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.

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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

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 $2.5x10^4$ 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

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

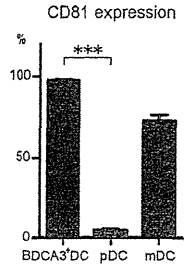

Figure S1. The degree of CD81 expression on BDCA3+DCs is higher than those on pDCs.
The PBMCs from healthy donors were stained with antibodies for DC phenotypes and CD81 as described in Materials and Methods. The percentage of CD81-positive cells in DCs was examined by FACS. Results are shown as the mean + SEM from 6 independent experiments. ***, $p < 0.0005$ by Kruskal-Wallis test.

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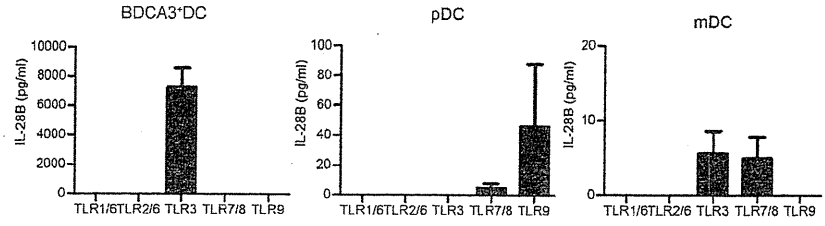

Figure S2. BDCA3+DCs, pDCs and mDCs distinctively respond to various TLR agonists for IL-28B production. Various DCs were placed at 2.5×104 cells/100 µl and were incubated with each TLR agonist; 50 ng/ml Pam3CSK4.3HCL (for TLR1/6), 25 µg/ml poly IC (TLR3), 50 ng/ml MALP-2 (TLR2/6), 1 µg/ml R848 (TLR7/8) 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.

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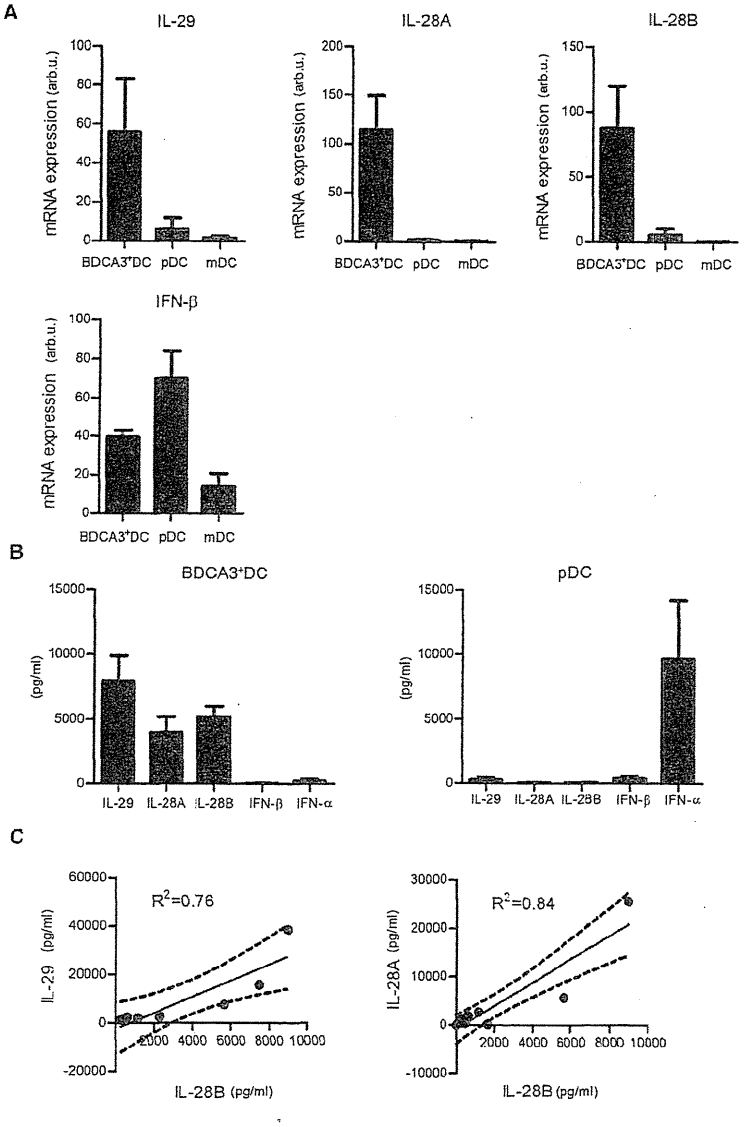

Figure S3. BDCA3+DCs express and produce IL-29, IL-28A and IL-28B in response to poly IC, the levels of which are positively correlated each other.

