

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,



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Hepatology



RESULTS

Human BDCA3⁺DCs are phenotypically distinct from pDCs and mDCs.

We defined BDCA3⁺DCs as LinHLA-DR⁺BDCA3^{high+} cells (Fig 1A, left, middle),

and pDCs and mDCs by the patterns of CD11c and CD123 expressions (Fig 1A, right). The

evel of CD86 on pDCs or mDCs is comparatively higher than those on BDCA3⁺DCs (Fig 1B).

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

CLEC9A, a member of C-type lectin, is expressed specifically on BDCA3⁺DCs as reported

elsewhere (16), but not on pDCs and mDCs (Fig 1B).

Liver BDCA3[†]DCs are more mature than the counterparts in the periphery.

BDCA3⁺DCs in infiltrated hepatic lymphocytes (IHLs) are all positive for CLEC9A,

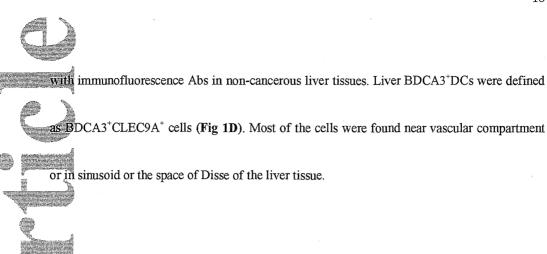
but liver pDCs or mDCs are not (data not shown). The levels of CD40, CD80, CD83 and CD86

on liver BDCA3 DCs are higher than those on the peripheral counterparts, suggesting that

BDCA3⁺DCs are more mature in the liver compared to those in the periphery (Fig 1C).

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

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BDCA3⁺DCs are scarce in PBMCs but more abundant in the liver.

The percentages of BDCA3⁺DCs in PBMCs were much lower than those of the other DC subsets (BDCA3⁺DCs, pDCs and mDCs, mean \pm SD [%], 0.054 ± 0.044 , 0.27 ± 0.21 and 1.30 ± 0.65) (Fig 2A). The percentages of BDCA3⁺DCs in IHLs were lower than those of the others (BDCA3⁺DCs, pDCs and mDCs, mean \pm SD [%], 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

(Supplementary 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- λ 1, IL-28A/IFN- λ 2,

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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 400ml of donated blood from healthy volunteers. We fixed

the number of DCs at 2.5×10⁴ cells/100 μl 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,

pDes 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- λ s 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).

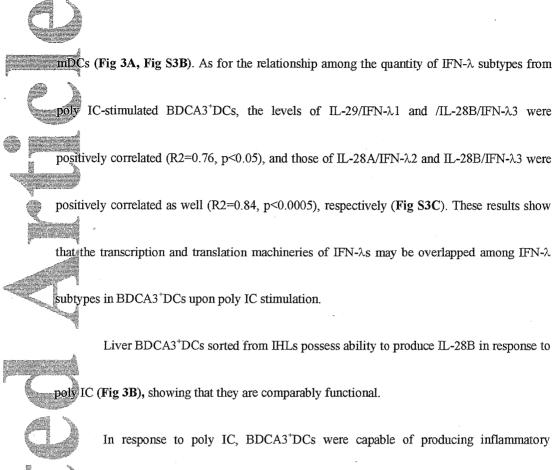
Similar results were obtained with the protein levels of IFN- λs , IFN- β and

IFN-a 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

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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.

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

Huh 7.5.1 cells.

We stimulated freshly isolated BDCA3⁺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 DCs 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

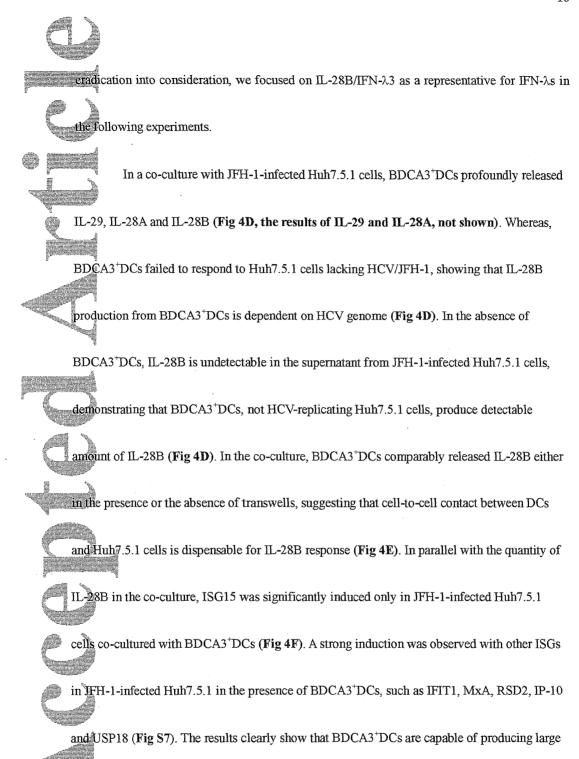
amounts of IFN- β and IFN- α than the other subsets (Fig 4B). Liver BDCA3⁺DCs were capable

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

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amount of IFN-λs 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

HCY-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).



