

with immunofluorescence Abs in non-cancerous liver tissues. Liver BDCA3<sup>†</sup>DCs were defined

as BDCA3+CLEC9A+ cells (Fig 1D). Most of the cells were found near vascular compartment

or in sinusoid or the space of Disse of the liver tissue.

### BDCA3<sup>+</sup>DCs are scarce in PBMCs but more abundant in the liver.

The percentages of BDCA3<sup>+</sup>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<sup>+</sup>DCs in IHLs were lower than those of the

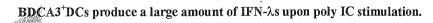
others (BDCA3<sup>+</sup>DCs, pDCs and mDCs, mean  $\pm$  SD [%], 0.29  $\pm$  0.25, 0.65  $\pm$  0.69 and 1.2  $\pm$ 

0.94) (Fig 2B). The percentages of BDCA3<sup>+</sup>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

(Supplementary Table 1).



We compared DC subsets for their abilities to produce IL-29/IFN-λ1, IL-28A/IFN-λ2,

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1L-28B/IFN- $\lambda$ 3, IFN- $\beta$ , and IFN- $\alpha$  in response to TLR agonists. Approximately  $4.0\times10^4$  of

BDCA3<sup>+</sup>DCs were recoverable from 400ml of donated blood from healthy volunteers. We fixed

the number of DCs at  $2.5 \times 10^4$  cells/100 µl for comparison in the following experiments.

BDCA3<sup>+</sup>DCs have been reported to express mRNA for TLR1, 2, 3, 6, 8, and 10 (17).

First, we quantified IL-28B/IFN-\(\lambda\)3 as a representative for IFN-\(\lambda\)s after stimulation of

BDCA3<sup>+</sup>DCs with relevant TLR agonists. We confirmed that BDCA3<sup>+</sup>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- $\lambda$ s and IFN- $\beta$  genes in response

to relevant TLR agonists. BDCA3+DCs expressed extremely high levels of IL-29, IL-28A and

IL- $\overline{28}$ B transcripts compared to other DCs, whereas pDCs induced a higher level of IFN- $\beta$  than

other DCs (Fig S3A).

Similar results were obtained with the protein levels of IFN- $\lambda$ s, IFN- $\beta$  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<sup>†</sup>DCs or

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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- $\lambda$ 1 and /IL-28B/IFN- $\lambda$ 3 were positively correlated (R2=0.76, p<0.05), and those of IL-28A/IFN- $\lambda$ 2 and IL-28B/IFN- $\lambda$ 3 were positively correlated as well (R2=0.84, p<0.0005), respectively (**Fig S3C**). These results show that the transcription and translation machineries of IFN- $\lambda$ s may be overlapped among IFN- $\lambda$ 

subtypes in BDCA3<sup>†</sup>DCs upon poly IC stimulation.

Liver BDCA3<sup>+</sup>DCs sorted from IHLs possess ability to produce IL-28B in response to poly IC (Fig 3B), showing that they are comparably functional.

In response to poly IC, BDCA3<sup>+</sup>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<sup>+</sup>DCs suppressed HCV replication in an IL-28B concentration dependent manner (**Fig S4B**). Therefore, poly IC-stimulated BDCA3<sup>+</sup>DCs are capable of producing biologically active substances suppressing HCV replication, some part of which may be mediated by IFN-λs.

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BDCA3<sup>+</sup>DCs produce IL-28B upon cell-cultured HCV or HCV/JFH-1-transfected

Huh7.5.1 cells.

We stimulated freshly isolated BDCA3<sup>+</sup>DCs, pDCs and mDCs with infectious viruses,

such as cell-cultured HCV (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<sup>+</sup>DCs produced a large amount of

IL-28B upon exposure to HCVcc and released a lower amount of IFN- $\alpha$  upon HCVcc or HSV

(Fig 4A). In contrast, pDCs produced a large amount of IFN- $\alpha$  in response to HCVcc and HSV

and a much lower level of IL-28B upon HCVcc (Fig S6). In mDCs, IL-28B and IFN- $\alpha$  were not

detectable with any of these viruses (data not shown).

BDCA3<sup>+</sup>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- $\beta$  and IFN- $\alpha$  than the other subsets (Fig 4B). Liver BDCA3<sup>†</sup>DCs were capable

of producing IL-28B in response to HCVcc (Fig 4C). These results show that, upon HCVcc

stimulation, BDCA3 $^{\dagger}$ DCs produce more IFN- $\lambda$ s and pDCs release more IFN- $\beta$  and IFN- $\alpha$  than

the other DC subsets, respectively. Taking a clinical impact of IL-28B genotypes on HCV

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eradication into consideration, we focused on IL-28B/IFN-λ3 as a representative for IFN-λs in

the following experiments.

