Fig. 1

559 with chronic hepatitis B or C infection underwent gadoxetic acid-enhanced MRI

432 were excluded
420 with presence or history of HCC
9 with Child-Pugh class C
3 dropped out

127 Included in this study

18 Hypovascular hypointense nodules in the liver on the hepatocyte phase Non-clean liver group

109 No hypovascular hypointense nodules in the liver on the hepatocyte phase Clean liver group

3-year follow up

8 without typical HCC

102 without typical HCC

10 were diagnosed as typical HCC
4 "de novo HCC"
6 HCC at the same site of the initial hypovascular hypointense nodule

7 were diagnosed as typical HCC
7 "de novo HCC"

hepr_12309_f1

Fig. 2

hepr_12309_f2

Fig. 3

No. of patients at risk

Non-Clean liver	18	18	15	14
Clean liver	109	109	103	102

hepr_12309_f3

Fig. 4

	HR (95% CI)	p value
Male	7.89 (1.96–31.74)	0.003
Female	18.54 (4.59–74.91)	<0.001
Age ≥ 65 years	13.56 (4.38–40.05)	<0.001
Age < 65 years	8.48 (1.19–60.28)	0.032
Cirrhosis	7.64 (2.83–20.65)	<0.001
Non-cirrhosis	37.23 (3.30–419.71)	0.003
HCV	14.23 (5.25–38.55)	<0.001
HBV	9.60 (0.86–106.18)	0.065
PLT ≥ 10 × 10⁴/μL	33.42 (6.69–166.94)	<0.001
PLT < 10 × 10⁴/μL	4.27 (1.14–15.97)	0.031
ALT ≥ 30 IU/L	15.32 (4.94–47.46)	<0.001
ALT < 30 IU/L	7.73 (1.08–54.97)	0.041
γ-GTP ≥ 40 IU/L	13.64 (3.79–49.05)	<0.001
γ-GTP < 40 IU/L	11.06 (2.46–49.61)	<0.001
AFP ≥ 10 ng/mL	12.90 (3.72–44.73)	<0.001
AFP < 10 ng/mL	9.28 (1.86–46.14)	0.006

Hazard ratio

hepr_12309_f4

Impaired induction of *IL28B* and expression of *IFNλ4* associated with non-response to interferon-based therapy in chronic hepatitis C

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ABSTRACT

Background: Interferon (IFN) λ plays an important role in innate immunity to protect against hepatitis C viral (HCV) infection. Single nucleotide polymorphisms (SNPs) near *IL28B* (*IFNλ3*) are strongly associated with treatment response to IFNα therapy in chronic hepatitis C (CHC) patients. Recently, IFNλ4 related to *IL28B*-unfavorable allele was discovered. However, the impact of IFNλs on CHC is unknown. We aimed to investigate the mechanism underlying responsiveness to IFN-based therapy in CHC associated with SNPs near *IL28B*.

Methods: We evaluated the basal mRNA levels and ex-vivo induction of *IFNλ* expression including *IFNλ4* in peripheral blood mononuclear cells (PBMCs) from 50 CHC patients treated with PEG-IFNα/RBV. Furthermore, we investigated the effect of *IFNλ4* on induction of *IL28B* in vitro.

Results: When PBMCs were stimulated with IFNα and poly(I:C), *IL28B* induction was significantly lower in patients with *IL28B*-unfavorable genotype (rs12979860 CT/TT) than those with *IL28B*-favorable genotype (rs12979860 CC; p = 0.049). *IL28B* induction was lower in non-responders than in relapsers (p = 0.04), and it was also lower in non-SVR patients for triple therapy including NS3 protease inhibitors. *IFNλ4* mRNA was detected in 12 of 26 patients with *IL28B*-unfavorable SNP and *IFNλ4* expression was associated with lower *IL28B* induction in patients with *IL28B*-unfavorable genotype (p = 0.04) and non-response to

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IFNα therapy (p = 0.003). Overexpression of *IFNλ4* suppressed *IL28B* induction and promoter activation.

Conclusions: Impaired induction of *IL28B,* related to *IFNλ4* expression in PBMCs of *IL28B*-unfavorable patients, is associated with non-response to IFNα-based therapy for HCV infection.

Keywords: hepatitis C virus, peripheral blood mononuclear cells, pegylated interferon, NS3 protease inhibitor, type III interferon

Abbreviations: HCV, Hepatitis C virus; IFN, interferon; CHC, chronic hepatitis C; PEG-, pegylated; RBV, ribavirin; DAA, direct-acting antiviral agents; SNP, single nucleotide polymorphism; IL, interleukin; TLR, Toll like receptor; RLR, RIG-I like receptor; ISG, IFN-stimulated gene; PBMC, peripheral blood mononuclear cells; SVR, sustained virological responder; VR, virological responder; NR, non-responder; poly (I:C), polyinosinic-polycytidylic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; BLC, immortalized B lymphocytes; IRF7, interferon regulatory transcription factor 7; ISRE, IFN-stimulated response element; STAT, signal transducers and activator of transcription; BDCA3, blood dendritic cell antigen 3; DC, dendric cell; ALT, alanine aminotransferase; γ-GTP, γ-glutamyl transpeptidase; LDL-C, low-density lipoprotein cholesterol; ISDR, IFN sensitivity determining region.