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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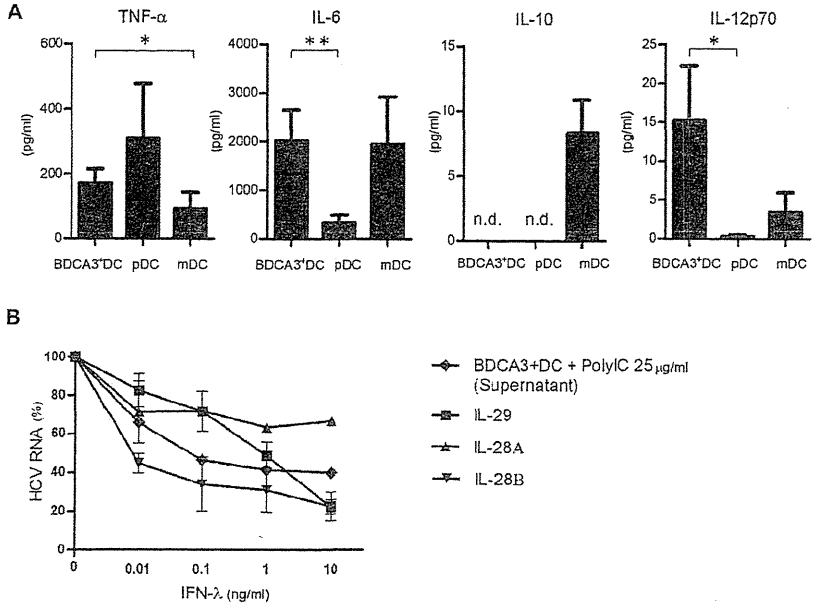

Figure S4. BDCA3+DCs produce various cytokines and IL-28B upon poly IC stimulation, exhibiting suppressive effect on HCV replication.

A. BDCA3+DCs and mDCs were placed at 2.5×104 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 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.

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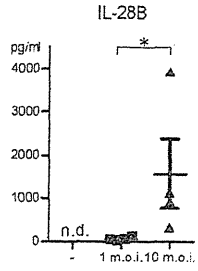

Figure S5. BDCA3+DCs produced IL-28B in response to HCVcc in an MOI-dependent manner. BDCA3+DCs were incubated for 24h with HCVcc-free medium (as depicted as -), HCVcc at an MOI of 1 or 10. The supernatants were examined for IL-28B. Results are shown as mean + SEM from 4 independent experiments. *, $p < 0.05$ by paired-t test

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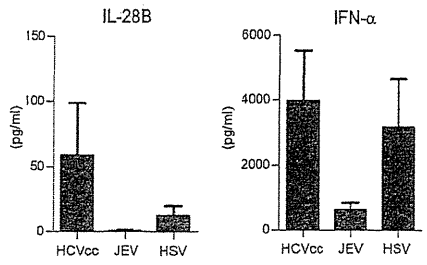

Figure S6. Plasmacytoid DCs produce IL-28B and IFN-α upon HCVcc stimulation.
Plasmacytoid DCs were cultured at 2.5x104 cells for 24 h with HCVcc, JEV or HSV at an MOI of 10. The levels of IL-28B and IFN-α in the supernatants were measured by ELISA. Results are shown as mean + SEM from 6 experiments.

