A

HCV RNA
(x10^6 copies/ml)
(x10^6 copies/μg RNA)

Extracellular   Intracellular

B

Infectivity
(FFU/ml)

Extracellular Intracellular

C

Specific Infectivity

BCAA (mM)

D

Secretion Rate
(Extra/Intra)

BCAA (mM)

E

Specific Infectivity

Val	–	+	–	–	+	+	–	+
Leu	–	–	+	–	+	–	+	+
Ile	–	–	–	+	–	+	+	+

F

Secretion Rate
(Extra/Intra)

Val	–	+	–	–	+	+	–	+
Leu	–	–	+	–	+	–	+	+
Ile	–	–	–	+	–	+	+	+

Fig. 4. Single-cycle virus production assay indicates a promoting effect of BCAA on virus formation. (A) Huh7-25 cells were transfected with in vitro-transcribed RNA of JFH-1, incubated in media with or without BCAA, followed by the RNA levels in the media or in the cells being calculated using the real-time quantitative RT-PCR method. (B) Infectivities in the media or in the cell lysates were measured. (C) Specific infectivities were calculated by dividing the infectivities by the HCV RNA amounts. (D) Secretion rates were calculated by dividing extracellular infectivities by intracellular infectivities. The data were presented as ratios defining the value of BCAA at 0 mM as 1. (E and F) Specific infectivities and secretion rates in the presence of valine (0.5 mM), leucine (0.5 mM), or isoleucine (0.5 mM). The data were presented as ratios defining the value with no BCAA as 1.

was translated in vitro and transfected into the Huh7-25 cells. The cells were cultured in media, with or without 1 mM of BCAA, with the RNA levels being monitored using quantitative RT-PCR. As observed in the experiment of replicon cells or virus particle formation-deficient viruses, the intracellular RNA level of HCV was suppressed by the presence of BCAA (Fig. 4A). However, the levels of extracellular HCV RNA were similar. Despite the suppression of intracellular HCV RNA levels by BCAA-containing medium, the infectivity titer of the intracellular virus in the cells treated with 1 mM BCAA was significantly higher than that of the cells with 0 mM BCAA (Fig. 4B). Extracellular infectivity titers were similar to those of intracellular infectivity. The specific infectivity of intracellular virus was calculated by dividing the infectivity titer by the HCV RNA level and this revealed that cultivation of the cells in a medium of 1 mM BCAA resulted in a 5.6-fold higher specific virus infectivity than that of 0 mM BCAA (Fig. 4C). Next, we measured

virus secretion rates by dividing extracellular infectivity titers by intracellular infectivity titers. There was a minimal difference between infectious virus particle secretions (Fig. 4D). Thus, these results indicated that the infectious virion production was promoted in the BCAA-supplemented medium, although the virus RNA replication was suppressed.

In the study using replicon cells, valine was shown amino acid responsible for regulating HCV RNA replication (Fig. 1E). Finally, we assessed the effect of individual BCAA on virus production. HCV infected cells were cultured in media containing each amino acid at 0.5 mM or a combination of them and subsequently specific infectivity and secretion rate were examined (Fig. 4E and F). Among the three BCAAs, valine promoted infectious virus production most effectively, while leucine and isoleucine promoted infectious virus production modestly. Secretion rates did not show a significant difference.

4. Discussion

In the present study, we investigated the role of BCAA in the HCV life cycle and discovered that these amino acids suppress HCV genome replication but promote viral particle formation. Thus far, many reports have indicated that various cellular factors are involved in the regulation of HCV. In particular, intracellular signaling pathways are important modulators for HCV genome replication [5–10]. BCAA, specifically leucine, were demonstrated to have a role in activating the mTOR pathway, leading to protein synthesis such as upregulation of albumin [4] and HGF production [19]. Recently, mTOR was reported to be involved in IFN-α signaling [17]. IFN-α induced phosphorylation of STAT1 was diminished by rapamycin (but not by LY294002, a PI3 kinase inhibitor). Consequently, rapamycin inhibited the IFN-stimulated regulatory element. Although we demonstrated that BCAA can activate mTOR (Fig. 2A), the inhibition of mTOR revealed that it was not the main pathway for the BCAA suppression of HCV replication. BCAA supplementation did not change the STAT1 phosphorylation status, nor did it induce ISG expression, indicating that the JAK/STAT pathway was not relevant for the suppression of HCV replication. Considering that leucine, the factor required for mTOR activation, did not actually take part in regulation of the HCV replicon (Fig. 1E), it was not surprising that mTOR was shown to not be the responsible molecule.

Very recently, Honda et al. demonstrated that STAT1 phosphorylation was increased by BCAA in a dose-dependent manner [20]. They showed that BCAA increased the phosphorylation levels of STAT1, Foxo3a and p70 S6 kinase leading to downregulation of Socs3 expression and HCV replication. The range of BCAA concentration examined in the present study was between 0 and 2 mM. We ranged the concentration of BCAA between 0 and 2 mM because its concentration in blood is approximately 1.6 mM after oral administration of 5 g of BCAA. However, in the Honda et al. study, the BCAA concentration at which STAT1 was efficiently phosphorylated was more than 4 mM, whereas at 2 mM or less, no obvious increase in phospho-STAT1 was observed. Therefore, we may have detected no change in phospho-STAT1 due to BCAA levels used in this study. Thus, BCAA may be capable of suppressing HCV genome replication at a low concentration by inhibiting HCV IRES activity while decreasing virus replication by augmentation of IFN signaling at high concentrations.