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Introduction

Hepatitis C virus (HCV) infection is a common cause of chronic hepatitis, which progresses to liver cirrhosis and hepatocellular carcinoma [1]. Interferon (IFN)-based therapy has been used to treat chronic hepatitis C (CHC) over the last two decades and the combination therapy with direct-acting antiviral agents (DAAs) improved the treating effect. However, non-responders [2] to previous pegylated interferon α (PEG-IFNα) plus ribavirin (RBV) therapy respond poorly to the triple therapy containing HCV NS3/4A serine protease inhibitors [3, 4]. Moreover, although IFN-free regimen using NS5A inhibitors or NS5B polymerase inhibitors is developed, triple or quadruple therapy including PEG-IFN may still be required to suppress DAA-resistant viruses or difficult-to-treat genotype. Therefore, IFNα responsiveness of host innate immunity remains essential for achieving a good prognosis, and determining the mechanisms responsible for non-response to IFNα is crucial.

In a recent genome-wide association study, single nucleotide polymorphisms (SNPs) located near *interleukin 28B* (*IL28B*) encoding type III IFN (IFNλ3) were found to be strongly associated with the virological response to PEG-IFNα/RBV therapy in CHC patients [5-8]. IFNλ3 is induced by viral infection through stimulation of Toll-like receptors (TLR) and RIG-I like receptors (RLR) [9-12], and it is also induced by type-I IFN signaling [13]. This interferon stimulates the expression of IFN-stimulated genes (ISGs), including numerous antiviral [13, 14] and immunoregulatory genes [15, 16]. Therefore, IFNλ3 induction may play essential roles in the innate antiviral response [17].

Recently, it was reported that high baseline expression levels of intrahepatic RLR, and lower responsiveness of ISGs to exogenous IFN, were significantly associated with unfavorable *IL28B* SNP and poor treatment outcome in CHC patients [18, 19, 20]. Furthermore, RNA sequencing using primary human hepatocytes revealed that unfavorable allele of dinucleotide polymorphisms near *IL28B* generate *IFNλ4* [21]. The ability of IFNλ4 to

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induce ISGs was reported in human liver tissue samples [22]. Based on these findings, we hypothesized that preactivation of IFN signaling by *IFNλ4* prevents further induction of antiviral genes by exogenous type I IFN, particularly the type I IFN-mediated induction of IFNλs. However, the expression of *IFNλ4* has never been documented in clinical blood samples from CHC patients.

The study aimed to determine the contribution of IFNλ family (IL29 [IFNλ1], IL28A [IFNλ2], IL28B [IFNλ3] and IFNλ4) to the poor response of CHC patients to anti-HCV therapy, and to clarify the mechanisms associated with SNPs near *IL28B*. Since peripheral blood mononuclear cells (PBMCs) are major sources of IFNλ [9, 10], we measured the expression level and investigated the ex vivo induction of *IFNλs* in PBMCs derived from CHC patients receiving PEG-IFNα/RBV therapy. Furthermore, we studied the impact of *IFNλ4* on *IL28B* expression in vitro.

Methods

Patients and Clinical samples. This study included 50 CHC patients with genotype 1b HCV treated with PEG-IFNα-2b/RBV at the Tokyo Medical and Dental University Hospital. Eleven of these patients were re-treated with telaprevir (TVR) or simeprevir (SMV). Exclusion parameters were alcoholic liver injury, autoimmune hepatitis, and decompensated liver cirrhosis. No patient tested positive for hepatitis B surface antigen or anti-human immunodeficiency virus antibody, or had received immunomodulatory therapy before enrollment. The clinical characteristics of the patients immediately before blood collection are shown in Table 1.Written informed consent was obtained from all patients, and this study was approved by the ethical committee of Tokyo Medical and Dental University in accordance with the Declaration of Helsinki.

Blood samples were collected from each patient during off-therapy periods for gene expression analysis. Human genomic DNA was extracted from whole blood, and SNPs located near the *IL28B* gene (rs8099917, rs12979860, and ss469415590) were analyzed using the TaqMan SNP genotyping assay (Applied Biosystems, Carlsbad, CA) [21, 23]. HCV core mutations and IFN-stimulated response element (ISDR) substitutions were determined before the therapy.

