in the cell lysates was the highest during co-transfection with CMV-HBV/C/core, followed by CMV-HBV/G/core, and CMV-HBV/A2/core (Fig. 4b). As anticipated, there was no difference in the expression levels of HBsAg in any co-transfection experiment (Fig. 4c).

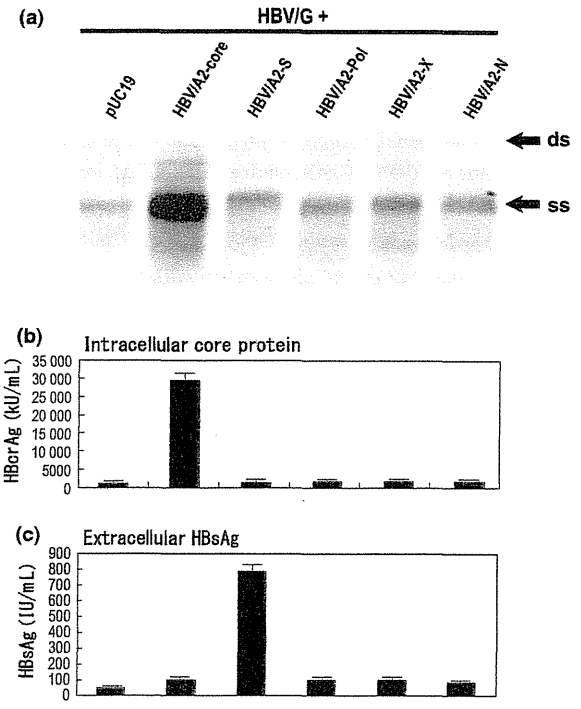

Fig. 3 (a) Southern blot analysis for replication competence of HBV/G clones co-transfected with each of the four HBV/A2 recombinant plasmids: HBV/A2-S, HBV/A2-core, HBV/A2-pol and HBV/A2-X selectively expressing only one of the four viral proteins (large surface, precore/core, polymerase or X protein, respectively). The 'HBV/A2-N' contained all the six mutations to be used as an experimental control. All of the above HBV/A2 recombinant plasmids had the 'packaging-negative' mutation in the ε signal to abrogate encapsidation. (b) Intracellular expression of core protein was measured as described in Fig. 2b. (c) The expression of HBsAg in the culture supernatant was detected as described in Fig. 2c.

A comparison of viral replication among HBV/G and recombinant HBV/G clones

To examine the effects of genetic recombination and the roles of the core promoter, precore and core genomic regions in the interaction of HBV/G and HBV/A2 during co-transfection, we employed three HBV/G and HBV/A2 chimaeric replicating constructs (see Materials and methods), which are shown in Fig. 1c. After the transfection experiment, Southern blot analysis of cell lysates indicated an abundant level of DNA expression in HBV/G/A2-CP/core–transfected cells compared with those in the cells transfected with HBV/G-wild type, HBV/G/A2-CP and HBV/G/A2-core (Fig. 5a). As shown in Fig. 5b, the highest