In order to compare the ability of BDCA3$^+$DCs to release IL-28B in healthy subjects between IL28B major (rs8099917, TT) and minor hetero (TG) genotypes, we stimulated BDCA3$^+$DCs of the identical subjects with poly IC (25μg/ml, 2.5μg/ml, 0.25μg/ml), HCVcc or JFH-1-infected Huh 7.5.1and subjected them for ELISA. The levels of IL-28B production by polyIC-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, 7C, 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 amount 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 for

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, 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 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 post-transcriptional 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 amount 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 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 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 co-culture with JFH-1-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 co-culture 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 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 crucial driver of ISG induction and subsequent HCV eradication. Besides, it is likely that BDCA3$^+$DCs, as a by-stander IFN-λ producer in the liver, give significant impact on hepatic ISG induction. In support for this possibility, we demonstrated in this study that BDCA3$^+$DCs are capable of producing large amount of IFN-λs in response to HCV, thereby inducing ISGs in the co-existing liver cells.

Controversial results have been reported regarding to 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 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 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 for the possibility that the 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 on the subsequent TLR3-TRIF-dependent responses. Certain unknown factors regulating such process may be linked to the IL-28B genotypes. For the comprehensive understanding of biological importance of IL-28B in HCV infection, such co-founding factors, if they exist, need to be explored.

In conclusion, human BDCA3$^+$DCs, having tendency of being accumulated 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 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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FIGURE LEGENDS

Figure 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**), respectively.

B. The expressions of CD40, CD80, CD83, CD86, CD81, and CLEC9A on each DC subset in peripheral blood are shown. Representative results of 5 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 co-stimulatory molecules on BDCA3$^+$DCs were compared between in PBMCs and in the liver. Results are shown as the percentage of positive cells. Results are the mean + SEM from 4 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 non-cancerous liver samples are shown.

BDCA, blood dendritic cell antigen; pDC, plasmacytoid DC; mDC, myeloid DC; CLEC9A, C-type lectin 9A

Figure 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. Results of 8 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.

Figure 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 $2.5x10^4$ cells with 25 μg/ml poly IC, and pDCs were with 5 μM CPG for 24 h. 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

$2.5x10^4$ cells with 25 μg/ml poly IC for 24 h. The samples of case #8 and case #9 were obtained from patients with non-B, non-C liver disease and that of case #17 was from a HCV-infected patient (**Supplementary table 1**).

Figure 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 $2.5x10^4$ cells for 24 h with HCVcc, JEV or HSV at an MOI of 10. Results are shown as mean + SEM from 6 experiments. n.d.; not detected

B. BDCA3$^+$DCs, pDCs, and mDCs were cultured at $2.5x10^4$ cells for 24 h with HCVcc at an MOI of 10. 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 $2.5x10^4$ cells for 24 h with HCVcc at an MOI of 10. Both of the samples (case #4 and case #5) were obtained from patients with non-B, non-C liver disease.

D. E. BDCA3$^+$DCs were co-cultured at $2.5x10^4$ cells with JFH-1-transfected (MOI=2) or – untransfected Huh7.5.1 cells for 24h. The supernatants of JFH-1-transfected Huh7.5.1.cells

without BDCA3$^+$DCs were also examined. In some experiments of the co-culture with JFH-1-transfected Huh7.5.1 cells and BDCA3$^+$DCs, transwells were inserted to the wells (E). Results are shown as mean + SEM from 5 experiments. *, $p < 0.05$ by paired-t test

F. BDCA3$^+$DCs were co-cultured at $2.5x10^4$ cells with JFH-1-transfected Huh7.5.1 cells (MOI=2) or –untransfected Huh7.5.1 cells for 24h. The Huh7.5.1.cells were harvested and subjected to real time RT-PCR analyses for ISG15 expression. Results are shown as mean + SEM from 5 experiments. *, $p < 0.05$ by paired-t test

HCVcc, cell-cultured HCV; JEV, Japanese encephalitis virus; HSV, herpes simplex virus

Figure 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 $2.5x10^4$ cells with HCVcc at an MOI of 10 (A) or poly IC (25ug/ml) (B). In some experiments, UV-irradiated HCVcc was used at the same MOI, and BDCA3$^+$DCs were treated with anti-CD81Ab (5μg/ml), chloroquine (10μM), or bafilomycin A1 (25nM). Results are expressed as ratios of IL-28B quantity with or without the treatments. They

are shown as mean + SEM from 5 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;