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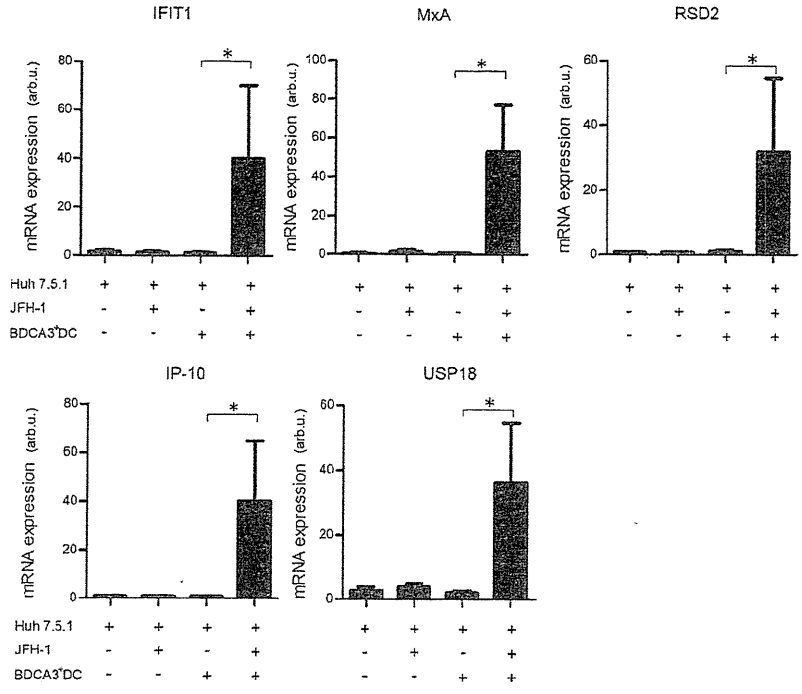

Figure S7. Various ISGs are induced in JFH-1-transfected Huh7.5.1 cells in the presence of BDCA3+DCs. BDCA3+DCs were co-cultured at 2.5x104 cells with JFH-1-transfected (M.O.I.=2) or -untransfected Huh7.5.1 cells for 24h. After non-adherent BDCA3+DCs were removed by extensive washing the culture wells, 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

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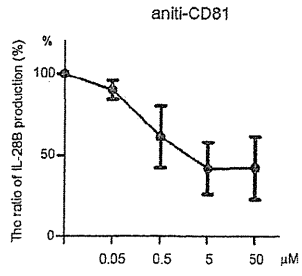

Figure S8: Anti-CD81 antibody inhibits HCVcc-induced IL-28B from BDCA3+DCs in a dose-dependent manner.
BDCA3+DCs were incubated for 24h with different concentrations of anti-CD81 antibody. The ratios of IL-28B levels are shown between the samples with various concentration of anti-CD81 antibody and those without. The horizontal bars indicate mean ± SD of 3 experiments.

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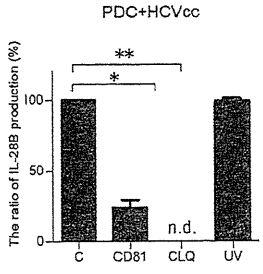

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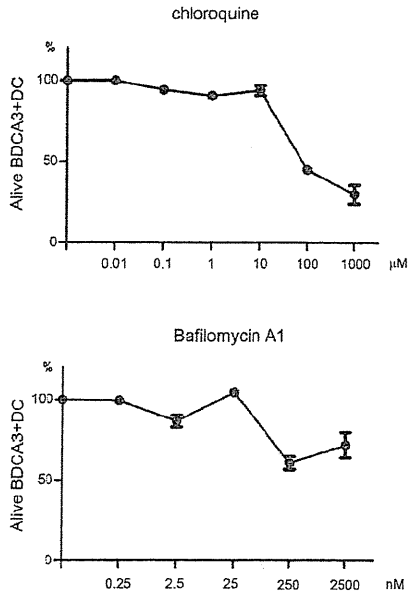

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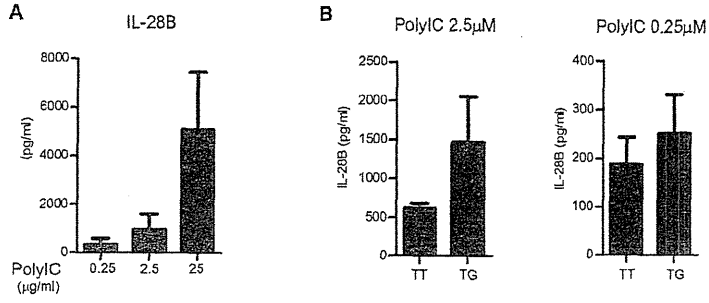

