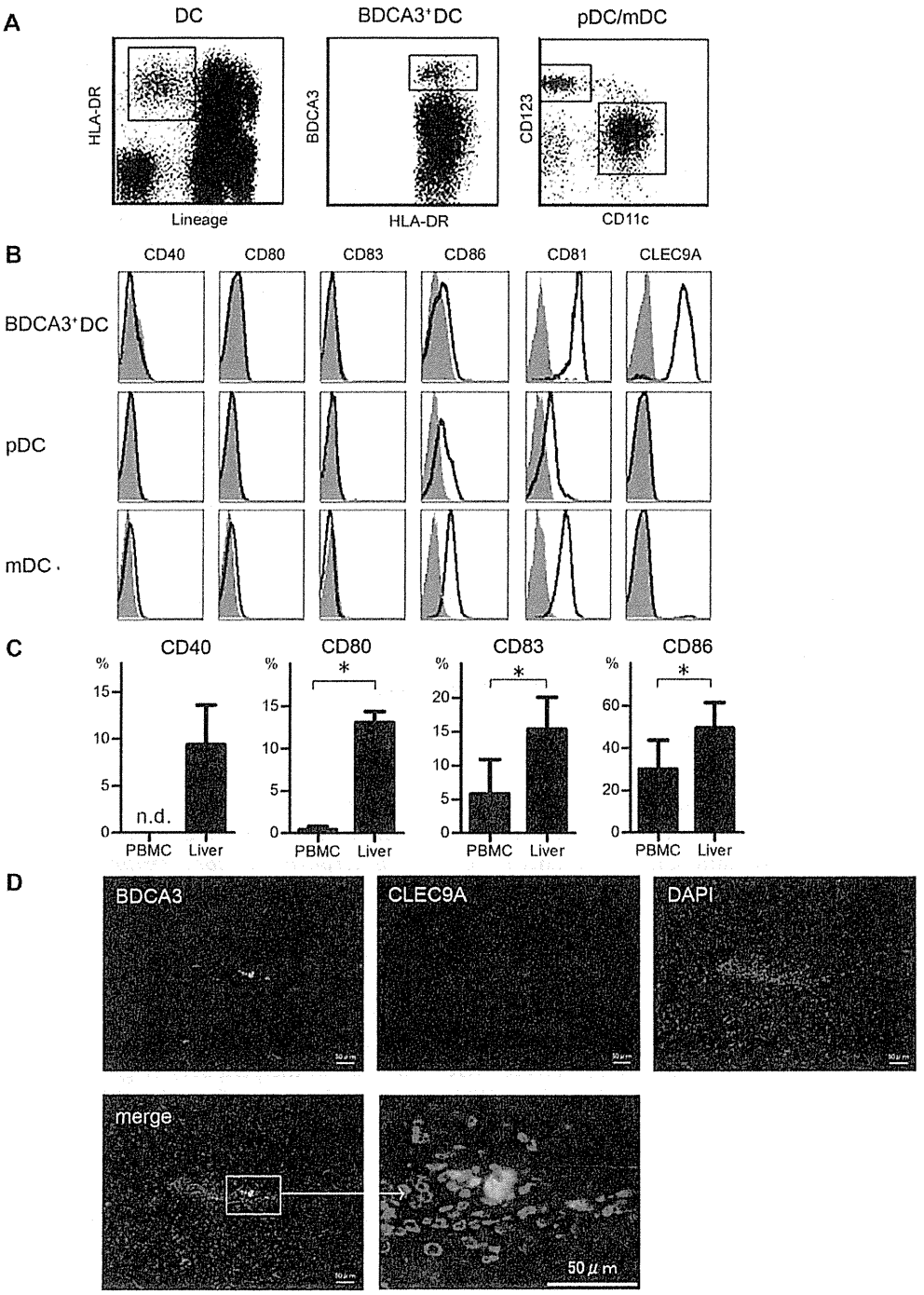

Fig. 1. Identification and phenotypic analyses of peripheral blood and intrahepatic BDCA3$^+$ DCs. (A) We defined BDCA3$^+$ DCs as Lineage$^-$HLA-DR$^+$BDCA3^{high+} cells (middle), pDCs as Lineage$^-$HLA-DR$^+$CD11c$^-$CD123^{high+} cells, and mDCs as Lineage$^-$HLA-DR$^+$CD11c$^+$CD123^{low+} cells (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.

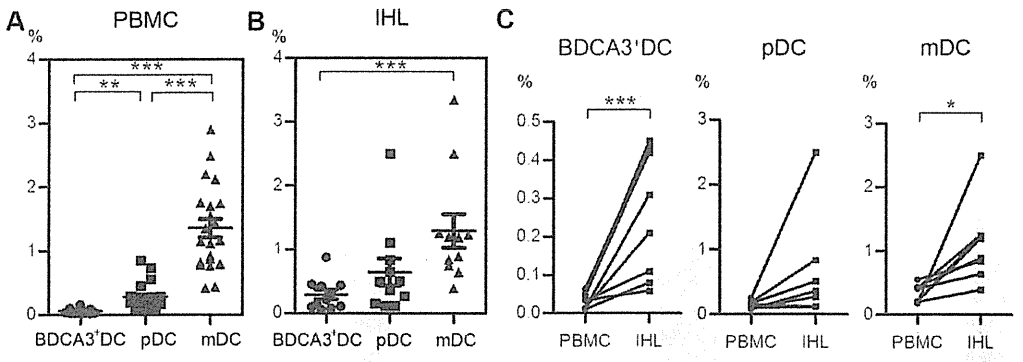

Fig. 2. Analysis of frequency of DC subsets in the peripheral blood and in the liver. Frequencies of BDCA3$^+$ DCs, pDCs, and mDCs in PBMCs (21 healthy subjects) (A) or in the intrahepatic lymphocytes (IHLs) (11 patients who had undergone surgical resection of tumors) (B) are shown. Horizontal bars depict 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

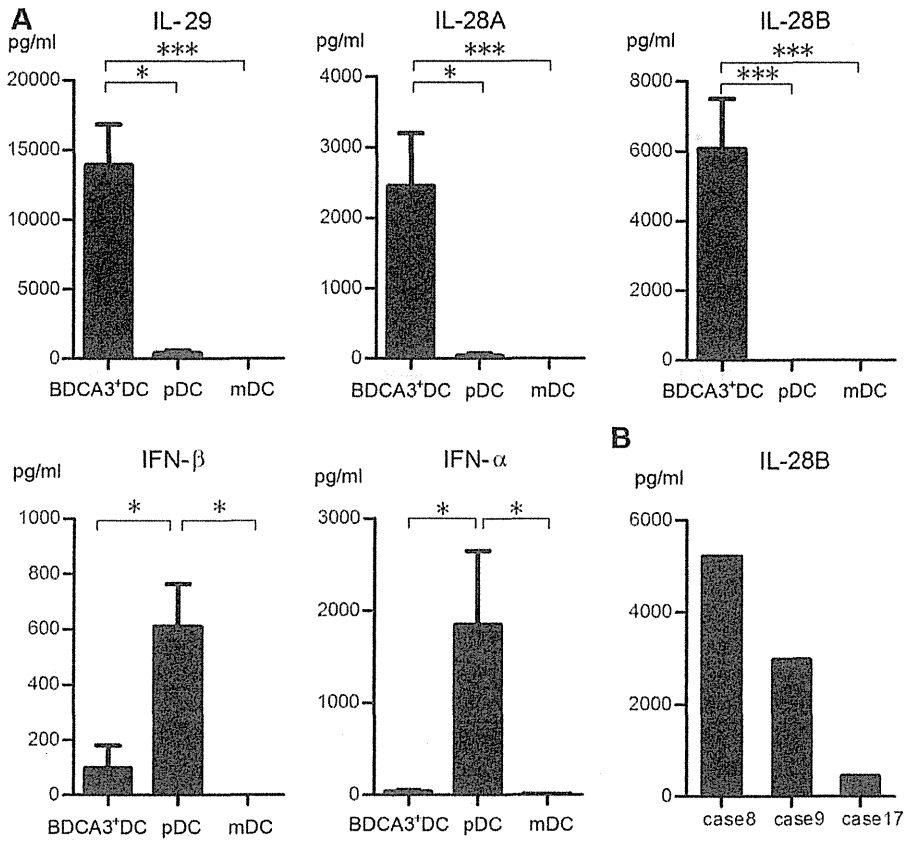

Fig. 3. BDCA3$^+$ DCs recovered from peripheral blood or intrahepatic lymphocytes produce large amounts of IL-29/IFN-λ1, IL-28A/IFN-λ2, and IL-28B/IFN-λ3 in response to poly IC. (A) BDCA3$^+$ DCs and mDCs were cultured at 2.5×10^4 cells with 25 mg/mL poly IC, and pDCs were with 5 mM CPG for 24 hours. 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.

