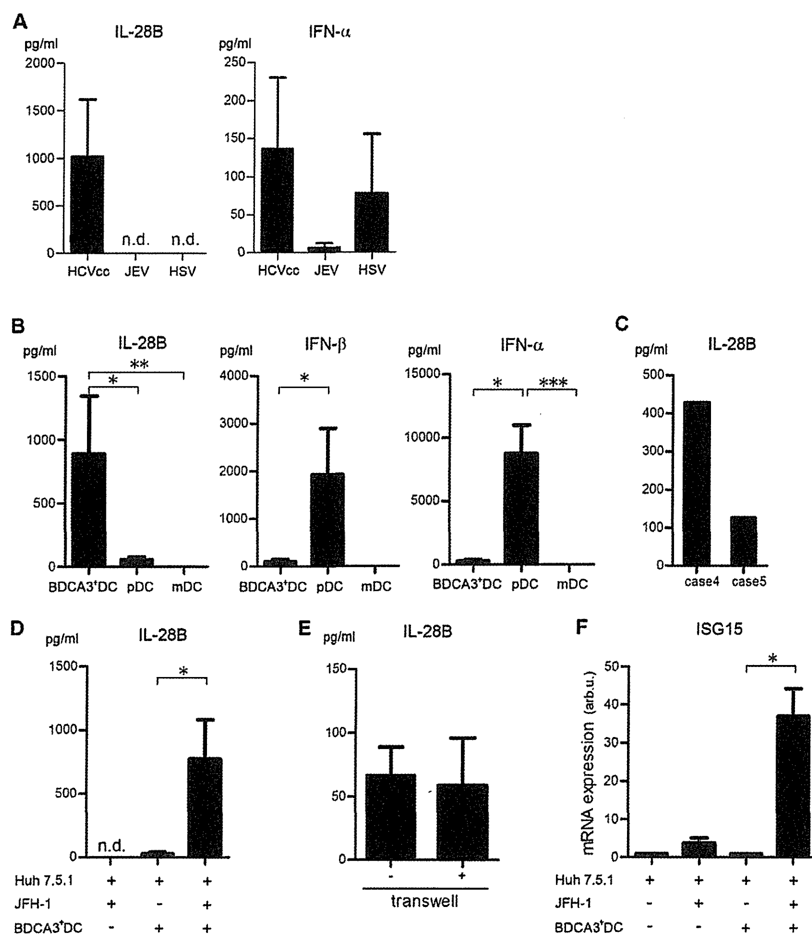

Figure 4. BDCA3+DCs produce IL-29, IL-28A and IL-28B upon cell-cultured HCV or HCV/JFH-1-transfected Huh7.5.1 cells, thereby inducing ISG.

A. BDCA3+DCs were cultured at 2.5x104 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 2.5x104 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 2.5x104 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 2.5x104 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 2.5x104 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

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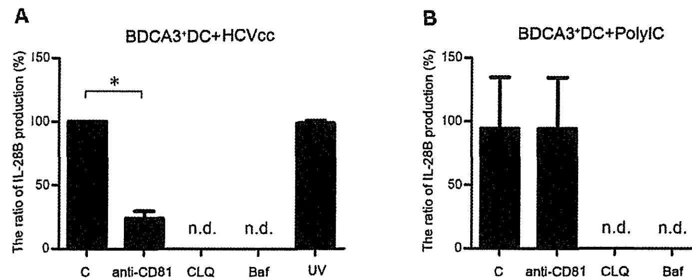

Figure 5: The CD81 and endosome acidification is involved in the production of IL-28B from HCV-stimulated BDCA3+DCs, but HCV replication is not necessary.
A.B. BDCA3+DCs were cultured at 2.5x104 cells with HCVcc at an MOI of 10 (A) or poly IC (25μg/ml) (B). In some experiments, UV-irradiated HCVcc was 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