Although BCAA suppressed replication of HCV replicon, they increased HCVcc production in infected cells. The life cycle of HCV has many steps that are required to achieve infection, such as attachment to the cell surface, endocytosis of the virus, uncoating and releasing genome RNA, RNA replication, polyprotein synthesis and processing, viral assembly, and release of progeny virus. Among these, the HCV replicon system only represents the steps of genome RNA replication and non-structural protein synthesis in the cells, and BCAA affects these by impairing protein synthesis via suppression of HCV IRES activity. However, HCVcc replication requires all of these steps. We assumed that the increase of HCVcc due to BCAA indicated that some step(s) must be upregulated by BCAA to the extent of overcoming the decreased genome replication. The study of particle formation-deficient viruses suggested that virus assembly or some downstream step in the virus life cycle was critical for the augmentation of HCVcc by BCAA. A single-cycle virus production assay indicated that the production of an infectious virus was prominent in the presence of BCAA, while virus secretion was not strongly affected. Although HCV genome replication was suppressed by BCAA, more infectious virus particles were secreted into the media, and they could have re-infected the Huh7 cells. We suggest that the abundant infectious HCV in BCAA-supplemented medium causes amplification of the virus during repe-

tition of such re-infection, which leads to an accumulation of HCV in the cells, and thus, the abundance of HCV RNA in the cells with BCAA medium overcomes that without BCAA. Further investigation is needed on the detailed mechanisms defining how BCAA regulates HCV particle formation. Clarification of this process could contribute to new insights into HCV replication and could also suggest a basis for treatment of HCV patients.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.06.051.