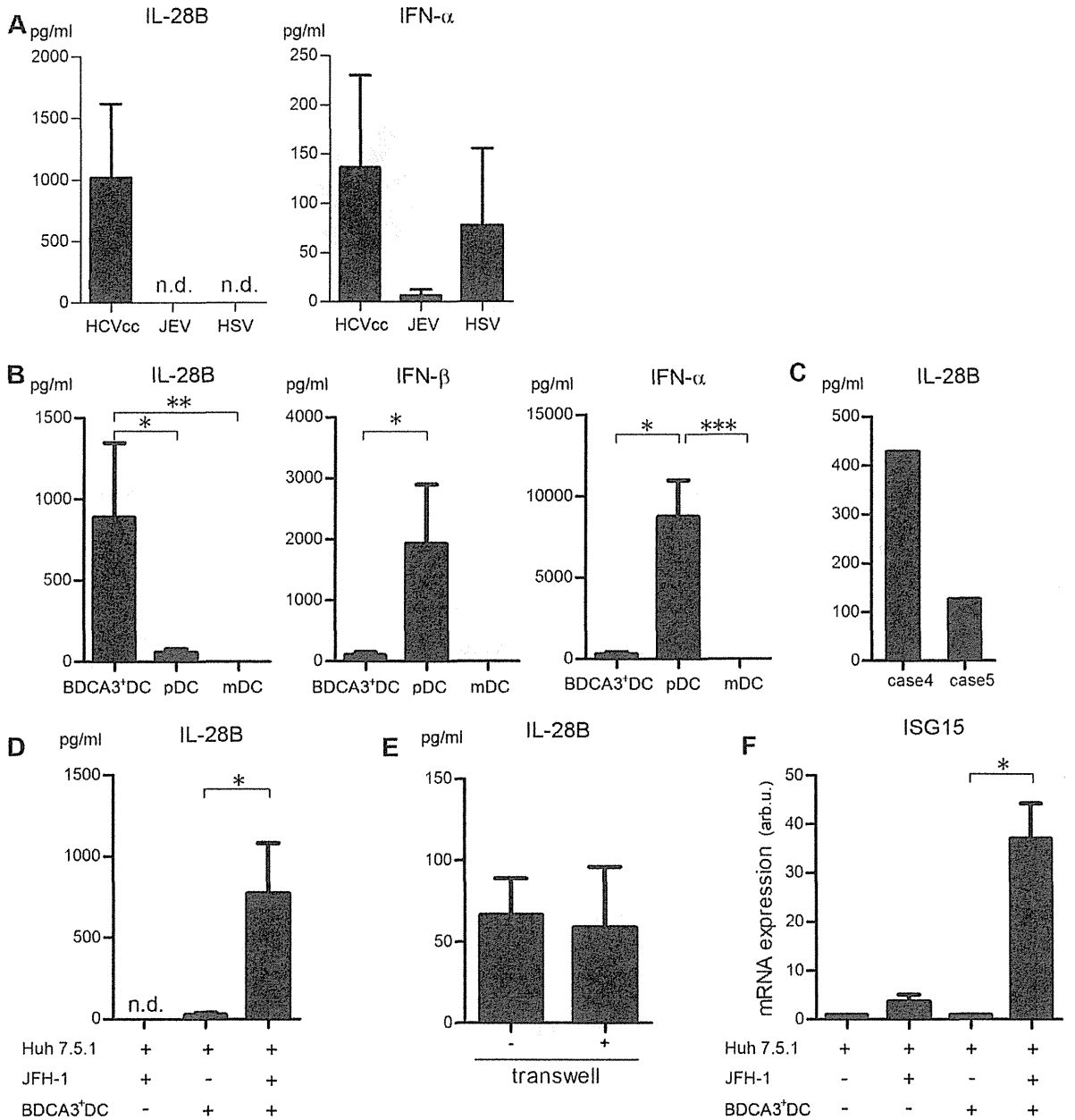

Fig. 4. BDCA3$^+$ DCs produce IL-29, IL-28A, and IL-28B upon cell-cultured HCV or HCV/JFH-1-transfected Huh7.5.1 cells, thereby inducing ISG. (A) BDCA3$^+$ DCs were cultured at 2.5×10^4 cells for 24 hours with HCVcc, JEV, or HSV at a multiplicity of infection (MOI) of 10. Results are shown as mean ± 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 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 ± 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.

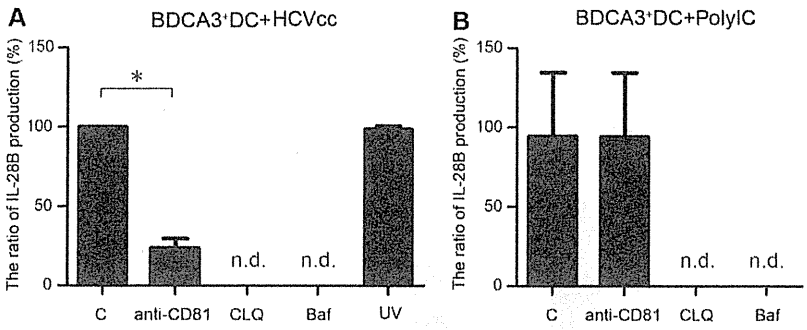

Fig. 5. The CD81 and endosome acidification is involved in the production of IL-28B from HCV-stimulated BDCA3$^+$ DCs, but HCV replication is not necessary. (A,B) BDCA3$^+$ DCs were cultured at 2.5×10^4 cells with HCVcc at an MOI of 10 (A) or poly IC (25 μg/mL) (B). In some experiments, UV-irradiated 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)

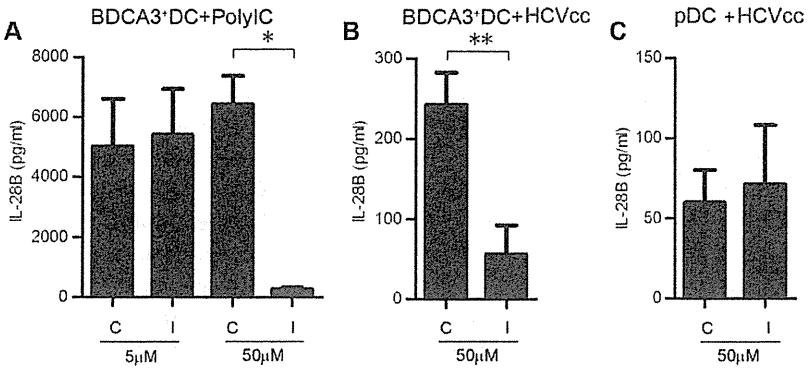

Fig. 6. BDCA3$^+$ DCs produce IL-28B upon HCVcc stimulation in a TRIF-dependent mechanism. BDCA3$^+$ DCs or pDCs had been treated with 5 or 50 mM TRIF inhibitory peptide or control peptide for 2 hours. Subsequently, BDCA3$^+$ DCs were stimulated with Poly IC (25 μg/mL) or HCVcc (MOI = 10), and pDCs were 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,