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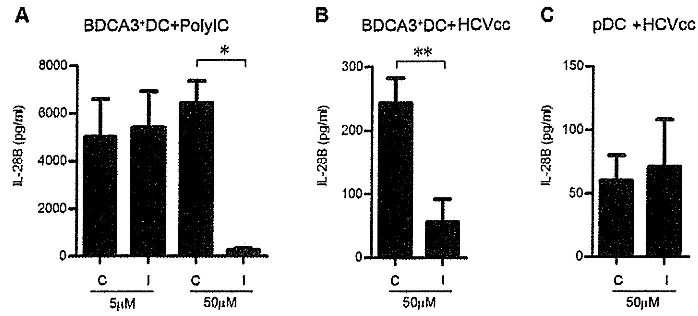

Figure 6: BDCA3+DCs produce IL-28B upon HCVcc stimulation in a TRIF-dependent mechanism. BDCA3+DCs or pDCs had been treated with 5 or 50 μM TRIF inhibitory peptide or control peptide for 2h. Subsequently, BDCA3+DCs were stimulated with PolyIC (25μg/ml) or HCVcc (M.O.I.=10), and pDCs were stimulated with 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.

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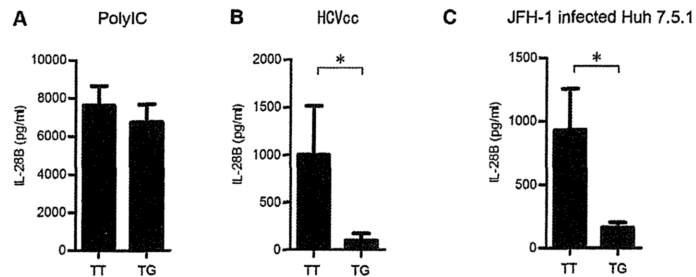

Figure 7. In response to HCVcc, BDCA3+DCs of healthy donors with IL-28B major genotype (rs8099917, TT) produced more IL-28B than those with minor type (TG).
BDCA3+DCs of healthy donors with IL-28B TT (rs8099917) or TG genotype were cultured at 2.5x104 cells with 25 μg/ml poly IC (A), with HCVcc at an 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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Supplementary materials

Human BDCA3+ dendritic cells are a potent producer of IFN-λ in response to hepatitis C virus

Sachiyo Yoshio[1], Tatsuya Kanto[1]*, Shoko Kuroda[1], Tokuhiro Matsubara[1], Koyo Higashitani[1], Naruyasu Kakita[1], Hisashi Ishida[1], Naoki Hiramatsu[1], Hiroaki Nagano[2], Masaya Sugiyama[3], Kazumoto Murata[3], Takasuke Fukuhara[4], Yoshiharu Matsuura[4] , Norio Hayashi[5], Masashi Mizokami[3], and Tetsuo Takehara[1]

Supplementary material and methods

Reagents.

Antibodies (Abs) to Lineage-1 (Lin-1) cocktail (CD3, CD14, CD16, CD19, CD20, and CD56), HLA-DR (clone, L243), CD123 (7G3), CD11c (B-ly6), CD40 (5C3), CD80 (L307.4), CD83 (HB15e), CD86 (2331), CD81 (JS-81), and isotypes were purchased from BD Bioscience Pharmingen (San Diego, CA). Anti-BDCA-3/CD141 (AD5-14H12) Ab, FcR-blocking, anti-CD3, anti-CD14, anti-CD19, and anti-CD56 micro-beads were obtained

from Miltenyi Biotec (Bergisch Gladbach, Germany). Anti-CLEC9A (8F9) Ab was from Biolegend (San Diego, CA). Poly IC was purchased from InvivoGen (San Diego, CA). CpG-DNA was from Sigma Aldrich (St. Louis, MO, USA). Synthetic agonists specific for TLR1/6 (Pam3CSK4.3HCL), TLR2/6 (MALP-2) or TLR7/8 (R848) were purchased from Imgenex (San Diego, CA). Chloroquine and Bafilomycin A1 were purchased from Sigma Aldrich. TRIF inhibitory peptide and control were from InvivoGen (San Diego, CA). Recombinant IL-29/IFN-λ1, IL-28A/IFN-λ2, and IL-28B/IFN-λ3 were obtained from R&D Systems.