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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

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. There-

fore, 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 $\pm$ standard deviation [SD], 37.3 $\pm$ 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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Potential conflict of interest: Nothing to report.
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-$\lambda3$ was quantified by a newly developed chemiluminescence enzyme immunoassay (CLEIA) system.[15] IL-29/IFN-$\lambda1$, IL-28A/IFN-$\lambda2$, 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-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 $\pm$ 0.044, 0.27 $\pm$ 0.21 and 1.30 $\pm$ 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 [%],
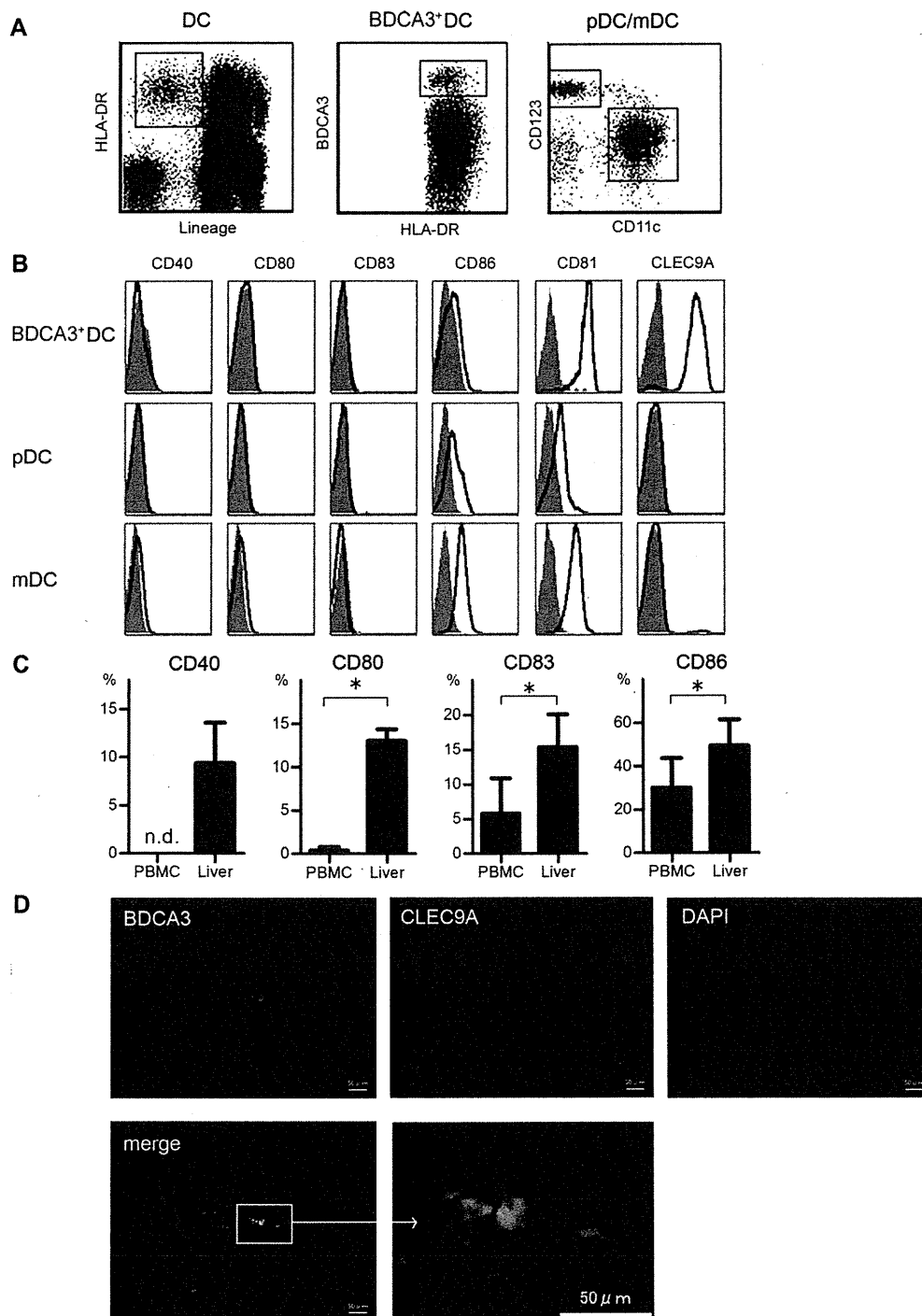
Fig. 1. Identification and phenotypic analyses of peripheral blood and intrahepatic BDCA3$^+$ DCs. (A) We defined BDCA3$^+$ DCs as Lineage$^-$HLA-DR$^+$BDCA3^{high+} cells (middle), pDCs as Lineage$^-$HLA-DR$^+$CD11c$^-$CD123^{high+} cells, and mDCs as Lineage$^-$HLA-DR$^+$CD11c$^+$CD123^{low+} cells (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.
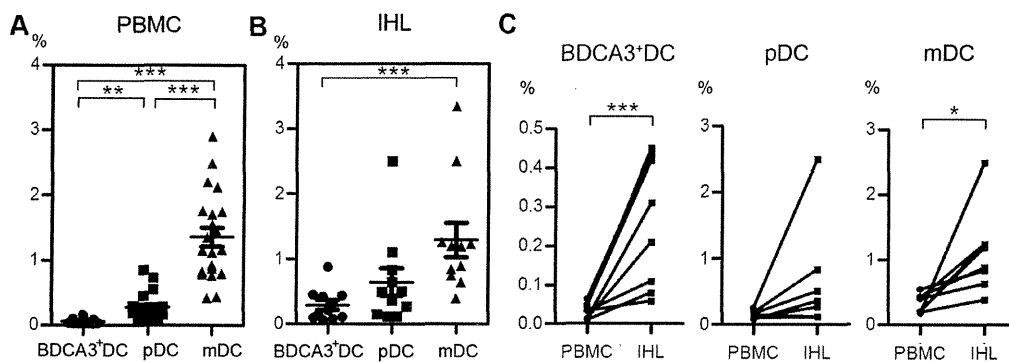
Fig. 2. Analysis of frequency of DC subsets in the peripheral blood and in the liver. Frequencies of BDCA3$^+$ DCs, pDCs, and mDCs in PBMCs (21 healthy subjects) (A) or in the intrahepatic lymphocytes (IHLs) (11 patients who had undergone surgical resection of tumors) (B) are shown. Horizontal bars depict the mean $\pm$ 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 $\pm$ 0.25, 0.65 $\pm$ 0.69 and 1.2 $\pm$ 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
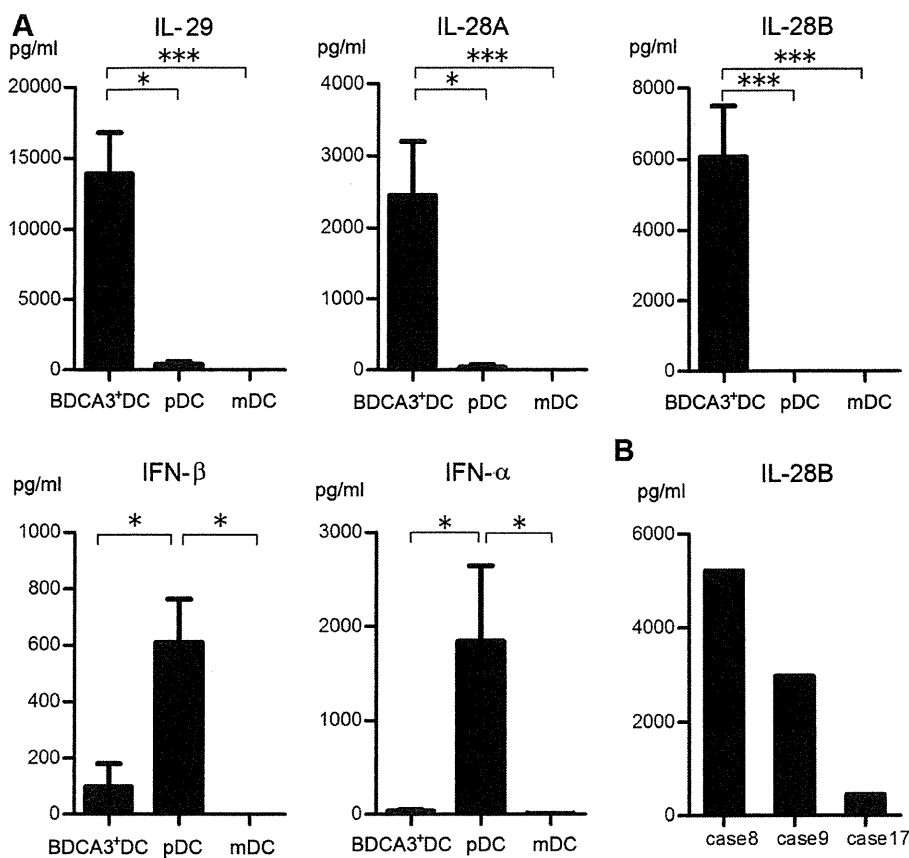
A

