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IL-28B/IFN- $\lambda$ 3 was quantified by a newly developed chemiluminescence enzyme immunoassay (CLEIA) system (15). IL-29/IFN- $\lambda$ 1, IL-28A/IFN- $\lambda$ 2 and IFN- $\beta$  were assayed by commercially available ELISA kits (eBioscience, R&D and PBL, respectively). IFN- $\alpha$  was measured by cytometric beads array kits (BD) according to the manufacturer's instructions.

#### Statistical analysis.

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

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## RESULTS

### **Human BDCA3<sup>+</sup>DCs are phenotypically distinct from pDCs and mDCs.**

We defined BDCA3<sup>+</sup>DCs as Lin<sup>-</sup>HLA-DR<sup>+</sup>BDCA3<sup>high</sup> cells (Fig 1A, left, middle), and pDCs and mDCs by the patterns of CD11c and CD123 expressions (Fig 1A, right). The level of CD86 on pDCs or mDCs is comparatively higher than those on BDCA3<sup>+</sup>DCs (Fig 1B).

The expression of CD81 is higher on BDCA3<sup>+</sup>DCs than on pDCs and mDCs (Fig 1B, Fig S1).

CLEC9A, a member of C-type lectin, is expressed specifically on BDCA3<sup>+</sup>DCs as reported elsewhere (16), but not on pDCs and mDCs (Fig 1B).

### **Liver BDCA3<sup>+</sup>DCs are more mature than the counterparts in the periphery.**

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

In order to confirm that BDCA3<sup>+</sup>DCs are localized in the liver, we stained the cells

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with immunofluorescence Abs in non-cancerous liver tissues. Liver BDCA3<sup>+</sup>DCs were defined as BDCA3<sup>+</sup>CLEC9A<sup>+</sup> 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<sup>+</sup>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<sup>+</sup>DCs in the liver over that in the periphery was observed regardless of the etiology of the liver disease (Supplementary Table 1).

**BDCA3<sup>+</sup>DCs produce a large amount of IFN- $\lambda$ s upon poly IC stimulation.**

We compared DC subsets for their abilities to produce IL-29/IFN- $\lambda$ 1, IL-28A/IFN- $\lambda$ 2,

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IL-28B/IFN- $\lambda$ 3, IFN- $\beta$ , and IFN- $\alpha$  in response to TLR agonists. Approximately  $4.0 \times 10^4$  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  $\mu$ l 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<sup>+</sup>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- $\beta$  than

other DCs (Fig S3A).

Similar results were obtained with the protein levels of IFN- $\lambda$ s, IFN- $\beta$  and

IFN- $\alpha$  released from DC subsets stimulated with TLR agonists. BDCA3<sup>+</sup>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- $\beta$  and IFN- $\alpha$  than BDCA3<sup>+</sup>DCs or

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mDCs (Fig 3A, Fig S3B). As for the relationship among the quantity of IFN- $\lambda$  subtypes from poly IC-stimulated BDCA3<sup>+</sup>DCs, the levels of IL-29/IFN- $\lambda$ 1 and IL-28B/IFN- $\lambda$ 3 were positively correlated ( $R^2=0.76$ ,  $p<0.05$ ), and those of IL-28A/IFN- $\lambda$ 2 and IL-28B/IFN- $\lambda$ 3 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- $\lambda$ 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- $\alpha$ , 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- $\lambda$ s.

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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<sup>+</sup>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<sup>+</sup>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- $\lambda$ 3 as a representative for IFN- $\lambda$ s in the following experiments.

In a co-culture with JFH-1-infected Huh7.5.1 cells, BDCA3<sup>+</sup>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 JFH-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.**

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<sup>+</sup>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 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

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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<sup>+</sup>DCs with chloroquine, bafilomycin A1 or NH<sub>4</sub>Cl significantly suppressed their IL-28B production either in response to HCVcc or poly IC (Fig 5A, 5B, NH<sub>4</sub>Cl, 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 μM) and bafilomycin A1 (25 nM) 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<sup>+</sup>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<sup>+</sup>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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In order to compare the ability of BDCA3<sup>+</sup>DCs to release IL-28B in healthy subjects between IL28B major (rs8099917, TT) and minor hetero (TG) genotypes, we stimulated BDCA3<sup>+</sup>DCs of the identical subjects with poly IC (25µg/ml, 2.5µg/ml, 0.25µg/ml), HCVcc or JFH-1-infected Huh 7.5.1 and subjected them for ELISA. The levels of IL-28B production by polyIC-stimulated BDCA3<sup>+</sup>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<sup>+</sup>DCs were significantly higher in subjects with IL-28B major than those with minor type (Fig 7B, 7C, S12).

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## DISCUSSION

In this study, we demonstrated that human BDCA3<sup>+</sup>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<sup>+</sup>DCs exist or not in the liver. We identified BDCA3<sup>+</sup>CLEC9A<sup>+</sup> 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<sup>+</sup>DCs are functional, capable of releasing IFN- $\lambda$ s in response to poly IC or HCVcc.

BDCA3<sup>+</sup>DCs were able to produce large amounts of IFN- $\lambda$ s but much less IFN- $\beta$  or IFN- $\alpha$  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- $\kappa$ B are involved in IFN- $\beta$  and IFN- $\lambda$ 1, while IRF-7 and NF- $\kappa$ B are involved in IFN- $\alpha$  and IFN- $\lambda$ 2/ $\lambda$ 3 (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- $\lambda$ s and IFN- $\alpha$  production by BDCA3<sup>+</sup>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<sup>+</sup>DCs were found to be more sensitive to HCVcc than JEV or HSV in IL-28B/IFN- $\lambda$ 3 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<sup>+</sup>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- $\kappa$ B 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- $\kappa$ B 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 occludin. 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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drawing much research attention. In primary human hepatocytes, it is reported that HCV primarily induces IFN- $\lambda$ , instead of type-I IFNs, subsequently enhancing ISG expression (7). Of particular interest is that the level of hepatic IFN- $\lambda$ s is closely correlated with the strength of ISG response (26). These reports strongly suggest that hepatic IFN- $\lambda$ s are crucial driver of ISG induction and subsequent HCV eradication. Besides, it is likely that BDCA3<sup>+</sup>DCs, as a by-stander IFN- $\lambda$  producer in the liver, give significant impact on hepatic ISG induction. In support for this possibility, we demonstrated in this study that BDCA3<sup>+</sup>DCs are capable of producing large amount of IFN- $\lambda$ s 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- $\lambda$  producer (BDCA3<sup>+</sup>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<sup>+</sup>DCs was observed only in response to HCV but not to poly IC.

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Since the pathways downstream of TLR3-TRIF leading to IL-28B in BDCA3<sup>+</sup>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- $\lambda$ 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<sup>+</sup>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<sup>+</sup>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

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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<sup>+</sup>DCs as IFN- $\lambda$  producer could provide useful information on the development of a natural adjuvant against HCV infection.

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