Quantitative RT-PCR

Total RNA was prepared using the RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Quantification of mRNA levels of IL-29/λ1, IL-28A/λ2, IL-28B/λ3 and IFN-β were performed by Light Cycler 480II (Roche Applied Science, Basel, Switzerland). Data were analyzed by absolute quantification using Light Cycler 480 software and normalized using β-actin. Specific primer sets for the detection of IFN-λ1, 2, or 3 cDNA and the PCR conditions specific for each were set as reported previously (1).

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Quantification of mRNA levels of ISG15, IFIT1, MxA, RSD2, IP-10, USP18 were performed using commercially available primers and probes according to the manufacturer's instructions (ABI system). The relative mRNA expression was compared using 18S as internal reference.

Analysis of Genetic Variations of IL28B

The IL-28B genotypes (rs 8099917 SNP) of healthy subjects were determined using ABI TaqMan SNP genotyping assays (Applied Biosystems).

Supplementary Figure Legends

Figure S1. The degree of CD81 expression on $BDCA3^{+}$DCs is higher than those on pDCs.

The PBMCs from healthy donors were stained with antibodies for DC phenotypes and CD81 as described in Materials and Methods. The percentage of CD81-positive cells in DCs was examined by FACS. Results are shown as the mean + SEM from 6 independent experiments. ***, $p < 0.0005$ by Kruskal-Wallis test.

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Figure S2. BDCA3⁺DCs, pDCs and mDCs distinctively respond to various TLR agonists for IL-28B production.

Various DCs were placed at 2.5×10^4 cells/100 μl and were incubated with each TLR agonist; 50 ng/ml Pam3CSK4.3HCL (for TLR1/6), 25 μg/ml poly IC (TLR3), 50 ng/ml MALP-2 (TLR2/6), 1 μg/ml R848 (TLR7/8) and 5 μM CPG-DNA (TLR9). After 24h incubation, the supernatants were examined for IL-28B. Results are shown as mean + SEM from 3 independent experiments.

Figure S3. BDCA3⁺DCs express and produce IL-29, IL-28A and IL-28B in response to poly IC, the levels of which are positively correlated each other.

BDCA3⁺DCs, and mDCs were placed at 2.5×10^4 cells/100 μl and were incubated with 25 μg/ml poly IC, and pDCs with 5μM CPG-DNA.

A. The expressions of IL-29, IL-28A, IL-28B and IFN-β mRNA were compared among the DC subsets after 4h. The relative mRNA expression (arbitrary unit) was compared using β-actin as internal reference. Results are shown as mean + SEM from 5 independent experiments. *, $p < 0.05$ by Kruskal-Wallis test

B. The levels of IL-29, IL-28A, IL-28B, IFN-β and IFN-α produced from poly IC-stimulated

BDCA3+DCs and CpG-stimulated pDCs were evaluated by ELISA. Results are shown as mean + SEM from 5 independent experiments.

C. D. A correlation was analyzed between the IL-28B and IL-29 (C) or IL-28B and IL-28A levels (D). IL-28B and IL-29, R^2=0.76, $p<0.05$, IL-28B and IL-28A, R2=0.84, $p<0.005$, by Spearmann's correlation coefficient.

Figure S4. BDCA3+DCs produce various cytokines and IL-28B upon poly IC stimulation, exhibiting suppressive effect on HCV replication.

A. BDCA3+DCs and mDCs were placed at 2.5×10^4 cells/100 μl and were incubated with 25 μg/ml poly IC, and pDCs with 5μM CPG-DNA. The supernatants were examined for TNF-α, IL-6, IL-10, and IL12p70. Results are shown as mean + SEM from 15 independent experiments. *, $p < 0.05$; **, $p < 0.005$ by Kruskal-Wallis test. n.d., not detected

B. The comparison of the suppressive effect on HCV replication of supernatants from poly IC-stimulated BDCA3+DCs and recombinant IFN-λs. As for an assessment of HCV replication, Huh7 cells transfected with pNNeo/3-5B harboring subgenomic replicon-(HCV-N strain) was used (2). The IL-28B concentration in the supernatants from

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BDCA3⁺DCs was determined by ELISA. HCV replicon-positive Huh7 cells were incubated with various concentrations of the supernatants adjusted by IL-28B level or recombinant IL-29 (rIL-29), rIL-28A or, rIL-28B. After 48 hrs, Huh7 cells were harvested and were subjected to real time PCR analysis for HCVRNA quantification as reported previously **(2)**. HCV RNA levels are shown as relative percentages of the untreated control. For each sample, RT-PCR was performed in triplicate. The mean value obtained from 3 independent experiments is plotted; error bars indicate the SEM.