B

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 × 10⁴ 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 ± 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 × 10⁴ 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.
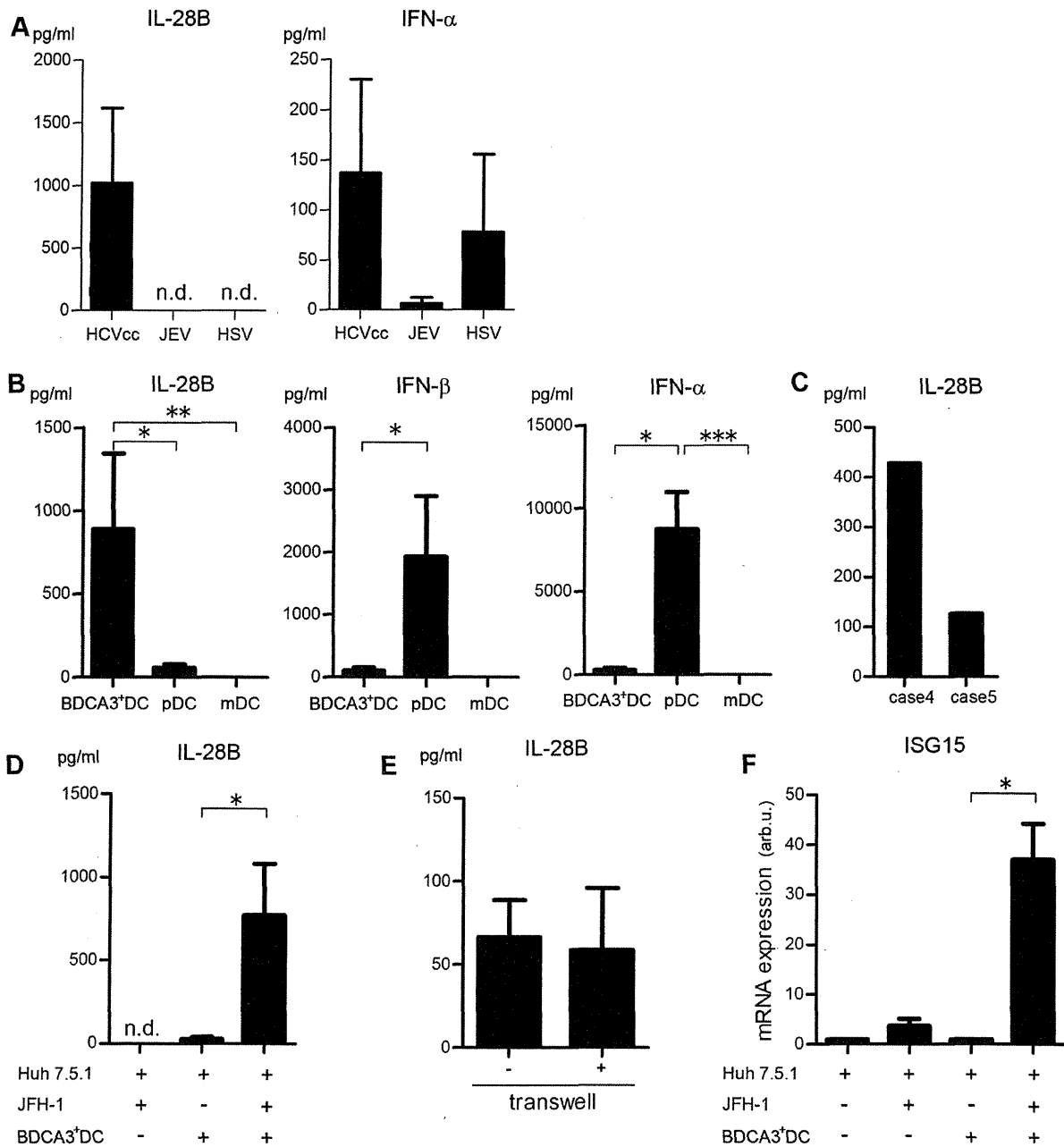
Fig. 4. BDCA3$^+$ DCs produce IL-29, IL-28A, and IL-28B upon cell-cultured HCV or HCV/JFH-1-transfected Huh7.5.1 cells, thereby inducing ISG. (A) BDCA3$^+$ DCs were cultured at 2.5 × 10^4 cells for 24 hours with HCVcc, JEV, or HSV at a multiplicity of infection (MOI) of 10. Results are shown as mean ± SEM from six experiments. n.d.; not detected. (B) BDCA3$^+$ DCs, pDCs, and mDCs were cultured at 2.5 × 10^4 cells for 24 hours with HCVcc at an MOI of 10. The results are shown as mean ± 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 × 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 × 10^4 cells with JFH-1-transfected (MOI = 2) or -untransfected Huh7.5.1 cells for 24 hours. The super-natants of JFH-1-transfected Huh7.5.1.cells without BDCA3$^+$ DCs were also examined. In some experiments of the coculture with JFH-1-trans-fected Huh7.5.1 cells and BDCA3$^+$ DCs, transwells were inserted into the wells (E). Results are shown as mean ± SEM from five experiments. *P < 0.05 by paired t test. (F) BDCA3$^+$ DCs were cocultured at 2.5 × 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 ± SEM from five experiments. *P < 0.05 by paired t test. HCVcc, cell-cultured HCV; JEV, Japanese encephalitis virus; HSV, herpes simplex virus.

A   BDCA3⁺DC+HCVcc   **B**   BDCA3⁺DC+PolyIC

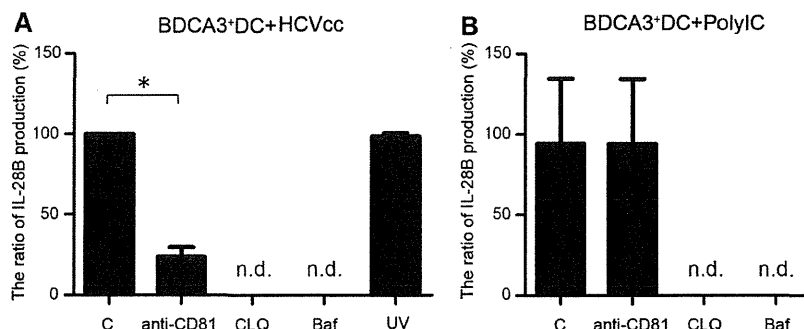Fig. 5. The CD81 and endosome acidification is involved in the production of IL-28B from HCV-stimulated BDCA3⁺ DCs, but HCV replication is not necessary. (A,B) BDCA3⁺ DCs were cultured at 2.5 × 10⁴ cells with HCVcc at an MOI of 10 (A) or poly IC (25 μg/mL) (B). In some experiments, UV-irradiated HCVcc 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 ± 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, bafilo-

mycin 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)

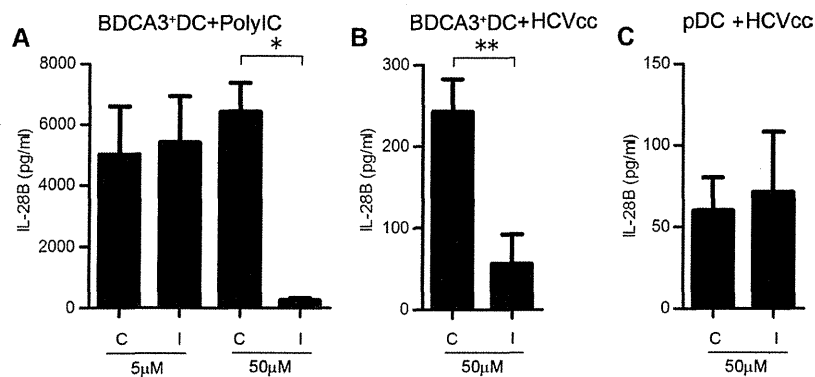**A** BDCA3⁺DC+PolyIC **B** BDCA3⁺DC+HCVcc **C** pDC +HCVcc

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,

A PolyIC   **B** HCVcc   **C** JFH-1 infected Huh 7.5.1
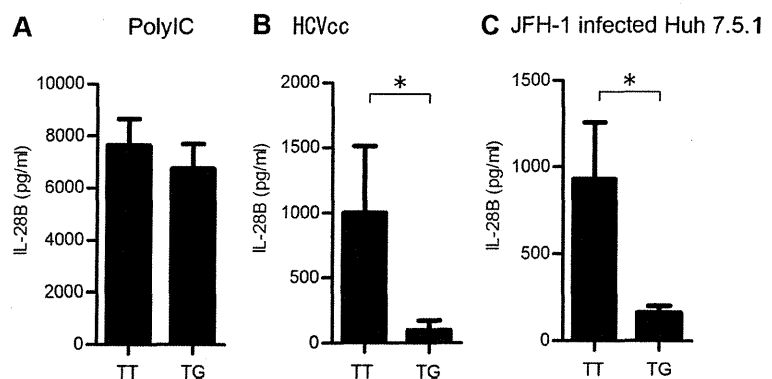
Fig. 7. In response to HCVcc, BDCA3⁺ DCs of healthy donors with IL-28B major genotype (rs8099917, TT) produced more IL-28B than those with minor type (TG). BDCA3⁺ DCs of healthy donors with IL-28B TT (rs8099917) or TG genotype were cultured at 2.5 × 10⁴ cells with 25 mg/mL poly IC (A), with HCVcc at 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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Special Report