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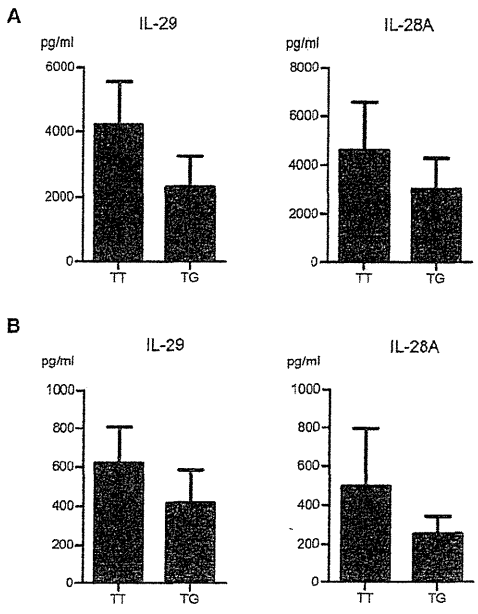

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BDCA3+DCs of healthy donors with the IL-28B major (rs8099917, TT) or the minor (TG) genotype were cultured at 2.5x104 cells with 25 μg/ml poly IC (A), or with JFH-1-infected- Huh 7.5.1 cells (B) for 24 h. The supernatants were subjected for IL-29 and IL-28A ELISA. The 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.

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Association of enhanced activity of indoleamine 2,3-dioxygenase in dendritic cells with the induction of regulatory T cells in chronic hepatitis C infection

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Abstract
Background Altered functions of dendritic cells (DCs) and/or increases of regulatory T cells (Tregs) are involved in the pathogenesis of chronic hepatitis C virus (HCV) infection. A tryptophan-catabolizing enzyme, indoleamine 2,3-dioxygenase (IDO), is reported to be an inducer of immune tolerance. Our aim was to clarify whether or not IDO is activated in chronic hepatitis C patients and its role in immune responses.
Methods This study enrolled 176 patients with chronic HCV infection and 37 healthy volunteers. Serum kynurenine concentration was evaluated by high-performance liquid chromatography, and its correlation with clinical parameters was examined. Monocyte-derived DCs were prepared from the subjects and subsequently stimulated with a combination of lipopolysaccharide and interferon-gamma to induce functional IDO (defined as IDO-DCs). The phenotypes, kynurenine or cytokine production, and T-cell responses with IDO-DCs were compared between the patients and healthy volunteers.
Results The serum kynurenine level in the patients was significantly higher than that in the healthy volunteers, and the level of serum kynurenine was positively correlated with the histological activity or fibrosis score. IDO activity in IDO-DCs from the patients was significantly higher than that in IDO-DCs from the volunteers. Furthermore, IDO-DCs from the patients induced more Tregs in vitro compared with those from the volunteers, and the frequency of induced Tregs by IDO-DCs was decreased with an IDO-specific inhibitor.
Conclusions Systemic IDO activity is enhanced in chronic hepatitis C patients in correlation with the degree of liver inflammation and fibrosis. In response to inflammatory stimuli, DCs from the patients tend to induce Tregs, with some of this action being dependent on IDO.

Keywords Hepatitis C virus · Dendritic cell · Regulatory T cell · Indoleamine 2,3-dioxygenase

Electronic supplementary material The online version of this article (doi:10.1007/s00535-012-0667-z) contains supplementary material, which is available to authorized users.

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Introduction

Hepatitis C virus (HCV) is a major cause of chronic liver disease worldwide. It is estimated that 170 million people

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are chronically infected with HCV and are at risk of developing liver cirrhosis and/or hepatocellular carcinoma [1]. Approximately 70 % of those exposed to HCV progress to a chronically infected state [2]. The mechanisms of HCV leading to persistent infection have been ascribed to escape mutations of the HCV genome and insufficient immune responses to HCV in hosts, but the precise mechanisms are still largely unknown.