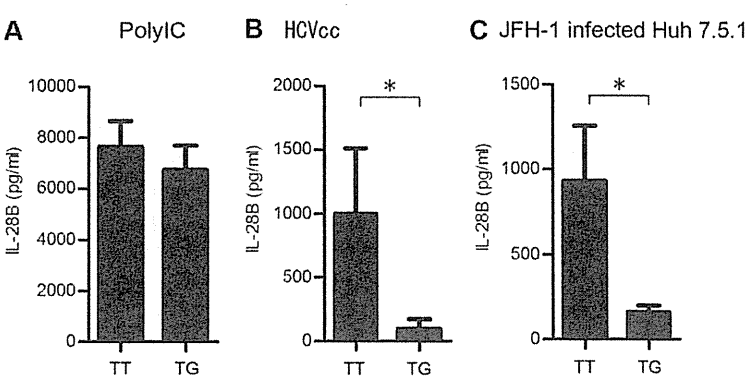

Fig. 7. In response to HCVcc, BDCA3$^+$ DCs of healthy donors with IL-28B major genotype (rs8099917, TT) produced more IL-28B than those with minor type (TG). BDCA3$^+$ DCs of healthy donors with IL-28B TT (rs8099917) or TG genotype were cultured at 2.5×10^4 cells with 25 mg/mL poly IC (A), with HCVcc 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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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Using early viral kinetics to predict antiviral outcome in response-guided pegylated interferon plus ribavirin therapy among patients with hepatitis C virus genotype 1

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Abstract
Background HCV kinetics during treatment demonstrated strong association with the antiviral outcome of patients treated with pegylated interferon (Peg-IFN) plus ribavirin. However, the relationship between HCV kinetics and pre-treatment factors remains unclear.
Methods Of 547 patients with HCV genotype 1 treated with Peg-IFN alfa-2b plus ribavirin, 401 completed the response-guided therapy and were assessed for per protocol analysis.
Results The sustained virologic response (SVR) rate was 53 % for all patients, 60 % for those with genotype TT, and 19 % for those with genotype TG/GG according to IL28B (rs8099917) single nucleotide polymorphisms. The SVR rates increased with HCV decrease at week 4; 4 % (2/56) with <1 $\log_{10}$ decrease, 13 % (7/56) with 1–2 $\log_{10}$ decrease, 51 % (44/87) with 2–3 $\log_{10}$ decrease, 64 % (56/87) with 3–4 $\log_{10}$ decrease, 88 % (72/82) with more than 4 $\log_{10}$ decrease but with detectable HCV RNA and 100 % (33/33) with undetectable HCV RNA ($p < 0.001$). Similarly, SVR rates increased step-by-step in proportion to HCV decrease in both IL28B TT and TG/GG groups, showing almost the same SVR rates for the same conditions. In multivariate analysis, age ($p = 0.005$) and the magnitude of HCV decrease at week 4 ($p < 0.001$) but not IL28B were associated with SVR. Advanced liver fibrosis ($p = 0.004$) and the magnitude of HCV decrease at week 4

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($p < 0.001$) but not IL28B were associated with non-response.

Conclusions The magnitude of the HCV decrease at week 4 seems to be the most reliable marker for predicting antiviral outcome after starting Peg-IFN plus ribavirin therapy.

Keywords Chronic hepatitis C · Pegylated interferon plus ribavirin · HCV decrease · IL28B

Introduction

Triple therapy with pegylated interferon (Peg-IFN), ribavirin plus telaprevir (TVR) can improve the antiviral effect for patients infected with hepatitis C virus (HCV) genotype 1 [1–8]. However, this triple therapy is not indicated for some patients due to adverse effects such as severe anemia progression, severe eruption and deterioration of renal function. On the other hand, 48–55 % of genotype 1 chronic hepatitis C (CH-C) patients have been shown to attain sustained virologic response (SVR) by response-guided Peg-IFN plus ribavirin combination therapy [9, 10]. Accordingly, naïve patients for whom there is concern about the possibility of enhanced adverse effects due to the addition of TVR, especially elderly patients, should first be given dual therapy with Peg-IFN plus ribavirin [11]. However, if patients having a high risk for hepatocellular carcinoma show poor antiviral response to this dual therapy, starting them on the triple therapy with Peg-IFN, ribavirin plus TVR should be considered. What is important is the ability to predict the antiviral outcome early during treatment in response-guided Peg-IFN plus ribavirin combination therapy.

The magnitude of the HCV RNA decrease or the timing of HCV RNA negativiation during the treatment has been reported to be strongly associated with SVR rates among genotype 1 CH-C patients treated with Peg-IFN plus ribavirin combination therapy [12–16]. During a 48-week treatment, SVR was attained in 70–80 % of patients with complete EVR (c-EVR), defined as undetectable HCV RNA at 12 weeks of treatment, and in about 90 % of patients with rapid virological response (RVR), defined as undetectable HCV RNA at 4 weeks of treatment [14–16], while those with less than a 2 $\log_{10}$ decrease in the HCV RNA level at 12 weeks mostly were non-SVR [12, 13, 16]. Currently, the magnitude of the HCV RNA decrease at 4 weeks of treatment has been attracting attention as a predictive factor for the antiviral outcome to this combination therapy [16, 17]. However, the aforementioned study was based on an existing method (lower limit 50 IU/ml) [17]. It is now possible to more precisely predict antiviral outcome thanks to progress in measurement of HCV RNA levels, which can now be quantitated with a higher degree of accuracy (lower limit 15 IU/ml) and for a broader range with 2 $\log_{10}$ differences.

A genetic polymorphism near the interleukin 28B (IL28B) gene has been reported to be a strong factor associated with antiviral outcome to the Peg-IFN plus ribavirin combination therapy [18–24]. Patients with the TT genotype of IL28B single nucleotide polymorphism (SNP) (rs8099917) can attain a higher virologic response during treatment (RVR, c-EVR) and a higher SVR rate in comparison with those with non-TT genotype [19, 23].

In the present study, we investigated the magnitude of impact of the early HCV RNA kinetics on predicting the antiviral outcome of response-guided Peg-IFN plus ribavirin combination therapy among genotype 1 CH-C patients. We also examined the relationship between the early viral kinetics and pre-treatment factors.

Patients and methods

Patients

The current study was a retrospective, multicenter trial conducted by Osaka University Hospital and other institutions participating in the Osaka Liver Forum. A total of 547 patients with CH-C treated with a combination of Peg-IFN alfa-2b plus ribavirin between January 2008 and August 2010 were enrolled in this study. Of the 547 patients, 401 completed the response-guided therapy and were assessed for per protocol analysis.

Eligible patients for this study were those who were infected with HCV genotype 1 and had a viral load of more than 10^5 IU/ml, but were negative for hepatitis B surface antigen and anti-human immunodeficiency virus. Patients were excluded from this study if they had decompensated cirrhosis or other forms of liver disease (alcohol liver disease, autoimmune hepatitis). Informed consent was obtained from each patient included in this study. This study was conducted according to the ethical guidelines of the 1975 Declaration of Helsinki amended in 2002.