Etiology and prognosis of fulminant hepatitis and late-onset hepatic failure in Japan: Summary of the annual nationwide survey between 2004 and 2009

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Aim: To summarize the annual nationwide survey on fulminant hepatitis (FH) and late-onset hepatic failure (LOHF) between 2004 and 2009 in Japan.

Methods: The annual survey was performed in a two-step questionnaire process to detail the clinical profile and prognosis of patients in special hospitals.

Results: Four hundred and sixty (*n* = 227 acute type; *n* = 233 subacute type) patients had FH and 28 patients had LOHF. The mean age of patients with FH and LOHF were 51.1 ± 17.0 and 58.0 ± 14.4 years, respectively. The causes of FH were hepatitis A virus in 3.0%, hepatitis B virus (HBV) in 40.2%, other viruses in 2.0%, autoimmune hepatitis in 8.3%, drug allergy-induced in 14.6% and indeterminate etiology in 29.6% of patients. HBV reactivation due to immunosuppressive therapy was observed in 6.8% of FH patients. The short-term survival rates of patients without liver transplantation (LT)

were 48.7% and 24.2% for the acute and subacute type, respectively, and 13.0% for LOHF. The prognosis was poor in patients with HBV reactivation. The implementation rate for LT in FH patients was equivalent to that in the previous survey. The short-term survival rates of total patients, including LT patients, were 54.2% and 40.8% for the acute and subacute type, respectively, and 28.6% for LOHF.

Conclusion: The demographic features and etiology of FH patients has gradually changed. HBV reactivation due to immunosuppressive therapy is problematic. Despite advances in therapeutic approaches, the prognosis of patients without LT has not improved.

Key words: acute liver failure, fulminant hepatitis, Japan, liver transplantation, viral hepatitis

INTRODUCTION

IN JAPAN, FULMINANT hepatitis (FH) is defined as having hepatitis, when grade II or worse hepatic

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encephalopathy develops within 8 weeks of the onset of disease symptoms, with a prothrombin time of 40% or less.[1,2] FH is further classified into two subtypes, acute and subacute types, in which encephalopathy occurs within 10 days and later than 11 days, respectively, of the onset of the disease symptoms. Patients showing a prothrombin time of 40% or less, with hepatic encephalopathy developing between 8 and 24 weeks of disease onset are classified as having late-onset hepatic failure (LOHF).[3] Etiologies with hepatitis present in the histology, such as viral infection, autoimmune hepatitis and drug allergy-induced liver injury are defined as causes of

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FH and LOHF. In contrast, acute liver failure due to other causes with the absence of hepatitis in the histology, such as drug toxicity, circulatory disturbance and metabolic disease, are excluded as causes of FH and LOHF. Recently, a novel diagnostic criteria for acute liver failure in Japan was established by the Intractable Hepato-Biliary Disease Study Group.[4,5] These criteria included other causes with liver damage without the absence of hepatitis in the histology in addition to the present criteria.

Among viral infection, hepatitis B virus (HBV) is a major cause of FH in Japan.[6,7] HBV infection is classified into transient HBV infection type and acute exacerbation in an HBV inactive carrier. With advances in cytotoxic chemotherapy and immunosuppressive therapy, reactivation of hepatitis B is becoming a clinical problem.[8] Moreover, recent introduction of rituximab plus steroid combination therapy for non-Hodgkin's lymphoma has been associated with HBV reactivation in transiently infected patients, namely, de novo hepatitis. However, the prevalence of HBV reactivation in patients with FH and LOHF is unknown.

Advances in therapeutic strategies for FH and LOHF have improved the prognosis. Since 1988, living-donor liver transplantation (LT) has been adopted in patients who are beyond the supportive care of a critical unit.[6] Recently, artificial liver support with high-flow or on-line hemodiafiltration (HDF) has been used. Since 2006, a nucleoside analog, entecavir, has been used as a substitute for lamivudine, as an antiviral agent for HBV. However, it is unknown whether these new treatments improve the prognosis of FH.

The Intractable Hepato-Biliary Diseases Study Group has annually performed a nationwide survey of patients with FH and LOHF since 1983.[6] Since 2000, approximately 600 hospitals have been enrolled in the survey. This report summarizes the results of the survey between 2004 and 2009 to addresses the trends in the etiology and prognosis of patients with FH and LOHF and compares them with the previous survey.[7]

METHODS

THE NATIONWIDE SURVEY was performed annually. The number of hospitals for survey has changed in each year. Maximum (608) was in 2007 and minimum (544) was in 2006, with active members of the Japan Society of Hepatology and the Japanese Society of Gastroenterology between 2005 and 2010. The survey was performed in a two-step questionnaire process to detail the clinical profile and prognosis of patients who were

diagnosed as FH or LOHF in the previous year. The recovery rate of the first and second questionnaire was 39–59% and 60–100%, respectively. Patients who met the diagnostic criteria for FH or LOHF were entered into the survey. Patients under 1 year of age, those with alcoholic hepatitis, those with chronic liver diseases and those with acute liver failure with no histological features of hepatitis were excluded from the analysis.