In a co-culture with JFH-1-infected Huh7.5.1 cells, BDCA3  $^{\mbox{\tiny T}}DCs$  profoundly released

IL-29, IL-28A and IL-28B (Fig 4D, the results of IL-29 and IL-28A, not shown). Whereas,

BDCA3<sup>+</sup>DCs failed to respond to Huh7.5.1 cells lacking HCV/JFH-1, showing that IL-28B

production from BDCA3<sup>+</sup>DCs is dependent on HCV genome (Fig 4D). In the absence of

BDCA3<sup>+</sup>DCs, IL-28B is undetectable in the supernatant from JFH-1-infected Huh7.5.1 cells,

demonstrating that BDCA3<sup>+</sup>DCs, not HCV-replicating Huh7.5.1 cells, produce detectable

amount of IL-28B (Fig 4D). In the co-culture, BDCA3<sup>+</sup>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 co-culture, ISG15 was significantly induced only in JFH-1-infected Huh7.5.1

cells co-cultured with BDCA3<sup>+</sup>DCs (Fig 4F). A strong induction was observed with other ISGs

in IFH-1-infected Huh7.5.1 in the presence of BDCA3<sup>+</sup>DCs, such as IFIT1, MxA, RSD2, IP-10

and USP18 (Fig S7). The results clearly show that BDCA3<sup>+</sup>DCs are capable of producing large

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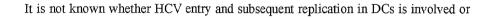
amount of IFN-\(\lambda\)s in response to cellular or cell-free HCV, thereby inducing various ISGs in

bystander liver cells.



CD81 and endosome acidification are involved in IL-28B production from

HCV-stimulated BDCA3<sup>+</sup>DCs, but HCV replication is not involved.



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

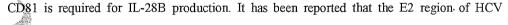
expression of NS5A after inoculation (data not shown). BDCA3<sup>+</sup>DCs produced comparable

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<sup>+</sup>DCs by



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

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BDCA3<sup>+</sup>DCs (Fig 1B, Fig S1). Masking of CD81 with Ab significantly impaired IL-28B

production from HCVcc-stimulated BDCA3<sup>+</sup>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<sup>+</sup>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, 5B, NH4Cl, data not shown). These results suggest that the

endosome acidification is involved in HCVcc- or poly IC-stimulated BDCA3<sup>+</sup>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  $\mu M$ ) and bafilomycin

A1\_25nM) did not reduce the viability of BDCA3<sup>+</sup>DCs (Fig S10).

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# BDCA3<sup>+</sup>DCs produce IL-28B in response to HCVcc by a 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<sup>+</sup>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<sup>†</sup>DCs (Fig 6A, 6B). 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 TRIF-dependent

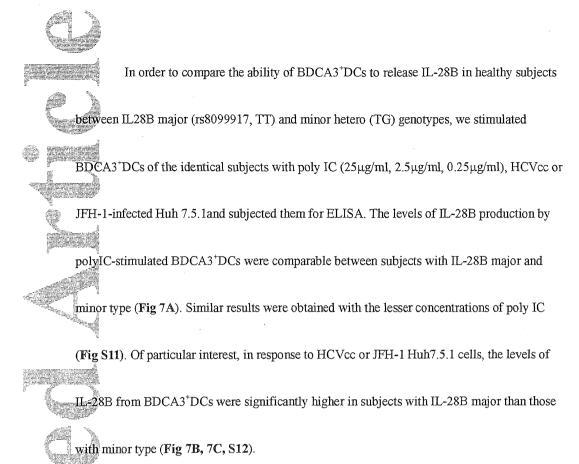
pathway to produce IL-28B. .



BDCA3<sup>†</sup>DCs in subjects with IL-28B major genotype produce more IL-28B in response to

HCV than those with IL-28B minor type.

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#### 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- $\lambda$ 1, IL-28A/IFN- $\lambda$ 2 and IL-28B/IFN- $\lambda$ 3 robustly in response to HCV, 3) recognize

HCV by a CD81-, endosome acidification and TRIF-dependent mechanism, and 4) produce

larger amount of IFN-\(\lambda\)s upon HCV stimulation in subjects with IL-28B major genotype

(rs8099917, TT). These characteristics of BDCA3<sup>+</sup>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<sup>+</sup>DCs between in PBMCs and in IHLs, the cells are more abundant in the liver. The

phenotypes of liver BDCA3<sup>†</sup>DCs were more mature than the PBMC counterparts. In support for

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our observations, a recent publication showed that CD141<sup>+</sup> (BDCA3<sup>+</sup>) 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-\(\lambda\)s in response to poly IC or

HCVcc.