Treatment

All patients received Peg-IFN alfa-2b (PEGINTRON; Merck & Co. Inc., Whitehouse Station, NJ, USA) plus ribavirin (REBETOL; Merck & Co. Inc.). Peg-IFN alfa-2b was given subcutaneously once weekly at a dosage of 60–150 μg/kg based on body weight (body weight 35–45 kg, 60 μg; 46–60 kg, 80 μg; 61–75 kg, 100 μg; 76–90 kg, 120 μg; 91–120 kg, 150 μg) and ribavirin was given orally twice a day at a total dose of 600–1000 mg/day based on body weight (body weight <60 kg, 600 mg;

60–80 kg, 800 mg; >80 kg, 1000 mg), according to a standard treatment protocol for Japanese patients.

Dose reduction

Dose modification followed, as a rule, the manufacturer's drug information according to the intensity of the hematologic adverse effects. The dose of Peg-IFN alfa-2b was reduced to 50 % of the assigned dose if the white blood cell (WBC) count declined to $<1500/mm^3$, the neutrophil count to $<750/mm^3$, or the platelet count to $<8 \times 10^4/mm^3$, and was discontinued if the WBC count declined to $<1000/mm^3$, the neutrophil count to $<500/mm^3$, or the platelet count to $<5 \times 10^4/mm^3$. Ribavirin was also reduced from 1000 to 600, or 800 to 600, or 600 to 400 mg if the hemoglobin (Hb) level decreased to <10 g/dl, and was discontinued if the Hb level decreased to <8.5 g/dl.

Histological evaluation

Pre-treatment liver biopsies were conducted within 6 months before the start of the combination therapy. Histopathological interpretation of the specimens was done by experienced liver pathologists who had no clinical, biochemical and virological information. The histological appearances, activity and fibrosis, were evaluated according to the METAVIR histological score [25].

IL28B genotyping

Human genomic DNA was extracted from a whole blood sample for each patient. Genetic polymorphism in SNPs located near the IL28B gene (rs8099917) was determined by a real-time PCR system. Each extracted DNA was used for PCR with primers and probes from a commercial kit (Taqman SNP Genotyping Assays, Applied Biosystems). The SNP of IL28B rs8099917 was amplified, and the results were analyzed by real-time PCR in a thermal cycler (7900 Real-time PCR System, Applied Biosystems). Homozygosity for TT genotype was defined as having the IL28B TT genotype, whereas homozygosity for GG or heterozygosity for TT (TG) genotype was defined as having the IL28B non-TT genotype.

Virologic assessment and definition of viral response

Serum HCV RNA level was quantified with the COBAS Taqman HCV test, version 2.0 (detection range 1.2–7.8 log IU/ml; Roche Diagnotics, Branchburg, NJ, USA). Serum HCV RNA level was assessed before treatment, every 4 weeks during treatment and 24 weeks after the therapy. The patients were divided into six groups according to the magnitude of the decrease in HCV RNA from baseline at treatment week 4, 8 and 12: <1 $\log_{10}$ decrease, 1 to <2 $\log_{10}$ decrease, 2 to <3 $\log_{10}$ decrease, 3 to <4 $\log_{10}$ decrease, ≥ 4 $\log_{10}$ (detectable), and undetectable. The case of serum HCV RNA level lower than 1.2 log IU/ml but positive for the amplification signal was defined as ≥ 4 $\log_{10}$ (detectable). A RVR was defined as undetectable serum HCV RNA at week 4, a c-EVR as undetectable serum HCV RNA at week 12, and a late virologic response (LVR) as detectable HCV RNA at week 12 but undetectable at week 36. SVR was defined as undetectable serum HCV RNA at 24 weeks after discontinuation of treatment. The treatment duration followed the response-guided therapy, i.e., patients with c-EVR were treated for 48 weeks and those with LVR for 72 weeks. Treatment was stopped for patients with detectable HCV RNA at week 36; they were considered to have experienced treatment failure (non-response, NR).

Definition of PPV and NPV

The positive predictive value (PPV) was defined as the probability that a certain outcome would occur in subjects on implementing the prediction criterion of interest, and a negative predictive value (NPV) was defined as the probability that a certain outcome would not occur in subjects if the prediction criterion of interest were not implemented.

Factors associated with SVR or NR on multivariate analysis

Factors associated with SVR or NR were assessed by multivariate analysis using two models; model 1 used the pre-treatment factors while model 2 also included the virologic response, i.e., the magnitude of the decrease in HCV RNA from baseline at treatment week 4, in addition to the factors used for model 1.

Statistical analysis

Baseline data for various demographic, biochemical, and virologic characteristics of the patients are expressed as mean ± SD or median. Viral response was evaluated using per protocol analysis. The difference between the two groups was assessed by Chi square test or t test and the significance trend was determined with the Mantel–Haenszel Chi square test. The factors associated with the virologic response were assessed by univariate and multivariate analyses using logistic regression analysis. A p value <0.05 was considered significant. Statistical analysis was conducted with SPSS version 19.0J (IBM, Armonk, NY, USA).

Results

The baseline characteristics of the 401 patients who completed the response-guided regimen are summarized in Table 1. All were assessed for the HCV RNA level at baseline and treatment weeks 4, 8 and 12. The IL28B genotype was assessed for 174 patients. The RVR rate was 8 % (33/401), the c-EVR rate was 41 % (164/401) and the LVR rate was 33 % (133/401). The NR rate was 26 % (104/401) and the SVR rate was 53 % (214/401).

Patient prevalence and SVR rates according to the magnitude of the decrease in HCV RNA from baseline at treatment week 4, 8 and 12

The patient prevalence for the different magnitudes of decrease in HCV RNA from baseline at treatment week 4, 8 and 12 are shown (Fig. 1a). The SVR rate on response-guided therapy was assessed for the six groups classified according to the magnitude of decrease in HCV RNA from the baseline (Fig. 1b). At week 4, the SVR rate significantly increased with the magnitude of the decrease in HCV RNA from the baseline; while all patients achieved SVR among those with undetectable HCV RNA (33/33), the SVR rates were very low among patients with <1 $\log_{10}$ decrease (4 %, 2/56). At week 8, none of the patients achieved SVR among those with <1 $\log_{10}$ decrease (0/32) and the SVR rates were very low among patients with 1 to <2 $\log_{10}$ decrease (6 %, 2/36). At week 12, none of the patients achieved SVR among those with <1 $\log_{10}$ decrease (0/32) or 1 to <2 $\log_{10}$ decrease (0/23) and the SVR rates were very low if HCV RNA did not decrease more than 4 $\log_{10}$ compared to the baseline (among patients with 2 to <3 $\log_{10}$ decrease, 8 %, 2/25, 3 to <4 $\log_{10}$ decrease, 7 %, 2/27).

Next, the relationship between the virologic response during the treatment (c-EVR, LVR and NR) and the magnitude of the decrease in HCV RNA from baseline were assessed (Fig. 2). The timing of HCV RNA negativiation tended to shift to an earlier time during the treatment with an increase in the magnitude of the decrease in HCV RNA from the baseline at any week (week 4, $p < 0.0001$, week 8, $p < 0.0001$, week 12, $p < 0.0001$, respectively). At week 4, 89 % of the patients with a <1 $\log_{10}$ decrease resulted in NR; none of those with a <2 $\log_{10}$ decrease achieved c-EVR. At week 8, all patients resulted in NR among those with a <1 $\log_{10}$ decrease (32/32, PPV for NR = 100 %); none of those with a < 3 $\log_{10}$ decrease achieved c-EVR. At week 12, all the patients with a <2 $\log_{10}$ decrease resulted in NR (55/55, PPV for NR = 100 %).