According to criteria described in previous reports,[7,9] the etiology of FH and LOHF was classified into five categories: (i) viral infection; (ii) autoimmune hepatitis; (iii) drug allergy-induced liver injury; (iv) indeterminate etiology despite sufficient examinations; and (v) unclassified due to insufficient examinations. Patients with viral infection consisted of those with hepatitis A virus (HAV), HBV, hepatitis C virus (HCV), hepatitis E virus (HEV) and other viruses. The patients with HBV infection were classified into three subgroups according to serum markers of HBV, hepatitis B core antibody (HBcAb) and immunoglobulin (Ig)M-HBcAb: (i) transient HBV infection; (ii) acute exacerbation in HBV carriers; and (iii) indeterminate infection patterns. In the present study, we classified acute exacerbation in HBV carriers into three subgroups according to the new criteria:[4,5] (i) inactive carriers, without drug exposure; (ii) reactivation in inactive carriers by immunosuppressant and/or anticancer drugs; and (iii) reactivation in transiently infected patients by immunosuppressant and/or anticancer drugs (i.e. de novo hepatitis). Because not every patient was examined for serological markers of transient HBV infection before the onset of FH and LOHF (with HBcAb and/or hepatitis B surface antigen [HBsAg] in serum), we defined HBV reactivation as that occurring in transiently infected patients when they developed HBV-related hepatitis due to immunosuppressive therapy or cytotoxic chemotherapy with reappearance of HBsAg in the serum and did not conform to the criteria of transient HBV infection.

The statistical significance of differences between groups was assessed using Student's *t*-test, Fisher's exact test or Kruskal–Wallis one-way ANOVA. Data are shown as mean ± standard deviation. The study was conducted with the approval of the Ethical Committee of Kagoshima University Graduate School of Medical and Dental Sciences.

RESULTS

Demographic features and survival rates

FROM 2004–2009, 582 PATIENTS were enrolled in the survey. Ninety-four patients were excluded from

the survey according to the exclusion criteria. Consequently, 460 patients ($n = 227$ acute type; $n = 233$ subacute type) were classified as having FH and 28 as having LOHF (Table 1). The incidence of the acute and subacute types of FH was similar and the incidence of LOHF was one-sixteenth of FH. The male : female ratio was higher for the acute type and lower for the subacute type of FH and LOHF. The mean age of patients was significantly higher for the subacute type of FH and LOHF than that for the acute type of FH. Almost half of the patients with FH and LOHF had complications which preceded the onset of acute liver failure. Furthermore, approximately 60% of patients with FH had received daily medication. This tendency for receiving medication was more obvious in patients with the subacute type of FH and LOHF.

The survival rates of patients without LT were 48.7% for the acute type and 24.2% for the subacute type of FH, and 13.0% for LOHF. The survival rates of the subacute type of FH and LOHF was worse than that of the acute type. The prognosis of both the acute type and the subacute type of FH appeared to be equivalent annually. The survival rates of patients with LT were 79.6% for FH and 100% for LOHF, with no difference in these rates among the disease types.

Clinical profile

Symptoms, imaging findings and complications are shown in Table 2. Since 2006, diagnostic criteria of systemic inflammatory response syndrome (SIRS) for fever, tachycardia and tachypnea have been adopted in the survey.[10] Icterus, flapping tremor, ascites, hepatic fetor, tachycardia, tachypnea and pretibial edema were frequently found. The frequency of patients with ascites and pretibial edema was significantly greater in the subacute type of FH and LOHF than in the acute type of FH. In contrast, fever appeared more frequently in patients with the acute type of FH. The frequency of liver atrophy was greater in the subacute type of FH, and even higher in LOHF, than in the acute type of FH.

With regard to complications, disseminated intravascular coagulation, renal failure and bacterial infection were found in more than 30% of patients with FH and LOHF. Brain edema was less frequent in the subacute type than in the acute type of FH.

Causes of FH and LOHF

The cause of FH was identified as viral infection in 46.1% of the patients (Table 3). The frequencies of viral infection were highest for the acute type of FH. HAV infection was found in 3% of patients with FH. HBV infection was found in 40.2% of patients with FH and 32.1% of patients with LOHF. Transient HBV infection was more frequent in the acute type than in the subacute type of FH, whereas the frequency of acute exacerbation in HBV carriers was greater in the subacute type than in the acute type of FH. HBV reactivation in inactive carriers and in transiently infected patients were found in 3.3% and 3.5% of patients with FH, respectively. With regard to underlying diseases in patients with HBV reactivation, non-Hodgkin's lymphoma/mucosa-associated lymphoid tissue lymphoma was most prevalent in 50% of inactive carriers and in 76% of those with transiently infected patients. Among patients with HBV

Table 1 Demographic features and survival rates of patients with fulminant hepatitis (FH) and late-onset hepatic failure (LOHF)

	FH			LOHF ($n = 28$)
	Total ($n = 460$)	Acute type ($n = 227$)	Subacute type ($n = 233$)	
Male/female	227/233	127/100	100/133**	9/19*
Age (years; mean ± SD)	51.1 ± 17.0	48.8 ± 16.9	53.4 ± 16.7**	58.0 ± 14.4**
HBV carrier (%)	13.1 (52/397)	10.5 (19/181)	15.3 (33/216)	22.2 (6/27)
Complications preceding acute liver failure (%)†	46.4 (208/448)	40.0 (88/220)	52.6 (120/228)**	50.0 (14/28)
History of medication (%)	59.9 (260/434)	51.2 (108/211)	68.2 (152/223)**	71.4 (20/28)*
Survival rates				
All patients	47.4 (218/460)	54.2 (123/227)	40.8 (95/233)**	28.6 (8/28)*
No LT	37.5 (132/352)	48.7 (93/191)	24.2 (39/161)**	13.0 (3/23)**
LT	79.6 (86/108)	83.3 (30/36)	77.8 (56/72)	100 (5/5)

$*P < 0.05$, $**P < 0.01$ vs acute type.
†Diseases such as metabolic syndrome, malignancy and psychiatric disorders.
Data in parenthesis indicate patient numbers.
HBV, hepatitis B virus; LT, liver transplantation; SD, standard deviation.