Figure S5. BDCA3$^+$DCs produced IL-28B in response to HCVcc in an MOI-dependent manner.

BDCA3$^+$DCs were incubated for 24h with HCVcc-free medium (as depicted as -), HCVcc at an MOI of 1 or 10. The supernatants were examined for IL-28B. Results are shown as mean + SEM from 4 independent experiments. *, $p < 0.05$ by paired-t test

Figure S6. Plasmacytoid DCs produce IL-28B and IFN-α upon HCVcc stimulation.

Plasmacytoid DCs were cultured at 2.5×10^4 cells for 24 h with HCVcc, JEV or HSV at an MOI

of 10. The levels of IL-28B and IFN-α in the supernatants were measured by ELISA. Results are shown as mean + SEM from 6 experiments.

Figure S7. Various ISGs are induced in JFH-1-transfected Huh7.5.1 cells in the presence of BDCA3$^+$DCs.

BDCA3$^+$DCs were co-cultured at 2.5x10^4 cells with JFH-1-transfected (M.O.I.=2) or –untransfected Huh7.5.1 cells for 24h. After non-adherent BDCA3$^+$DCs were removed by extensive washing the culture wells, Huh7.5.1 cells were harvested and were subjected to real time RT-PCR for the quantification of IFIT1, MxA, RSD2, IP-10 and USP18. The relative mRNA expression (arbitrary unit) was compared using 18S as internal reference. The assays were performed according to the manufacturer's instructions. Results are shown as mean + SEM from 5 experiments. *, $p < 0.05$ by paired-t test

Figure S8: Anti-CD81 antibody inhibits HCVcc-induced IL-28B from BDCA3$^+$DCs in a dose-dependent manner.

BDCA3$^+$DCs were incubated for 24h with different concentrations of anti-CD81 antibody. The

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ratios of IL-28B levels are shown between the samples with various concentration of anti-CD81 antibody and those without. The horizontal bars indicate mean ± SD of 3 experiments.

Figure S9: The CD81 and endosome acidification is involved in the production of IL-28B from HCV-stimulated pDCs, but HCV replication is not necessary.

Plasmacytoid DCs were cultured at 2.5 x 10^4 cells with HCV at an MOI of 10. As the same as the experiments with $BDCA3^+$DCs, UV-irradiated HCVcc, the treatments with anti-CD81Ab (5µg/ml) or chloroquine (10µM) were performed. The supernatants were examined for IL-28B. Results are expressed as ratios of IL-28B quantity between samples with or without the treatments. The values are shown as mean + SEM from 5 independent experiments. *, $p < 0.05$ by paired-t test

C, UV, CD81, CLQ, see Figure 5. n.d, not detected

Figure S10. The relationships between concentrations of chloroquine, bafilomycin A1 and the viability of $BDCA3^+$DCs.

$BDCA3^+$DCs were incubated in the presence of different concentrations of chloroquine or

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bafilomycin A1. After 24h, the viability of BDCA3+DCs was evaluated by a trypan blue dye-exclusion test. The values are expressed as the ratios of live cells in samples with or without the treatments. The horizontal bars indicate means ± SD of 3 experiments.

Figure S11. The quantity of IL-28B from poly IC-stimulated BDCA3+DCs were comparable regardless of the IL-28B genotype, even at the lower concentrations of poly IC.

BDCA3+DCs were incubated for 24h with various concentrations of Poly IC. The levels of IL-28B are quantified by ELISA. The values are shown as mean + SEM from 3 independent experiments.