Table 1 Baseline characteristics of patients

Factor	Number or mean ± SD
Number	401
Age (y.o)	56.0 ± 10.6
Sex: male/female	190/211
Past history of IFN therapy[a]: naïve/experienced	316/66
Liver histology (METAVIR)[b]	
Activity, A0–1/2–3	188/120
Fibrosis, F0–2/3–4	265/42
IL28B SNP[c]: TT/TG/GG	131/40/3
HCV RNA (log IU/ml)	6.5 ± 0.6
White blood cells (/mm^3)	5152 ± 1486
Hemoglobin (g/dl)	13.8 ± 1.4
Platelets ($\times 10^4$/mm^3)	16.9 ± 5.5
ALT (IU/l)	65 ± 47
γGTP (IU/l)	62 ± 68

ALT alanine aminotransferase, *γGTP* γ-glutamyl transferase

[a] Past history of IFN was unknown in 19 patients

[b] 94 missing

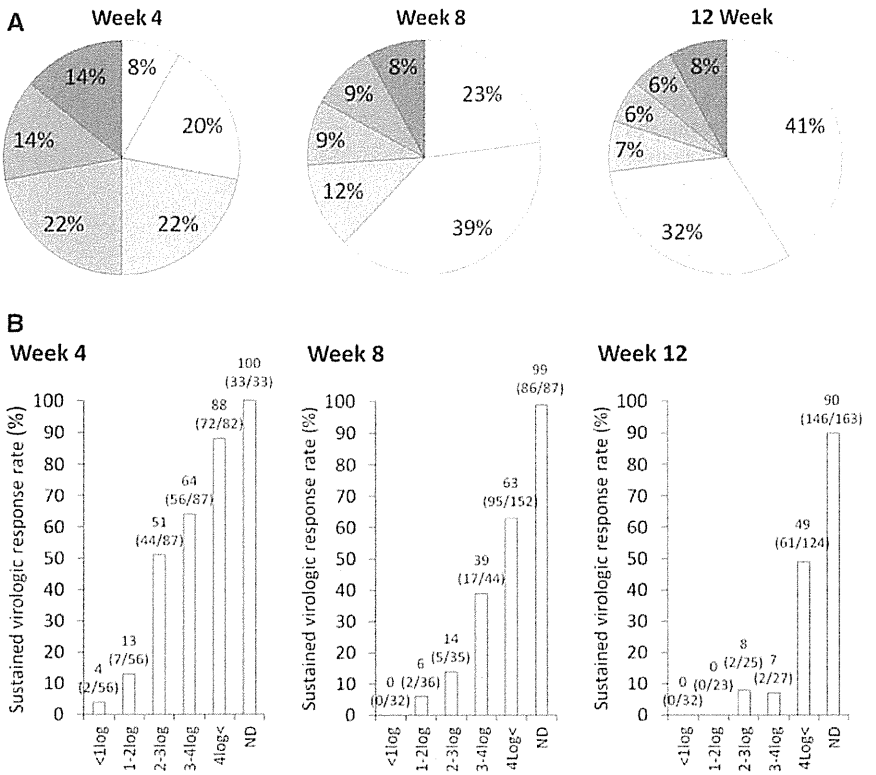
[c] Single nucleotide polymorphism of IL28B gene determined for rs8099917, 227 missing

Patient prevalence and virologic responses according to IL28B genotype

Among the patients who were assessed for their IL28B genotype, 131 had the IL28B TT genotype and 25 had the IL28B non-TT genotype (Fig. 3A). The SVR rate for response-guided therapy was significantly higher in patients with the IL28B TT genotype than those with the non-TT genotype ($p < 0.001$) (Fig. 3B). The treatment responses distributing c-EVR, LVR, and NR between the two groups were assessed. The c-EVR rate was significantly higher in patients with the IL28B TT genotype and the NR rate was significantly higher in those with the IL28B non-TT genotype ($p < 0.001$) (Fig. 3C).

Patient prevalence and SVR rates according to the magnitude of the decrease in HCV RNA from baseline at treatment week 4, 8 and 12 among patients with IL28B genotype TT or non-TT genotype

The patient prevalence and SVR rates according to the magnitude of the decrease in HCV RNA from baseline at treatment week 4, 8 and 12 are shown by IL28B genotype (Figs. 4a, 5a). The magnitudes of the decrease in HCV RNA from baseline at treatment week 4, 8 and 12 were significantly higher in patients with the IL28B TT genotype than in those with the IL28B non-TT genotype. The SVR rate for response-guided therapy was assessed according to



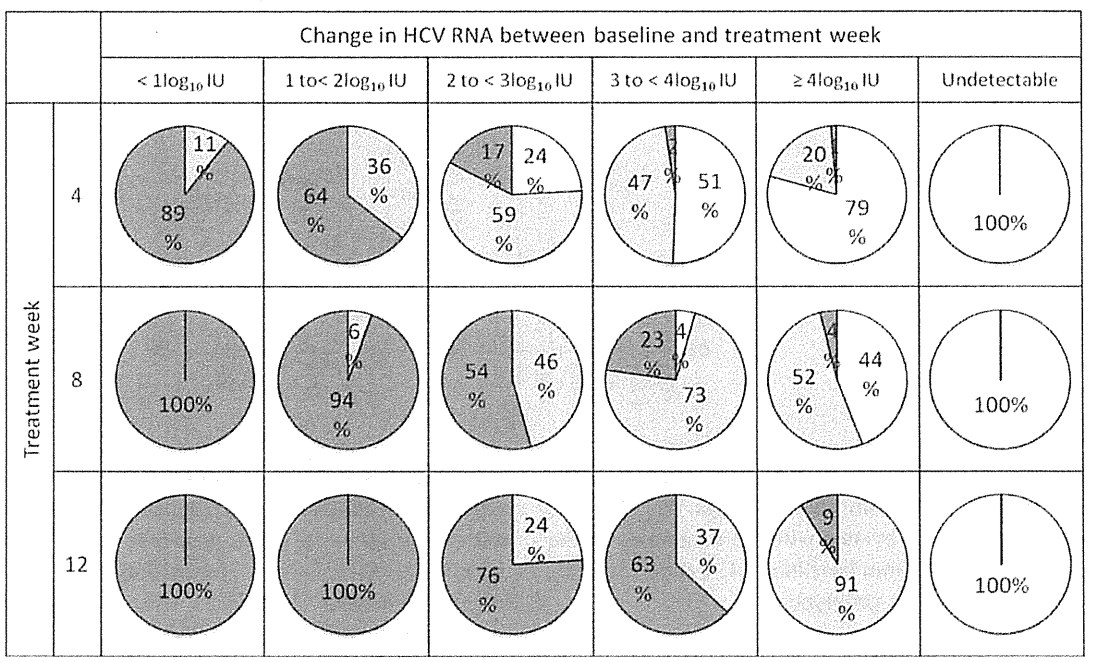


Fig. 1 **a** Patient prevalence according to the magnitude of HCV RNA decrease from baseline. ■ <1 $\log_{10}$ decrease. ▩ 1 to <2 $\log_{10}$ decrease. ▨ 2 to <3 $\log_{10}$ decrease. ▨ 3 to <4 $\log_{10}$ decrease. ▨ ≥4 $\log_{10}$ (detectable). □ undetectable. **b** SVR rate according to the magnitude of the decrease in HCV RNA from baseline

Fig. 2 Relationship between the virologic response during treatment and the magnitude of the decrease in HCV. RNA from baseline. □ early virologic response. ▨ late virologic response. ■ non-response