Table 2 Symptoms, imaging findings and complications of patients with fulminant hepatitis (FH) and late-onset hepatic failure (LOHF)

		FH		LOHF ($n = 28$)
	Total ($n = 460$)	Acute type ($n = 227$)	Subacute type ($n = 233$)	
(a) Symptoms at diagnosis				
Fever†	13.0 (42/322)	17.5 (28/160)	8.6 (14/162)*	0 (0/23)*
Icterus	96.8 (427/441)	95.0 (208/219)	98.6 (219/222)*	96.4 (27/28)
Ascites	57.2 (237/414)	45.2 (88/204)	71.0 (149/210)**	81.5 (22/27)**
Convulsion	5.2 (22/422)	6.7 (14/210)	3.8 (8/212)	0 (0/27)
Tachycardia‡	36.7 (117/319)	39.5 (62/157)	34.0 (55/162)	47.8 (11/23)
Tachypnea§	34.5 (87/252)	39.1 (52/133)	29.4 (35/119)	31.6 (6/19)
Flapping tremor	79.0 (309/391)	75.8 (144/190)	82.1 (165/201)	80.8 (21/26)
Hepatic fetor	46.6 (146/313)	49.0 (73/149)	44.5 (73/164)	42.1 (8/19)
Pretibial edema	35.5 (127/358)	24.1 (42/174)	46.2 (85/184)**	75.0 (15/20)**,***
(b) Imaging findings				
Liver atrophy¶	58.8 (255/434)	45.6 (98/215)	71.7 (157/219)**	92.6 (25/27)**,***
(c) Complications				
Infection	34.8 (149/428)	32.9 (68/207)	36.7 (81/221)	51.9 (14/27)
Brain edema	18.5 (71/384)	24.1 (46/191)	13.0 (25/193)**	22.7 (5/22)
Gastrointestinal bleeding	13.2 (59/446)	11.0 (24/219)	15.4 (35/227)	20.0 (5/25)
Renal failure	38.9 (177/455)	40.9 (92/225)	37.0 (85/230)	39.3 (11/28)
DIC	34.6 (150/433)	35.7 (76/213)	33.6 (74/220)	53.8 (14/26)
Congestive heart failure	7.3 (31/427)	8.9 (19/214)	5.6 (12/213)	12.0 (3/25)

*$P < 0.05$, **$P < 0.01$ vs acute type, ***$P < 0.05$ vs subacute type.
†Temperature: >38°C or <36°C.
‡Heart rate: >90 beats/min.
§Respiratory rate: >20 breaths/min or $PaCO_2$: <32 Torr.
† ‡ § Cases between 2005 and 2009.
¶Liver atrophy detected by ultrasound and/or computed tomography imaging.
Data in parentheses indicate patient numbers.
DIC, disseminated intravascular coagulation.

reactivation, rituximab plus steroid combination chemotherapy was administered to 35% of patients in inactive carriers and to 59% of those with transiently infected patients. HCV and HEV infection were less frequently found. In the survey, Epstein–Barr virus, herpes simplex virus and human herpes virus type-6 were found as other causes of viral hepatitis.

Autoimmune hepatitis was frequently observed in patients with the subacute type of FH and LOHF. Drug allergy-induced liver injury was observed in approximately 10–20% of patients irrespective of disease types. Anti-tuberculosis agents, non-steroidal anti-inflammatory drugs, anticancer agents, drugs for metabolic syndrome, and various herbal and natural remedies were the probable causative agents for this liver injury in the survey. Notably, the etiology was indeterminate in approximately 40% of patients with the subacute type of FH.

Therapies

For artificial liver support, plasma exchange and HDF were performed in most patients with FH (Table 4). Conventional HDF and continuous HDF (CHDF) were performed in 22.5% and 51.8% of patients with FH, respectively. A more powerful method, high-flow HDF (HF-HDF), high-flow CHDF (HF-CHDF) and on-line HDF were performed in 2.6%, 11.7% and 1.8% of the patients, respectively. The nucleoside analogs lamivudine and entecavir were used in approximately a quarter of patients with FH. Entecavir were used more frequently than lamivudine since 2007. Glucocorticosteroid, mainly as steroid pulse therapy, were administrated in more than 70% of patients with FH and LOHF. Anti-coagulation therapy were performed in approximately 40–50% of patients with FH and LOHF. Glucagon/insulin, branched-chain amino acid-rich solution,

Table 3 Causes of fulminant hepatitis (FH) and late-onset hepatic failure (LOHF)

	FH			LOHF (n = 28)
	Total (n = 460)	Acute type (n = 227)	Subacute type (n = 233)	
Viral infection	46.1 (212)	62.6 (142)	30.0 (70)	32.1 (9)
HAV	3.0 (14)	5.7 (13)	0.4 (1)	0 (0)
HBV	40.2 (185)	54.2 (123)	26.6 (62)	32.1 (9)
(1) Transient infection	19.6 (90)	35.2 (80)	4.3 (10)	3.6 (1)
(2) Acute exacerbation in HBV carrier	14.1 (65)	7.9 (18)	20.2 (47)	25.0 (7)
(i) Inactive carrier, without drug exposure	7.4 (34)	6.2 (14)	8.6 (20)	3.6 (1)
(ii) Reactivation in inactive carrier†	3.3 (15)	1.8 (4)	4.7 (11)	17.9 (5)
(iii) Reactivation in transiently infected patient‡	3.5 (16)	0 (0)	6.9 (16)	3.6 (1)
(3) Indeterminate infection patterns	6.5 (30)	11.0 (25)	2.1 (5)	3.6 (1)
HCV	1.1 (5)	0.9 (2)	1.3 (3)	0 (0)
HEV	0.9 (4)	0.9 (2)	0.9 (2)	0 (0)
Other viruses	0.9 (4)	0.9 (2)	0.9 (2)	0 (0)
Autoimmune hepatitis	8.3 (38)	2.2 (5)	14.2 (33)	32.1 (9)
Drug allergy-induced liver injury	14.6 (67)	13.7 (31)	15.5 (36)	17.9 (5)
Indeterminate§	29.6 (136)	19.4 (44)	39.5 (92)	17.9 (5)
Unclassified¶	1.5 (7)	2.2 (5)	0.9 (2)	0 (0)

†Reactivation in inactive carrier by immunosuppressant and/or anticancer drugs.
‡Reactivation in transiently infected patients by immunosuppressant and/or anticancer drugs (de novo hepatitis).
§Indeterminate etiology despite sufficient examinations.
¶Unclassified due to insufficient examinations.
Data in parentheses indicate patient numbers.
HAV, hepatitis A virus; HBV, hepatitis B virus; HCV, hepatitis C virus; HEV, hepatitis E virus.

cyclosporin A and prostaglandin E_1 therapy were administrated less frequently compared with the previous survey.