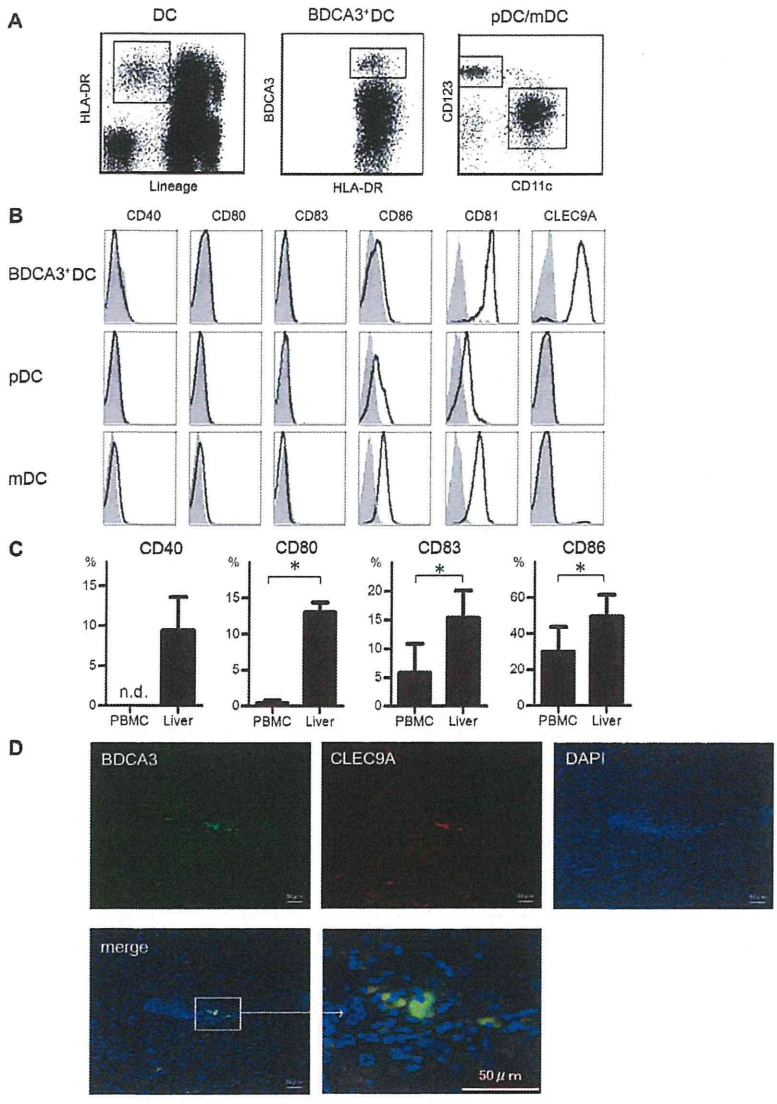
Figure 6: BDCA3$^+$DCs produce IL-28B upon HCVcc stimulation in a TRIF-dependent mechanism.

BDCA3$^+$DCs or pDCs had been treated with 5 or 50 μM TRIF inhibitory peptide or control peptide for 2h. Subsequently, BDCA3$^+$DCs were stimulated with PolyIC (25ug/ml) or HCVcc (M.O.I.=10), and pDCs were stimulated with HCVcc (M.O.I.=10), respectively. IL-28B was quantified by ELISA. They are shown as mean + SEM from 5 experiments. *, $p < 0.05$ by paired-t test.

C, TRIF control peptide; I, TRIF inhibitory peptide.

Figure 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 $2.5x10^{4}$ cells with 25 μg/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 h. The supernatants were subjected for 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.



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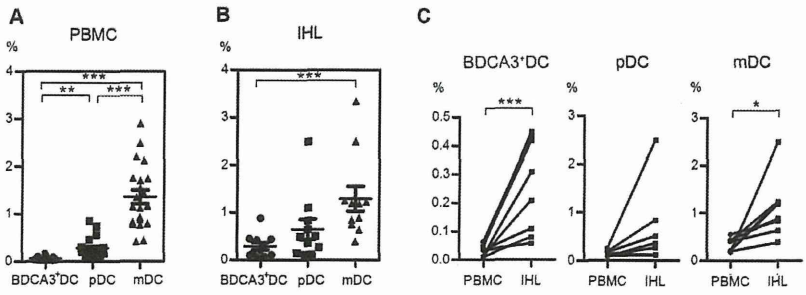

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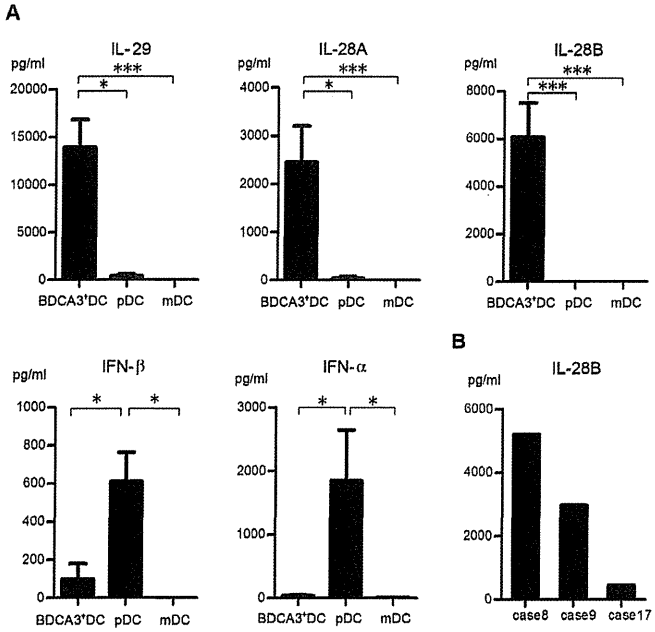

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