Figure S12. The quantity of IL-29 and IL-28A produced from BDCA3+DCs stimulated with poly IC- or JFH-1-infected Huh 7.5.1 .

BDCA3+DCs of healthy donors with the IL-28B major (rs8099917, TT) or the minor (TG) genotype were cultured at 2.5×10^4 cells with 25 μg/ml poly IC (**A**), or with JFH-1-infected- Huh 7.5.1 cells (**B**) for 24 h. The supernatants were subjected for IL-29 and IL-28A ELISA. The

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results are the mean + SEM from 15 donors with TT and 8 with TG (A), and from 8 donors with TT and 7 with TG (B), respectively.

Supplementary Reference

1. Sugiyama M, Kimura T, Naito S, Mukaide M, Shinauchi T, Ueno M, Ito K, et al. Development of specific and quantitative real-time detection PCR and immunoassays for lambda3-interferon. Hepatology research : the official journal of the Japan Society of Hepatology 2012.

2. Yi M, Ma Y, Yates J, Lemon SM. Compensatory mutations in E1, p7, NS2, and NS3 enhance yields of cell culture-infectious intergenotypic chimeric hepatitis C virus. Journal of virology 2007;81:629-638.

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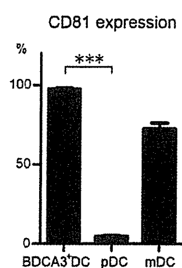
Supplementary Table 1: Clinical backgrounds of the subjects for analysis of liver samples

						$BDCA3^{+}$DCs (%)	
case	**sex**	**Age**	**etiology**	**tumor type**	**ALT (U/L)**	**in PBMC**	**in IHL**
1	M	82	nonBnonC	angiosarcoma	12	0.05	0.42
2	M	77	nonBnonC	liver metastasis	18	-	0.25
3	M	42	nonBnonC	liver metastasis	16	-	0.15
4	F	62	nonBnonC	liver metastasis	12	-	-
5	M	62	nonBnonC	liver metastasis	18	-	-
6	F	28	nonBnonC	hemangioma	70	-	0.88
7	M	68	nonBnonC	HCC	113	0.033	0.11
8	M	80	nonBnonC	HCC	22	-	-
9	M	79	nonBnonC	HCC	16	-	-
10	M	67	nonBnonC	CCC	8	0.01	0.08
11	M	79	nonBnonC	CCC	10	-	-
12	F	39	nonBnonC	Post-transplant lymphoproliferative disorders	8	-	0.10
13	M	81	HCV	HCC	27	0.03	0.06
14	F	64	HCV	HCC	71	0.01	0.29
15	M	59	HCV	HCC	33	0.06	0.44
16	M	72	HCV	HCC	21	-	-
17	M	82	HCV	HCC	37	-	-
18	F	65	HBV	HCC	10	0.044	0.45
19	M	64	HBV	CCC	27	0.01	0.31
20	F	67	HBV	HCC	30	-	-

DC, dendritic cell; ALT, alanine aminotransferase;
PBMC, peripheral blood mononuclear cell; IHL, intrahepatic lymphocyte
HCC, hepatocellular carcinoma; CCC, cholangiocellular carcinoma
HCV, hepatitis C virus; HBV, hepatitis B virus



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The PBMCs from healthy donors were stained with antibodies for DC phenotypes and CD81 as described in Materials and Methods. The percentage of CD81-positive cells in DCs was examined by FACS. Results are shown as the mean + SEM from 6 independent experiments. ***, $p < 0.0005$ by Kruskal-Wallis test.

