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Ⅳ. 研究成果の刊行物・別刷

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

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pose a conflict of interest.

Abbreviations : Ab, antibody; HCV, hepatitis C virus; HCVcc, cell-cultured hepatitis C virus; HSV, herpes simplex virus; IHL, intrahepatic lymphocyte; IRF, interferon regulatory factor; ISGs, interferon-stimulated genes; JEV, Japanese encephalitis virus; Lin, lineage; mDC, myeloid DC; MOI, multiplicity of infection; PBMC, peripheral blood mononuclear cell; pDC, plasmacytoid DC; Poly IC, polyinosine-polycytidylic acid; RIG-I, retinoic acid-inducible gene-I; SNPs, single nucleotide polymorphisms; TLR, Toll-like receptor; TRIF, TIR-domain-containing adapter-inducing interferon-β

ABSTRACT

The polymorphisms in *IL-28B* (IFN-λ3) gene are strongly associated with the efficacy of HCV clearance. Dendritic cells (DCs) sense HCV and produce IFNs, thereby playing some cooperative roles with HCV-infected hepatocytes in the induction of interferon-stimulated genes (ISGs). BDCA3$^+$ DCs are discovered as a producer of IFN-λ upon toll-like receptor 3 (TLR3) agonist. We thus aimed to clarify the roles of BDCA3$^+$DCs in anti-HCV innate immunity. Seventy healthy subjects and 20 patients with liver tumors were enrolled. BDCA3$^+$DCs, in comparison with plasmacytoid DCs and myeloid DCs, were stimulated with TLR agonists, cell-cultured HCV (HCVcc) or Huh7.5.1 cells transfected with HCV/JFH-1. BDCA3$^+$DCs were treated with anti-CD81 antibody, inhibitors for endosome acidification, TRIF-specific inhibitor or ultraviolet-irradiated HCVcc. The amounts of IL-29/IFN-λ1, IL-28A/IFN-λ2 and IL-28B were quantified by subtype-specific ELISA. The frequency of BDCA3$^+$DCs in PBMC was extremely low but higher in the liver. BDCA3$^+$DCs recovered from PBMC or the liver released large amounts of IFN-λs, when stimulated with HCVcc or HCV-transfected Huh7.5.1.

BDCA3$^+$DCs were able to induce ISGs in the co-existing JFH-1-positive Huh7.5.1 cells. The treatments of BDCA3$^+$DCs with anti-CD81 antibody, cloroquine or bafilomycin A1 reduced HCVcc-induced IL-28B release, whereas BDCA3$^+$DCs comparably produced IL-28B upon replication-defective HCVcc. The TRIF-specific inhibitor reduced IL-28B release from HCVcc-stimulated BDCA3$^+$DCs. In response to HCVcc or JFH-1-Huh7.5.1, BDCA3$^+$DCs in healthy subjects with IL-28B major (rs8099917, TT) released more IL-28B than those with IL-28B minor genotype (TG). ***Conclusion:*** Human BDCA3$^+$DCs, having tendency of being accumulated in the liver, recognize HCV by a CD81-, endosome- and TRIF-dependent manner and produce substantial amounts of IL-28B/IFN-λ3, the ability of which is superior in subjects with IL-28B major genotype.

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INTRODUCTION

Hepatitis C virus (HCV) infection is one of the most serious health problems in the world. More than 170 million people are chronically infected with HCV and are at high risk of developing liver cirrhosis and hepatocellular carcinoma. Genome-wide association studies have successfully identified the genetic polymorphisms (single nucleotide polymorphisms, SNPs) upstream of the promoter region of the IL-28B/IFN-λ3 gene which are strongly associated with the efficacy of pegylated interferon-α (PEG-IFN-α) and ribavirin therapy or spontaneous HCV clearance (1-4).

IFN-λs, or types III IFNs, comprise a family of highly homologous molecules consisting of IFN-λ1 (IL-29), IFN-λ2 (IL-28A) and IFN-λ3 (IL-28B). In clear contrast with type I IFNs, they are released from relatively restricted types of cells, such as hepatocytes, intestinal epithelial cells or dendritic cells (DCs). Also, the cells that express hetero-dimeric IFN-λ receptors (IFN-λR1 and IL-10R2) are restricted to cells of epithelial origin, hepatocytes or DCs (5). Such limited profiles of cells expressing IFN-λs and their receptors define the biological uniqueness of IFN-λs. It has been shown that IFN-λs convey anti-HCV activity by inducing

various interferon-stimulated gens (ISGs) (5), the profiles of which were overlapped but others were distinct from those induced by IFN-α/β. Some investigators showed that the expression of IL-28 in PBMC was higher in subjects with IL-28B major than those with minor, however the levels of IL-28 transcripts in liver tissue were comparable regardless of IL-28B genotype (2, 6).

At the primary exposure to hosts, HCV maintains high replicative levels in the infected liver, resulting in the induction of IFNs and ISGs. In a case of successful HCV eradication, it is postulated that IFN-α/β and IFN-λ cooperatively induce anti-viral ISGs in HCV-infected hepatocytes. It is of particular interest that, in primary human hepatocytes or chimpanzee liver, IFN-λs, but not type-I IFNs, is primarily induced after HCV inoculation, the degree of which is closely correlated with the levels of ISGs (7). These results suggest that hepatic IFN-λ could be a principal driver of ISG induction in response to HCV infection. Nevertheless, the possibility remains that DCs, as a prominent IFN producer in the liver, play significant roles in inducing hepatic ISGs and thereby suppressing HCV replication.