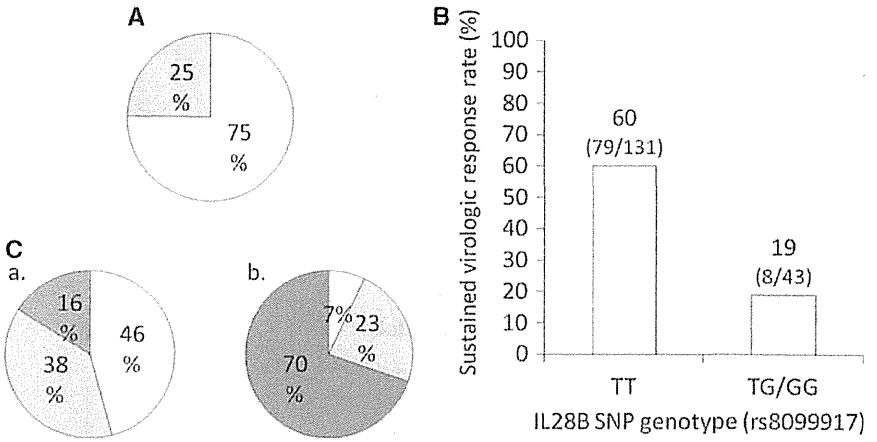


Fig. 3 **A** Patient prevalence according to IL28B genotype. □ TT genotype. ▒ non-TT genotype (TG of GG genotype). **B** SVR rate according to IL28B genotype. **C** Relationship between the virologic response during treatment and IL28B genotype. *a* Patients with IL28B TT genotype. *b* Patients with IL28B non-TT genotype. □ early virologic response. ▒ late virologic response. ■ non-response

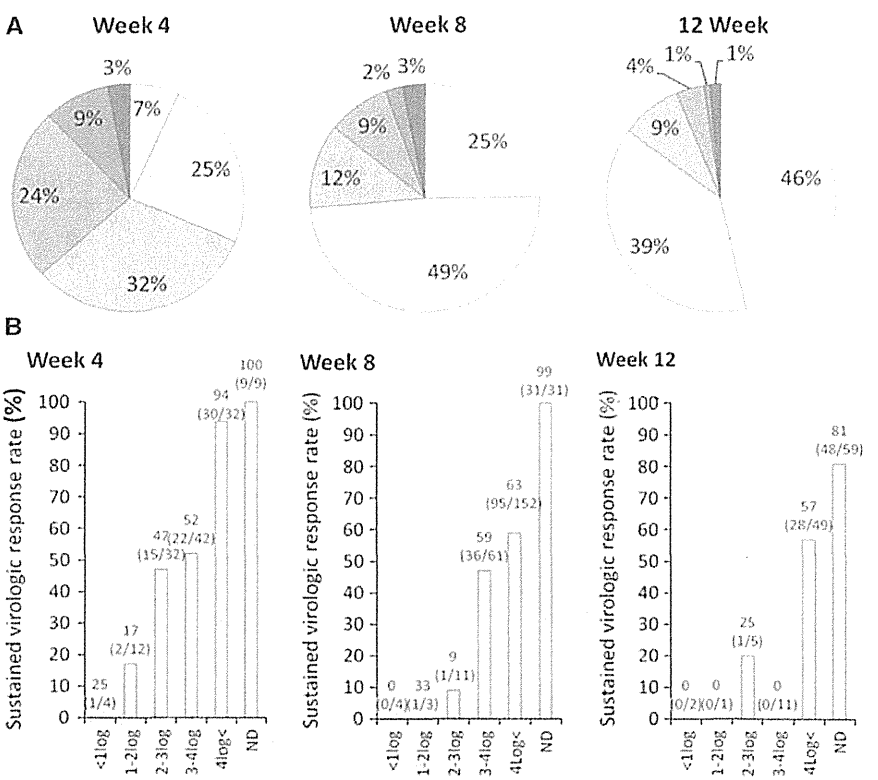


Fig. 4 **a** Patient prevalence according to the magnitude of HCV RNA decrease from baseline among patients with IL28B TT genotype. ■ <1 $\log_{10}$ decrease. ▒ 1 to <2 $\log_{10}$ decrease. ▒ 2 to <3 $\log_{10}$ decrease. ▒ 3 to <4 $\log_{10}$ decrease. ▒ ≥4 $\log_{10}$ (detectable). □ undetectable. **b** SVR rate according to the magnitude of the decrease in HCV RNA from baseline among patients with IL28B TT genotype

the six-group classification of the magnitude of the decrease in HCV RNA from baseline (Figs. 4b, 5b). The SVR rate increased step-by-step with the magnitude of the decrease in HCV RNA from baseline at 4, 8 and 12 weeks, irrespective of the IL28B genotype.

Factors associated with SVR

The factors associated with SVR were assessed for the variables shown in Table 1 and the magnitude of the decrease in HCV RNA from baseline at week 4 (Table 2). The factors selected as significant by univariate analysis (age, stage of liver fibrosis, platelet count, IL28B genotype and the magnitude of the decrease in HCV RNA from baseline at week 4) were evaluated by multivariate logistic regression analysis. In model 1, which consisted of only the pre-treatment factors, the IL28B genotype was the most powerful independent factor for SVR (Odds ratio, OR; 6.92, $p < 0.001$), apart from age (OR; 0.96, $p = 0.048$) and the stage of liver fibrosis (OR; 0.23, $p = 0.021$). However, in model 2, which included the parameter of viral kinetics at week 4, the magnitude of the decrease in HCV RNA

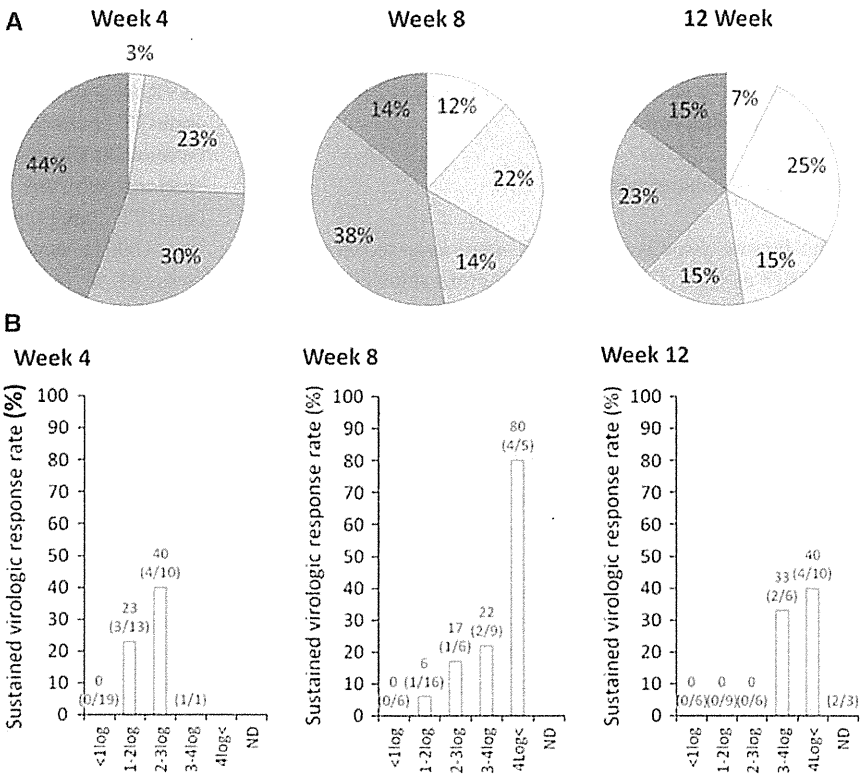


Fig. 5 **a** Patient prevalence according to the magnitude of HCV RNA decrease from baseline among patients with IL28B non-TT genotype. ■ <1 $\log_{10}$ decrease. ▩ 1 to <2 $\log_{10}$ decrease. ▨ 2 to <3 $\log_{10}$ decrease. ▧ 3 to <4 $\log_{10}$ decrease. ▫ ≥4 $\log_{10}$ (detectable). □ undetectable. **b** SVR rate according to the magnitude of the decrease in HCV RNA from baseline among patients with IL28B non-TT genotype