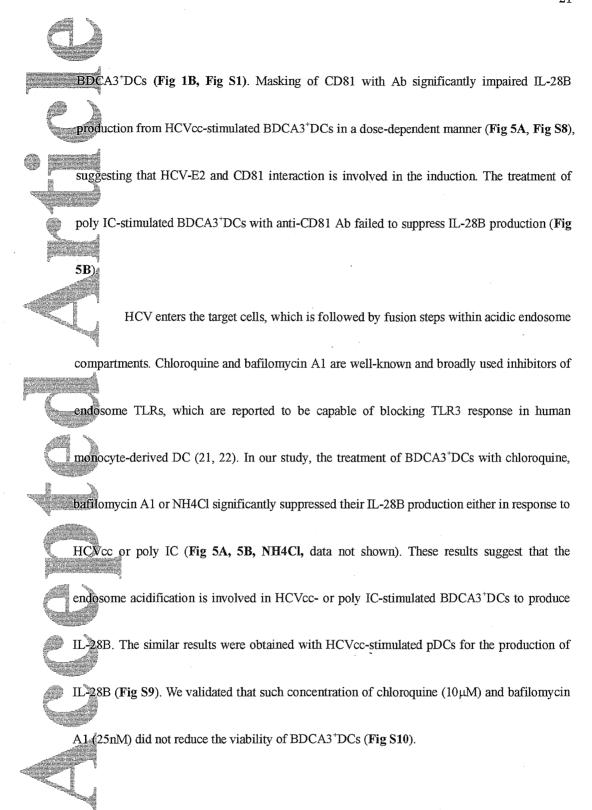
We next examined whether or not the association of HCVcc with BDCA3+DCs by

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

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BDCA3⁺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 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, 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

patliway. These results show that BDCA3 DCs may recognize HCVcc by way of TRIF-dependent

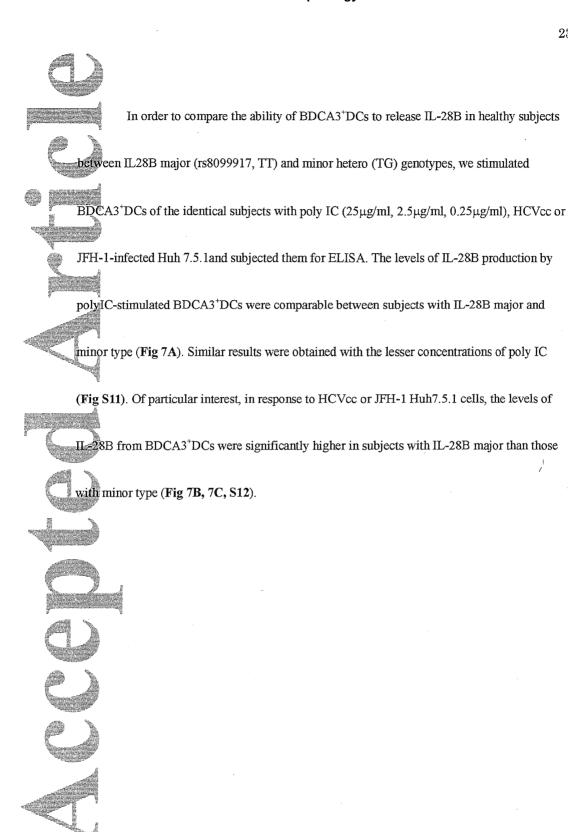
pathway to produce IL-28B. .



BDCA3⁺DCs in subjects with IL-28B major genotype produce more IL-28B in response to

HCV than those with IL-28B minor type.

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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- λ 1, IL-28A/IFN- λ 2 and IL-28B/IFN- λ 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-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

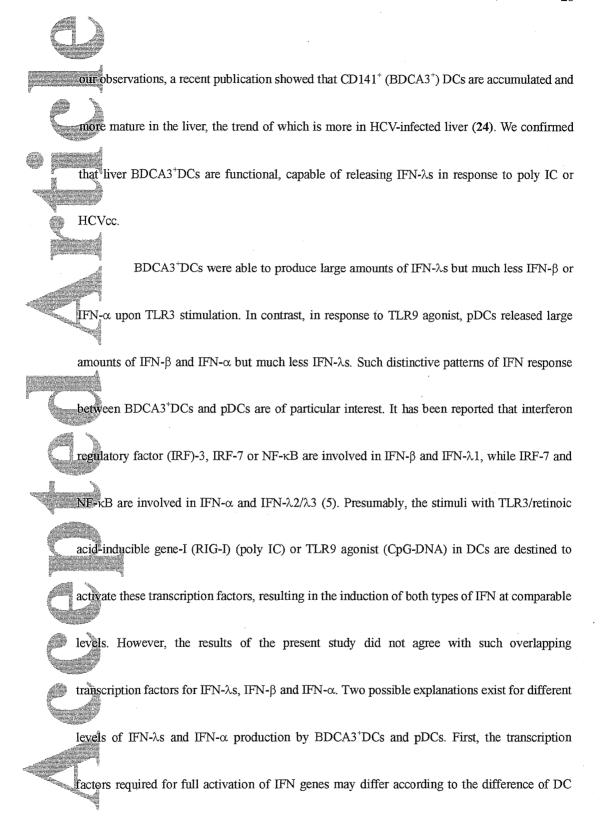
remains obscure whether functional BDCA3*DCs exist or not in the liver. We identified