Dendritic cells (DCs), as immune sentinels, sense specific genomic and/or structural components of pathogens with various pattern recognition receptors and eventually release IFNs and inflammatory cytokines (8). In general, DCs migrate to the organ where inflammation or

cellular apoptosis occurs and alter their function in order to alleviate or exacerbate the disease conditions. Therefore, the phenotypes and/or capacity of liver DCs are deemed to be influenced in the inflamed liver. In humans, the existence of phenotypically and functionally distinct DC subsets has been reported: myeloid DC (mDC) and plasmacytoid DC (pDC) (9). Myeloid DCs predominantly produce IL-12 or TNF-α following pro-inflammatory stimuli, while pDCs release considerable amounts of type I IFNs upon virus infection (9). The other type of mDCs, mDC2 or BDCA3$^+$(CD141) DCs, have been drawing much attention recently, since human BDCA3$^+$DCs are reported to be a counterpart of murine CD8α$^+$DCs (10). Of particular interest is the report that BDCA3$^+$DCs have a potent capacity of releasing IFN-λ in response to toll-like receptor 3 (TLR3) agonist (11). However, it is still largely unknown whether human BDCA3$^+$DCs are able to respond to HCV.

Taking these reports into consideration, we hypothesized that human BDCA3$^+$DCs, as a producer of IFN-λs, have crucial roles in anti-HCV innate immunity. We thus tried to clarify the potential of BDCA3$^+$DCs in producing type III IFNs, by using cell-cultured HCV (HCVcc) or hepatoma cells harboring HCV as stimuli. Our findings show that BDCA3$^+$DCs are quite unique DC subset, characterized by a potent and specialized ability to secrete IFN-λs in

response to HCV. The ability of BDCA3$^+$DCs to release IL-28B upon HCV is superior in subjects with IL-28B major (rs8099917, TT) to those with minor (TG or GG) genotype, suggesting that BDCA3$^+$DCs are one of the key players in IFN-λ-mediated innate immunity.

MATERIALS AND METHODS

Subjects.

This study enrolled 70 healthy volunteers (male / female; 61 / 9) (age; mean ± SD, 37.3 ± 7.8 yrs.) and 20 patients who underwent surgical resection of liver tumors at Osaka University Hospital (**Supplementary table 1).** The study was approved by the ethical committee of Osaka University Graduate School of Medicine. Written informed consent was obtained from all of them. All healthy volunteers are negative for HCV, hepatitis B virus (HBV) and human immunodeficiency virus (HIV) and had no apparent history of liver, autoimmune or malignant diseases.

Reagents.

The specifications of all antibodies used for FACS or cell sorting TLR-specific synthetic agonists, pharmacological reagents and inhibitory peptides are listed in the supplementary materials.

Separation of dendritic cells from PBMC or intra-hepatic lymphocytes.

We collected 400 mL of blood from each healthy volunteer and processed for PBMCs. Non-cancerous liver tissues were obtained from patients who underwent resection of liver tumors (**Supplementary table**). For the collection of intrahepatic lymphocytes (IHLs), liver tissues were washed thoroughly with phosphate-buffered saline to remove the peripheral blood adhering to the tissue and ground gently. After Lin-negative ($CD3^-$, $CD14^-$, $CD19^-$ and $CD56^-$) cells were obtained by the MACS system, each DC subset with the defined phenotype was sorted separately under FACS Aria (BD). The purity was more than 98%, as assessed by FACS Canto II (BD). Sorted DCs were cultured at $2.5x10^4$/well on 96-well culture plates.

Immunofluorescence staining of human liver tissue

Tissue specimens were obtained from surgical resections of non-cancerous liver from the patients as described above. Briefly, the 5μm sections were incubated with the following antibodies: mouse biotinylated anti-human BDCA3 antibody (Miltenyi-Biotec), and mouse anti-human CLEC9A antibody (Biolegend) and subsequently with secondary goat anti-rabbit Alexa Fluor®488 or goat anti-mouse Alexa Fluor®594 (Invitrogen, Molecular Probes) antibodies. Cell nuclei were counterstained with Dapi-Fluoromount-GTM (SouthernBiotech,

Birmingham, AL). The stained tissues were analyzed by fluorescence microscopy (Model BZ-9000; Keyence, Osaka, Japan).

Cells and viruses.

The *in vitro* transcribed RNA of the JFH-1 strain of HCV was introduced into FT3-7 cells (12) or Huh7.5.1 cells. The stocks of cell-cultured HCV (HCVcc) were generated by concentration of the medium from JFH-1-infected FT3-7 cells. The virus titers were determined by focus forming assay (13). The control medium was generated by concentration of the medium from HCV-uninfected FT3-7 cells. Infectious JEVs were generated from the expression plasmid (pMWJEATG1) as reported previously (14). HSV (KOS) was a generous gift from Dr. K. Ueda (Osaka University). Huh7.5.1 cells transduced with HCV JFH-1 strain was used for the co-culture with DCs. The transcripts of interferon-stimulated genes (ISGs) in Huh7.5.1 were examined by RT-PCR methods using gene-specific primers and probes (Applied Biosystems, Foster City, CA).

Secretion assays.

IL-28B/IFN-λ3 was quantified by a newly developed chemiluminescence enzyme immunoassay (CLEIA) system (15). IL-29/IFN-λ1, IL-28A/IFN-λ2 and IFN-β were assayed by commercially available ELISA kits (eBioscience, R&D and PBL, respectively). IFN-α 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).

RESULTS

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

We defined BDCA3$^+$DCs as Lin$^-$HLA-DR$^+$BDCA3^{high+} 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$^+$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