Table 2 Factors associated with SVR

Factor	Category	Univariate analysis			Multivariate analysis					
					Model 1			Model 2		
		OR	95 % CI	*p* value	OR	95 % CI	*p* value	OR	95 % CI	*p* value
Age		0.967	0.95–0.99	0.001	0.963	0.93–1.00	0.048	0.936	0.89–0.98	0.005
Sex	M/F	0.679	0.46–1.01	0.055						
Activity	A0–1/A2–3	0.671	0.42–1.06	0.090						
Fibrosis	F0–2/F3–4	0.301	0.15– 0.62	0.001	0.231	0.07–0.80	0.021	–	–	NS
HCV-RNA	By 1 log	0.657	0.53–1.03	0.075						
WBC		1.000	1.00–1.00	0.167						
Hb		1.080	0.94–1.24	0.272						
PLT		1.069	1.03–1.11	0.001	–	–	NS	–	–	NS
ALT		0.999	0.96–1.00	0.756						
γGTP		0.998	0.99–1.00	0.160						
IL28	TG or GG/TT	6.647	2.86–15.5	<0.001	6.924	2.60–18.4	<0.001	–	–	NS
Change of HCV RNA at 4 weeks	By 1 log increase	3.472	2.74–4.40	<0.001				3.338	2.20–5.06	<0.001

Model 1 consisted of pre-treatment factors. Model 2 consisted of pre-treatment factors and virologic response, that is the magnitude of the decrease in HCV RNA from baseline at treatment week 4

OR Odds ratio, *SVR* sustained virologic response, *WBC* white blood cells, *Hb* hemoglobin, *PLT* platelets, *ALT* alanine aminotransferase, *γGTP* γ-glutamyltransferase

from baseline at week 4 was the most powerful independent factor for SVR (OR; 3.4, $p < 0.001$) apart from age (OR; 0.936, $p = 0.005$), and the factor of IL28B was not a significant independent factor.

Factors associated with NR

The factors associated with NR were assessed in the same manner as SVR (Table 3). The factors selected as significant by the univariate analysis were evaluated by multivariate logistic regression analysis: grade of liver activity, stage of liver fibrosis, WBC count, platelet count, serum γ-glutamyltransferase level, IL28B genotype and the magnitude of the decrease in HCV RNA from baseline at week 4. In model 1, IL28B genotype was the most powerful independent factor for NR (OR; 39.75, $p < 0.001$), apart from the degree of liver fibrosis (OR; 10.31, $p = 0.021$) and platelet count (OR; 0.84, $p = 0.01$). However, in model 2, the magnitude of the decrease in HCV RNA from baseline at week 4 was the most powerful independent factor for NR (OR; 9.29, $p < 0.001$) apart from the degree of liver fibrosis (OR; 14.48, $p = 0.004$). IL28B was not selected as a significant independent factor.

SVR rate according to IL28B genotype and the timing of HCV RNA negativiation during treatment

Lastly, the SVR rate according to the IL28B genotype and the timing of HCV RNA negativiation during the treatment were assessed (Supplementary Figure 1). RVR was attained in nine patients with the IL28B TT genotype, and SVR was attained by all, but none of those with the IL28B non-TT genotype attained RVR. Among the patients without RVR, the SVR rate was significantly higher in the patients with the IL28B TT genotype than those with the non-TT genotype (57 %, 70/122 vs. 19 %, 8/43, $p < 0.001$). Among the patients with c-EVR, the SVR rate was 81 % (48/59) in the patients with the IL28B TT genotype and 67 % (2/3) in those with the non-TT genotype ($p = 0.48$). On the other hand, among the patients without c-EVR, the SVR rate was significantly higher in the patients with the IL28B TT genotype than those with the non-TT genotype (43 %, 30/70 vs. 16 %, 6/37, $p < 0.01$). Among the patients with LVR, the SVR rate was 60 % in both patients with the IL28B TT genotype (30/50) and the non-TT genotype (6/10) ($p = 1.00$). Next, the HCV RNA decrease among the patients without RVR or c-EVR was examined. The results revealed a significantly greater HCV RNA decrease in the IL28 TT group compared with the non-TT group among the patients without RVR (3.3 ± 1.2 $\log_{10}$ IU/ml vs. 1.3 ± 0.9 $\log_{10}$ IU/ml, $p < 0.001$) and those without c-EVR (4.7 ± 1.5 $\log_{10}$ IU/ml vs. 2.6 ± 1.5 $\log_{10}$ IU/ml, $p < 0.001$). The mean level of HCV RNA was significantly lower in the IL28 TT group compared with the non-TT group among the patients without RVR (3.3 ± 1.3 vs. 5.1 ± 1.2 $\log_{10}$ IU/ml, $p < 0.001$) and those without c-EVR (2.0 ± 1.4 vs. 3.9 ± 1.8 $\log_{10}$ IU/ml, $p < 0.001$).

Table 3 Factors associated with NR

Factor	Category	Univariate analysis			Multivariate analysis					
					Model 1			Model 2		
		OR	95 % CI	*p* value	OR	95 % CI	*p* value	OR	95 % CI	*p* value
Age		1.015	0.99–1.04	0.185						
Sex	M/F	1.187	0.76–1.86	0.455						
Activity	A0–1/A2–3	2.186	1.23–3.71	0.004	–	–	NS	–	–	NS
Fibrosis	F0–2/F3–4	5.079	2.53–10.0	<0.001	10.306	2.17–48.9	0.003	18.482	2.5–136.2	0.004
HCV-RNA	By 1 log	1.371	0.93–2.02	0.111						
WBC		1.000	1.00–1.00	0.004	–	–	NS	–	–	NS
Hb		0.947	0.81–1.10	0.498						
PLT		0.887	0.85–0.93	<0.001	0.844	0.74–0.96	0.01	–	–	NS
ALT		0.985	0.99–1.01	0.985						
γGTP		1.003	1.00–1.01	0.035	–	–	NS	–	–	NS
IL28	TT/TG or GG	12.088	5.4–26.9	<0.001	39.750	10.3–153.5	<0.001	–	–	NS
Change of HCV RNA at 4 weeks	By 1 log decrease	6.717	4.56–9.88	<0.001				9.292	3.95–21.8	<0.001

Model 1 consisted of pre-treatment factors. Model 2 consisted of pre-treatment factors and virologic response, that is the magnitude of the decrease in HCV RNA from baseline at treatment week 4

OR Odds ratio, *SVR* sustained virologic response, *WBC* white blood cells, *Hb* hemoglobin, *PLT* platelets, *ALT* alanine aminotransferase, *γGTP* γ-glutamyltransferase

Discussion

The importance of the timing of HCV RNA negativiation for the prediction of SVR has been well recognized [12–16]. In this study, all of the patients with RVR achieved SVR and those with c-EVR achieved 90 % of SVR by 48 weeks of treatment. However, only 8 % of the patients achieved RVR and about 40 % achieved c-EVR. This means that there is a need for another predictor of the effects of the response-guided therapy for the remaining population in which RVR or c-EVR were not attained. At present, triple therapy with Peg-IFN, ribavirin plus TVR, which can improve the antiviral outcome, is an option and the next generation of direct-acting antivirals is coming [1–8, 26–29]. Thus, the decision of "to treat or not to treat" during Peg-IFN plus ribavirin combination therapy should be carefully made with consideration of predictable antiviral outcomes and the degree of the adverse effect. In the present study, we focused on the early HCV RNA dynamics during the first 12 weeks of Peg-IFN plus ribavirin combination therapy.