BDCA3 $^{+}$ DCs were able to produce large amounts of IFN- $\lambda$ s but much less IFN- $\beta$  or

FN<sub>-</sub>α upon TLR3 stimulation. In contrast, in response to TLR9 agonist, pDCs released large

amounts of IFN- $\beta$  and IFN- $\alpha$  but much less IFN- $\lambda$ s. Such distinctive patterns of IFN response

between BDCA3<sup>+</sup>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-kB 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- $\lambda s$ , IFN- $\beta$  and IFN- $\alpha$ . 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

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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<sup>+</sup>DCs and pDCs.

Comprehensive analysis of gene profiles downstream of TLRs or RIG-I in BDCA3<sup>+</sup>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<sup>+</sup>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-\(\lambda\)s 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<sup>+</sup>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<sup>+</sup>DCs through

CD81 and subsequent endosome acidification are critically involved in IL-28B responses.

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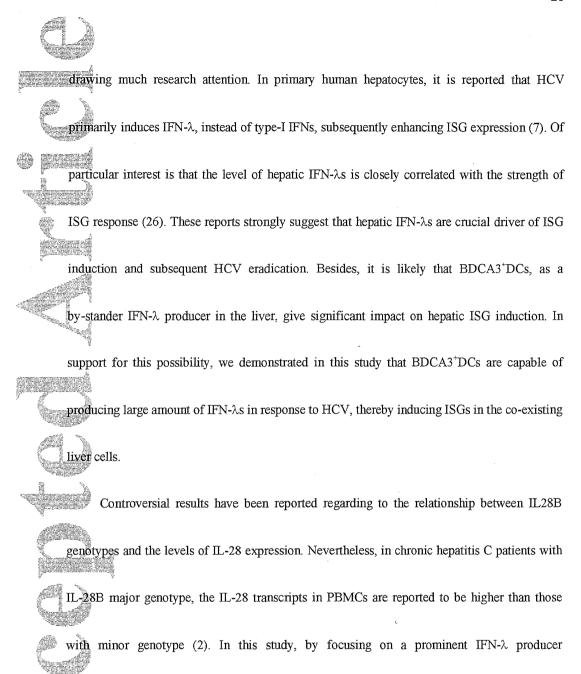
Involvement of TRIF-dependent pathways in IL-28B production was shown by the significant inhibition of IL-28B with TRIF inhibitor. Nevertheless, active HCV replication in the cells is not required. Based on our data, we considered that BDCA3<sup>+</sup>DCs recognize HCV genome mainly by endosome and 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<sup>+</sup>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<sup>+</sup>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<sup>+</sup>DCs.

The relationship between IL-28B expression and the induction of ISGs has been

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IL-28B major genotype could respond to HCV by releasing more IL-28B. Of interest, such superior capacity of BDCA3<sup>+</sup>DCs was observed only in response to HCV but not to poly IC.

(BDCA3 DCs) and using the assay specific for IL-28B, we showed that the subjects with

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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-\(\lambda\)1 production (27). This report supports for the possibility that the similar epigenetic machineries could be operated as well in HCV-induced IFN-\(\lambda\)s 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

In conclusion, human BDCA3 $^{+}$ DCs, having tendency of being accumulated in the liver, recognize HCV and produce large amounts of IFN- $\lambda$ s. An enhanced IL-28B/IFN- $\lambda$ 3 response of

comprehensive understanding of biological importance of IL-28B in HCV infection, such

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co-founding factors, if they exist, need to be explored.



BDCA3<sup>+</sup>DCs to HCV in subjects with IL-28B major genotype suggests that BDCA3<sup>+</sup>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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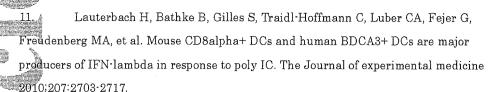
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### FIGURE LEGENDS

Figure 1: Identification and phenotypic analyses of peripheral blood and intrahepatic



A\_We defined BDCA3<sup>+</sup>DCs as Lineage HLA-DR<sup>+</sup>BDCA3<sup>high+</sup> cells (**middle**), pDCs as

Lineage HLA-DR CD11c CD123 tight cells and mDCs as

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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.

5. 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

DeThe staining for BDCA3 (green), CLEC9A (red) identifies BDCA3 DCs (merge,

BDCA3<sup>+</sup>CLEC9A<sup>+</sup>) 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

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