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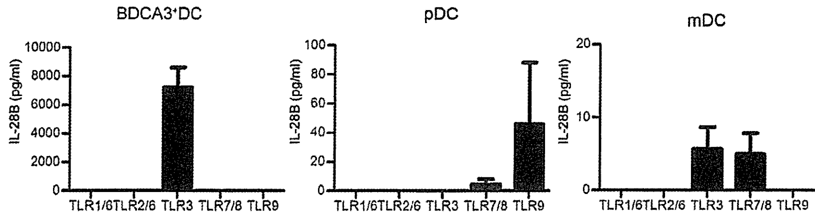

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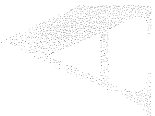

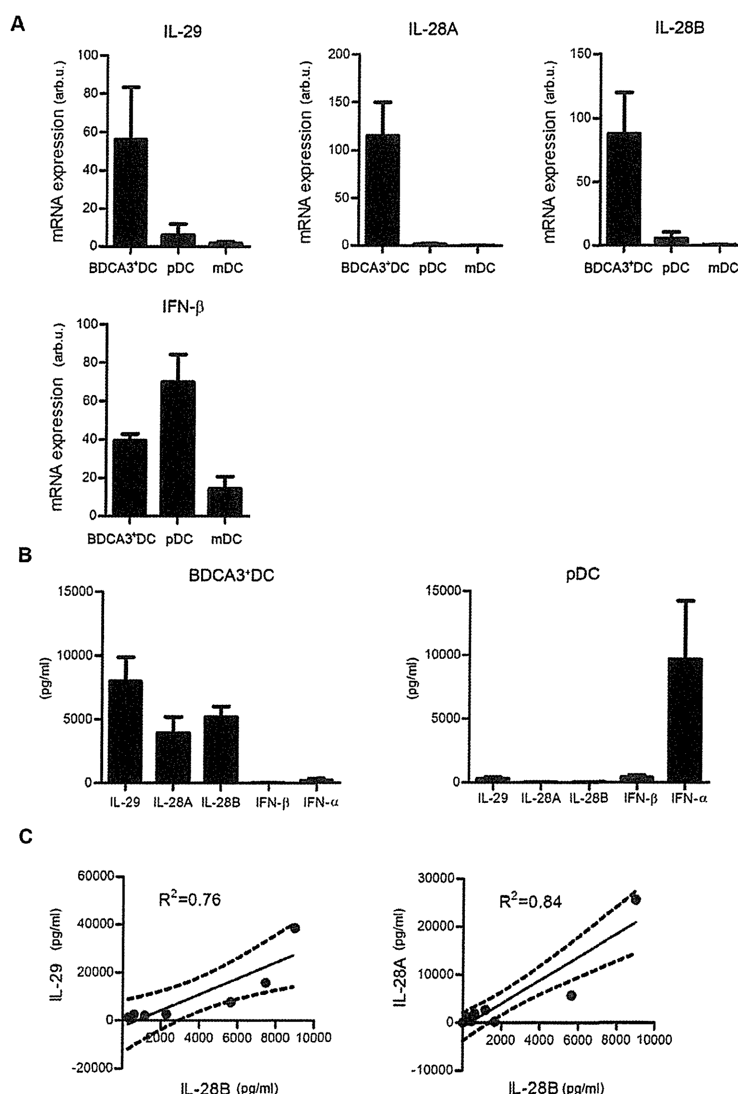

Figure S3. BDCA3+DCs express and produce IL-29, IL-28A and IL-28B in response to poly IC, the levels of which are positively correlated each other.
BDCA3+DCs, and mDCs were placed at 2.5×104 cells/100 μl and were incubated with 25 μg/ml poly IC, and pDCs with 5μM CPG-DNA.
A. The expressions of IL-29, IL-28A, IL-28B and IFN-β mRNA were compared among the DC subsets after 4h. The relative mRNA expression (arbitrary unit) was compared using β-actin as internal reference. Results are shown as mean + SEM from 5 independent experiments. *, $p < 0.05$ by Kruskal-Wallis test
B. The levels of IL-29, IL-28A, IL-28B, IFN-β and IFN-α produced from poly IC-stimulated BDCA3+DCs and CpG-stimulated pDCs were evaluated by ELISA. Results are shown as mean + SEM from 5 independent experiments.
C. D. A correlation was analyzed between the IL-28B and IL-29 (C) or IL-28B and IL-28A levels (D). IL-28B and IL-29, R2=0.76, p<0.05, IL-28B and IL-28A, R2=0.84, p<0.005, by Spearmann's correlation coefficient.

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