Liver transplantation was performed in 23.5% and 17.9% of patients with FH and LOHF, respectively. Two patients received deceased-donor LT and 111 patients received living-donor LT. The frequency of LT was significantly greater in the subacute type than in the acute type of FH.

Prognosis

The prognosis of patients with FH and LOHF differed depending on the etiology (Table 5). Prognosis was good in patients with HAV infection. The prognosis was fair in patients with transient HBV infection. In contrast, the prognosis was poor in acute exacerbation in HBV carriers. The prognosis was extremely poor in patients with HBV reactivation, either from inactive carriers or transiently infected patients. Patients with the subacute type of FH and LOHF caused by autoimmune hepatitis, drug allergy-induced liver injury and indeterminate etiology also showed a poor prognosis.

The clinical features of the patients appeared to be associated with the prognosis. In the acute type of FH with no LT, the frequency of patients with SIRS (tachycardia or tachypnea) was greater in patients who died than in surviving patients ($P < 0.05$). Liver atrophy on ultrasound and/or computed tomography imaging was an important factor in predicting the prognosis of FH and LOHF with no LT. The frequencies were 25.0% and 64.5% in patients with the acute type ($P < 0.01$) and 55.6% and 78.1% in those with the subacute type of FH in surviving patients and those who died, respectively ($P < 0.05$).

Prognosis also appeared to be affected by complications. Any of the complications significantly decreased survival rate (data not shown). Furthermore, the number of these complications affected the prognosis. The survival rate of patients with the acute type of FH was greater than 80% in those with no complications, while it was less than 30% in those with two or more complications. The survival rate of patients with the subacute type of FH was decreased in proportion to the number of complications.

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Table 4 Therapies for patients with fulminant hepatitis (FH) and late-onset hepatic failure (LOHF)

	FH			LOHF (n = 28)
	Total (n = 460)	Acute type (n = 227)	Subacute type (n = 233)	
Plasma exchange	90.9 (418/460)	92.5 (210/227)	89.3 (208/233)	71.4 (20/28)**,***
Hemodiafiltration	75.0 (342/456)	75.1 (169/225)	74.9 (173/231)	57.1 (16/28)
Glucocorticosteroids	72.4 (333/460)	68.3 (155/227)	76.4 (178/233)	89.3 (25/28)*
Glucagon/insulin	14.6 (67/459)	13.7 (31/227)	14.7 (34/232)	17.9 (5/28)
BCAA-rich solution	19.1 (87/456)	14.3 (32/223)	23.6 (55/233)*	39.3 (11/28)**
Prostaglandin E_1	7.0 (32/458)	6.7 (15/225)	7.3 (17/233)	3.6 (1/28)
Cyclosporin A	10.0 (46/460)	7.0 (16/227)	12.9 (30/233)*	10.7 (3/28)
Interferon	14.1 (65/460)	15.4 (35/227)	12.9 (30/233)	10.7 (3/28)
Nucleoside analog	38.9 (179/460)	50.9 (115/226)	27.5 (64/233)**	32.1 (9/28)
Lamivudine	25.5 (116/455)	40.0 (76/224)	30.4 (40/231)	12.5 (6/28)
Entecavir†	22.4 (70/312)	27.7 (41/148)	17.7 (29/164)	33.3 (5/15)
Anticoagulation therapy‡	47.2 (216/458)	43.2 (98/227)	51.1 (118/231)	39.3 (11/28)
Liver transplantation	23.5 (108/460)	15.9 (36/227)	30.9 (72/233)	17.9 (5/28)

*P < 0.05, **P < 0.01 vs acute type, ***P < 0.05 vs subacute type.
†Cases between 2006 and 2009.
‡Drugs such as antithrombin III concentrate and protease inhibitor compounds, gabexate mesylate and nafamostat mesilate.
Data in parentheses indicate patient numbers.
BCAA, branched-chain amino acid.

DISCUSSION

IN THIS SURVEY, 488 patients were enrolled over 6 years. In the previous 6-year survey, 697 patients (634 for FH and 64 for LOHF) were enrolled.[7] The incidence ratio of LOHF to FH was decreased from 9:1 to 16:1. In national epidemiology research, the annual incidence of FH was estimated at 1050 cases in 1996 and 429 cases in 2004.[11] Therefore, the incidence of FH and LOHF could be decreasing longitudinally. In this survey, the mean age of patients with FH and LOHF was older than that in the previous survey. More patients with complications received daily medication compared with the previous survey. Changes in demographic features of the patients may affect the etiology and prognosis of FH. A relationship between daily dose of oral medication and idiosyncratic drug-induced liver injury has been reported.[12] Additionally, older age is considered a poor prognostic factor in acute liver failure and may be considered a relative contraindication for LT.[13,14]

The current study showed that HBV still remains a major cause of FH and LOHF. Notably, almost half of acute exacerbations in HBV carriers occurred in patients with HBV reactivation owing to immunosuppressive or cytotoxic therapy. Approximately 80% of patients with transiently infected patients had received rituximab plus steroid combination therapy for non-Hodgkin's lym-

phoma. This combination therapy has been identified as a risk factor for HBV reactivation in HBsAg positive/ negative patients with non-Hodgkin's lymphoma.[15,16] Our survey revealed that careful attention is necessary for transiently infected patients, as well as for inactive HBV carriers using intensive immunosuppressive agents.

The frequency of HAV infection in patients with FH was decreased compared with the previous survey. This result is compatible with no occurrence of outbreak of acute hepatitis A during this period. In Japan, zoonotic transmission from pigs, wild boar and deer, either foodborne or otherwise, is the cause of HEV infection.[17,18] In the currently studied survey, two-thirds of the patients were from endemic areas (Hokkaido Island and the northern part of mainland Honshu) in Japan.

The other principal finding in this survey was that the etiology was indeterminate in approximately 40% of patients with FH. One of the reasons for this result may be the failure of diagnosis for autoimmune hepatitis or drug-induced liver injury. Although the diagnosis of autoimmune hepatitis relies on the presence of serum autoantibodies, with higher IgG levels (>2 g/dL), acute-onset autoimmune hepatitis does not always show typical clinical features.[19–21] Additionally, the sensitivity of the drug-induced lymphocyte stimulation test for diagnosis is not completely reliable.