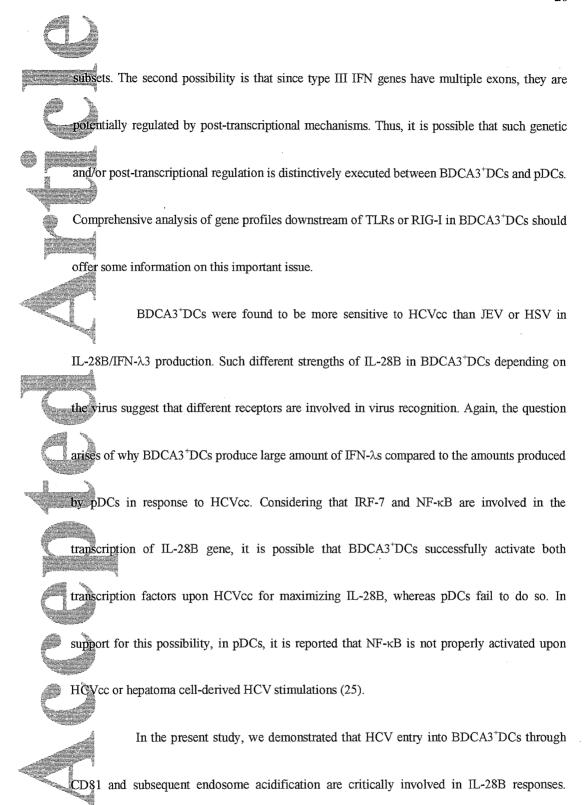
 $B\bar{D}CA3^{+}CLEC9A^{+}$ cells in the liver tissue (Fig 1D). In a paired frequency analysis of

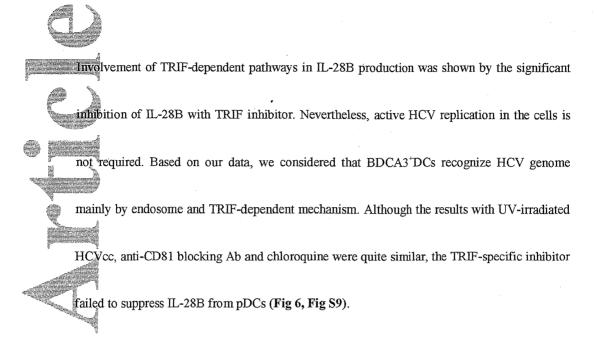
BDCA3+DCs between in PBMCs and in IHLs, the cells are more abundant in the liver. The

phenotypes of liver BDCA3 DCs were more mature than the PBMC counterparts. In support for

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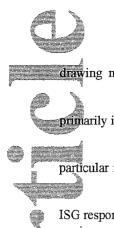




In the co-culture with JFH-1-transfected Huh7.5.1 cells, BDCA3*DCs presumably receive some signals for IL-28B production by way of cell-to-cell dependent and independent mechanisms. In the present study, most of the stimuli to BDCA3*DCs for IL-28B production may be the released HCVcc from Huh7.5.1 cells, 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 BD€A3*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- 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.

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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 L-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.

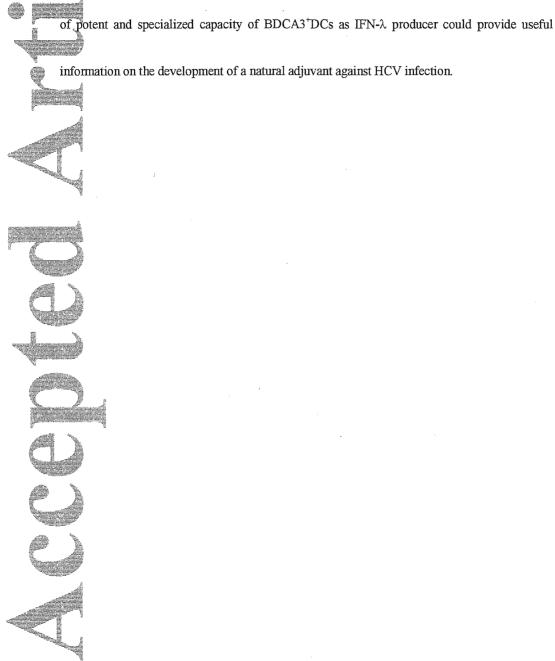
In conclusion, human BDCA3⁺DCs, having tendency of being accumulated in the liver,

recognize HCV and produce large amounts of IFN-\(\lambda\)s. An enhanced IL-28B/IFN-\(\lambda\)3 response of

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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



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