First, we assessed the relationship between the magnitude of the decrease in HCV RNA from the baseline and SVR. At week 4, the patients without RVR were categorized into five groups, almost equally (Fig. 1a). The SVR rate increased step-by-step in proportion to the magnitude of the HCV RNA decrease at week 4, 8 and 12 (Fig. 1b). The SVR rates were very low (0–8 %) in patients with <1 $\log_{10}$ decrease at week 4, <2 $\log_{10}$ decrease at week 8 or <4 $\log_{10}$ decrease at week 12; NR rates were very high (63–100 %) in these patient groups (Fig. 2). All patients with <1 $\log_{10}$ decrease at week 8 or with <2 $\log_{10}$ decrease at week 12 were NR. These results could be useful for preparing guidelines on when to stop the response-guided therapy. Similar results were obtained from stratified analysis according to the IL28B genotype; SVR rates increased step-by-step in proportion to HCV RNA decrease in both the IL28B TT group and the non-TT group (Figs. 4, 5). Both groups showed almost the same SVR rates with the same level of HCV RNA decrease over the same number of weeks after the start of treatment, although few patients with non-TT showed a good response (marked HCV RNA decrease) to the treatment. This means that early HCV RNA decrease can predict SVR irrespective of the IL28B status.

In order to examine in more detail whether early HCV RNA dynamics can predict antiviral outcome irrespective of IL28B status, we investigated the predictive factors for treatment response before and after the start of treatment by multivariate logistic regression analysis. As a result, the IL28B genotype was found to be the strongest predictive factor for pre-treatment prediction in response-guided therapy (Table 2, model 1). However, on analysis including the HCV RNA decrease from the baseline at week 4, the magnitude of the HCV RNA decrease at week 4 was shown to be the best predictor for SVR (Table 2, model 2). Multivariate analysis for NR gave similar results; the IL28B genotype was the strong predictive factor for pre-treatment prediction and the HCV RNA decease at week 4 for post-treatment (Table 3). These results indicate that the information of the IL28B genotype is very useful for predicting the antiviral response before treatment, however, once the treatment is initiated, that of the HCV RNA decrease at week 4 can replace the IL28B status for predicting the antiviral outcome.

Recently, the relationship between the timing of HCV RNA negativiation and IL28B status for predicting the antiviral effect has been reported [21, 24]. No significant difference of the SVR rates was shown among patients with the same on-treatment virologic response, such as RVR or c-EVR, regardless of the IL28B genotype. However, the IL28B status was shown to affect the antiviral outcome among patients who could not attain RVR or c-EVR; higher SVR rates were obtained for patients with a favorable IL28B genotype. With the cohort enrolled in this study, we assessed stratified analysis according to the IL28B genotype and the timing of HCV RNA negativiation in order to determine whether the IL28B genotype directly affects the antiviral outcome or whether the difference of HCV RNA decrease caused by IL28B status affects the antiviral outcome (Supplementary Figure 1). The results showed that the IL28B genotype was associated with SVR among the patients without RVR or c-EVR, while nearly equal SVR rates were attained regardless of the IL28B genotype among the patients with HCV RNA negativiation over the same period of time. These results correspond to those of a previous study [21, 24], and the IL28B genotype was concluded to affect the antiviral outcome among the patients without RVR or c-EVR. However, examination of the HCV RNA decrease among the patients without RVR or c-EVR in our cohort showed that HCV RNA significantly decreased in the IL28 TT group compared with the non-TT group. These results suggest that the IL28B status does not directly affect the antiviral outcome and that the difference of HCV RNA decrease caused by the IL28B status affects the antiviral outcome among the patients without RVR or c-EVR. In sum, the HCV decrease at week 4 can be used in place of the IL28B status for predicting the antiviral outcome after the start of treatment, as shown by the multivariate analysis in the present study.

The limitation of this study was that pre-treatment viral factors were not assessed due to the small numbers of patients. Among the patients enrolled in this study, the amino acid (aa) sequences at position 70 in the HCV core protein were examined in some cases (126 patients). The analysis for SVR and NR including the factor of aa

substitution of core 70, the same results were obtained. That is, the results of the factor of IL28B in model 1 and the HCV RNA decrease at week 4 in model 2 were the significant predictive factors for SVR and NR but the factor of aa substitution of core 70 was not significantly associated with SVR by univariate analysis and NR by multivariate logistic regression analysis. Further examination is needed to clarify the usefulness of the factor of aa substitution of core 70 as a predictor for SVR or NR in this combination therapy.

In conclusion, the IL28B genotype is a very strong predictive factor in pre-treatment prediction. After the start of treatment, the magnitude of the HCV RNA decrease at week 4 seems to be the most reliable marker for predicting the antiviral outcome among patients treated with response-guided Peg-IFN plus ribavirin combination therapy.

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Valine, the branched-chain amino acid, suppresses hepatitis C virus RNA replication but promotes infectious particle formation

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ABSTRACT

Background & aims: Concentrations of the branched-chain amino acid (BCAA) in the serum of patients with liver cirrhosis correlate with their liver function. Oral administration of BCAA can ameliorate hypoalbuminemia and hepatic encephalopathy. In this study, we aim to clarify the role of BCAA in regulating the replication of the hepatitis C virus (HCV).
Methods: HCV sub-genomic replicon cells, genome-length replicon cells, and cells infected with cell culture-infectious HCV (HCVcc) were cultured in media supplemented with various concentrations of BCAA, followed by evaluation of the replicon or HCV abundance.
Results: BCAA was capable of suppressing the HCV replicon in a dose-dependent manner and the effect was independent of the mTOR pathway. Of the three BCAAs, valine was identified as being responsible for suppressing the HCV replicon. Surprisingly, an abundance of HJ3-5(YH/QL), an HCVcc, in Huh7 cells was augmented by BCAA supplementation. In contrast, BCAA suppressed an abundance of HJ3-5(wild), an HCVcc that cannot assemble virus particle in Huh7 cells. Internal ribosome entry site of HCV was shown to be a target of BCAA. Single-cycle virus production assays using Huh7-25 cells, which lacked CD81 expression, revealed that BCAA, especially valine, promoted infectious virus particle formation with minimal effect on virus secretion. Thus, BCAA was found to have two opposing effects on HCV production: suppression of the HCV genome RNA replication and promotion of infectious virus formation.
Conclusions: BCAA accelerates HCV production through promotion of infectious virus formation in infected cells despite its suppressive effect on HCV genome replication.

1. Introduction

Persistent infection of hepatitis C virus (HCV) causes progressive liver disease in humans. Chronic inflammation in the liver leads to the accumulation of fibrosis and an eventual progression to liver cirrhosis. In patients with decompensated liver cirrhosis, a change in plasma amino acid composition is frequently observed. In particular, the ratio of branched-chain amino acid (BCAA) to aromatic amino acid (AAA), known as Fischer's ratio, decreases as the liver function deteriorates [1]. In such cirrhotic patients, hypoalbuminemia occurs, and it has been shown that oral administration of BCAA can ameliorate hypoalbuminemia and hepatic encephalopathy.

Three amino acids valine, leucine, and isoleucine are BCAAs, which are considered to be essential for protein anabolism. In addition to the role of acting as nutrient substrates, recent studies have demonstrated that BCAA also serve as physiologically active substances. BCAA have been shown to have pharmacological effects, such as induction of protein synthesis [2] and glucose metabolism [3]. In rat primary hepatocytes, albumin synthesis is significantly increased by BCAA administration, which is dependent on activation of the mammalian target of rapamycin (mTOR), mainly induced by leucine [4].

HCV replication is controlled by intracellular signaling pathways. In addition to the interferon (IFN)-induced JAK/STAT pathway, which activates interferon-stimulated genes, leading to strong anti-viral activity, activation of ERK [5], PI3 kinase/Akt [6,7], smad [8], PKC [9], and p38 [10], have been shown to be capable of regulating HCV replication. mTOR, one of the downstream molecules of Akt, phosphorylates the two substrates p70 S6 kinase and eukaryotic translation initiation factor 4E binding protein 1

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