Dendritic cells (DCs) are key regulators of the immune system and are capable of promoting or suppressing T-cell responses depending on their environment [3, 4]. One of the crucial machineries of HCV-induced immune dysfunction is impaired abilities of DCs. Several research groups, including ours [5, 6] have demonstrated that DCs from chronically HCV-infected patients have lower ability to stimulate T cells and to drive T-helper 1 (Th1) polarization than those from healthy controls [7, 8]. Regulatory T cells (Tregs) are specialized suppressor cells that maintain immune tolerance against auto-reactive T cells or against pathogens [9]. In patients with chronic HCV infection, the frequency of Tregs in peripheral blood mononuclear cells (PBMCs) is higher than that in healthy individuals, suggesting the active roles of Tregs in immune alteration or alleviation of inflammation [10, 11]. However, the mechanisms of DC dysfunction or Treg expansion in chronic HCV infection have not been completely elucidated.

Indoleamine 2,3-dioxygenase (IDO) is an enzyme that catalyzes the initial and rate-limiting steps in the catabolism of the essential amino acid tryptophan (Trp), resulting in the generation of kynurenine (Kyn). IDO is widely expressed in human tissues [12] and cell subsets [13] and is induced during inflammation by interferon-gamma (IFN-γ) and/or other inflammatory cytokines [14–16]. Recent studies have demonstrated a crucial role of IDO in the induction of immune tolerance during infection, pregnancy, transplantation, autoimmunity, and cancers [17–21]. IDO expressed by DCs promotes immune tolerance by inhibiting T-cell activation and proliferation or by inducing Tregs through Trp starvation and/or the accumulation of Trp catabolites, such as Kyn, 3-hydroxykynurenine, and 3-hydroxyanthranilic acid [22–25]. With respect to chronic HCV infection, a small-sized study showed that IDO expression was up-regulated in the liver and was associated with increased serum IDO activity [26]. However, the functions of IDO in immune cells in HCV infection still remain obscure.

In this study, we aimed to clarify whether or not IDO in DCs has a role in chronic HCV infection. We found that systemic IDO activity was enhanced in chronic hepatitis C patients. By comprehensively comparing the function of IDO-expressing DCs between the patients and healthy volunteers, we showed that IDO in DCs may be related to the induction of Tregs.

Subjects, materials, and methods

Subjects

This study enrolled 176 patients chronically infected with HCV serotype 1 (CHC group) who had been followed at Osaka University Hospital (Suita, Japan), National Hospital Organization Osaka National Hospital (Osaka, Japan), or Ikeda Municipal Hospital (Ikeda, Japan). All of them were confirmed to be positive for both serum anti-HCV antibody and HCV-RNA but were negative for other viral infections, including hepatitis B virus (HBV) and human immunodeficiency virus. The presence of other liver diseases, such as alcoholic, metabolic, or autoimmune hepatitis was ruled out, and the presence of liver cirrhosis and hepatocellular carcinoma was excluded by the use of laboratory and imaging analyses. As controls, we examined 37 healthy volunteers (HV group), working as medical staff at Osaka University Hospital, who were negative for HCV and HBV markers. As disease controls, 13 patients with chronic HBV infection followed at National Hospital Organization Osaka National Hospital were also enrolled. They were positive for hepatitis B surface (HBs) antigen and had abnormal levels of alanine aminotransferase (ALT). The characteristics of the group were: male/female 10/3, hepatitis B envelope (HBe) antigen-positive/HBe antigen-negative 6/7, mean age 43.9 $\pm$ 15.0 years, mean serum ALT level 218.7 $\pm$ 282.5 IU/L, and mean HBV-DNA level [assayed by the COBAS AmpliPrep™/COBAS TaqMan™ HBV test (Roche, Branchburg, NJ, USA)] 6.1 $\pm$ 2.3 Log copies/mL. At enrollment, written informed consent was obtained from each subject. The study protocol was approved by the ethics committee of each institution.

In this study, because of the limitations of sampling from multiple centers, the conditions for blood collection and preservation differed among the facilities. Thus, for the precise comparison of IDO activity between the patients and healthy volunteers, firstly, we examined the samples collected and preserved under the same conditions at Osaka University Hospital (Cohort I, Table 1). Secondly, because liver biopsy was not carried out in Cohort I patients, we used another cohort (Cohort II, Table 1) for our analysis of the correlation between IDO activity and clinical parameters. Cohort II consisted of the remaining 127 patients, whose samples were collected at National Hospital Organization Osaka National Hospital or Ikeda Municipal Hospital. Histological examination was performed according to the METAVIR scoring system. The clinical backgrounds of the patients in Cohorts I and II, except for HCV-RNA quantity, were not different.