Definitions of responsiveness to therapy. The present study used the definition of response to therapy outlined by the AASLD Practice Guideline for Diagnosis, Management, and Treatment of Hepatitis C [2, 3].

Generation of *IL28B* mRNA-specific RT-qPCR systems. For the quantification of *IL28B* mRNA expression, we developed an original real-time quantitative PCR assay that distinguishes *IL28B* from *IL28A*. Gene-specific PCR primers were designed to anneal directly to the cDNA sequences of each gene (Supplementary Table 1).

Real-time detection RT-PCR analysis for *IL28A*, *IL28B*, and *IL29*. Immediately after blood collection, PBMCs were separated by gradient centrifugation with Ficoll-Conray, and incubated in the RPMI 1640 medium (Sigma, St. Louis, MO) with 10% fetal calf serum at $37^\circ C$ under 5% CO_2. The cells were treated with recombinant IFNα-2b (100 IU/ml) (Schering-Plough, Kenilworth, NJ) for 12 h prior to polyinosinic-polycytidylic acid (poly(I:C)) (Sigma) treatment (10 μg/ml) for 8 h. PBMC RNA was extracted using the RNeasy Mini Kit (Quiagen, Valencia, CA). Total cell RNA (200 ng) was used to generate 10 μl of cDNA from each sample using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). The mRNA expression levels were measured using a ABI 7500 real-time PCR system (Applied Biosystems), and a QuantiTect SYBR Green PCR kit (Quiagen) or TaqMan Universal PCR Master Mix (Applied Biosystems). Expression levels were normalized to the

expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β-actin. The

sequences of the primer sets are provided in Supplementary Table 1.

Analysis of *IFNλ4* mRNA expression. Total cell RNA was pre-treated with DNase-I

(Nippon Gene, Tokyo, Japan), followed by RT with SuperScript II, and PCR analysis was

performed for 45 cycles using 4 sets of primers (Supplementary Table 1). Primer set #1 could

detect 1 copy of IFNλ4 per assay, whereas primer sets #2, 3, and 4 could detect 10 copies of

IFNλ4 per assay (Supplementary Fig. 1). The PCR products corresponding to the size of

spliced *IFNλ4* mRNA were extracted and the sequences were confirmed. Only the amplicon

with ss469415590-ΔG (*) is defined as IFNλ4 (Supplementary Figs. 1, 2).

Generation of the *IL28B* promoter-reporter and stably expressing cell lines. The

promoter sequences of human *IL28B* (-1129/+111) were subcloned and the DNA fragment

was inserted into the pGL3-basic vector (Invitrogen). The reporter plasmid was transfected

into HEK293 cells with pcDNA3.1 (Invitrogen). After cell culture in the presence of the

selective antibiotic G418 (Nacalai Tesque, Kyoto, Japan), transfected colonies were isolated

to establish a cell line stably expressing the IL28B-Fluc-reporter (HEK293/ IL28B-luc).

Cell culture. HEK293T, Huh7, HepG2, and HeLa cells were maintained in Dulbecco's

modified Eagle's Medium (Sigma) supplemented with 10% fetal calf serum (37°C; 5% CO_2).

The maintenance medium for the IL28B-promoter-reporter-harboring cell line

(HEK293/IL28B-luc) was supplemented with 500 μg/ml of G418 (Nacalai Tesque).

Immortalized B lymphocytes (BLC) were generated in-house from human PBMCs by EBV

transformation, and maintained in RPMI1640 medium (Sigma) with 10% feral calf serum and

200 ng/ml Cyclosporin-A (Sigma). The HuS/E-2 cells were kindly provided by Dr. Hijikata

(Kyoto University, Kyoto, Japan) and cultured as previously described [24].

Expression plasmids and transfections. The expression construct for IFNλ4 (p179) was

kindly provided by Dr. Prokunina-Olsson (National Cancer Institute, Bethesda, MD). The

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DNA fragments of IRF7 were inserted into the vector pcDNA4/TO/myc-His (Invitrogen). The expression plasmids for p50 and p65 were kindly provided by Dr. Rongtuan Lin (Lady Davis Institute for Medical Research, Baltimore, MD). pcDNA4/TO/myc-His vector (Invitrogen) was used as control for mock transfection.

BLC were transfected with *IFNλ4* plasmids or control plasmids by electroporation using Gene Pulser Xcell Electroporation System (BIO RAD, Hercules, CA). After 24 h, cells were treated with mock, recombinant IFNα-2b (100 IU/ml) (Schering-Plough) for 24h. *IFNλ4* plasmids and *IRF7* plasmids or control plasmids were co-transfected into HEK293T cells with Lipofectamine LTX reagent (Invitrogen) and Opti-MEM medium, according to the manufacturer's instructions. Total RNA was extracted and quantified by real-time qRT-PCR.

Luciferase assays. *IFNλ4* or control plasmids were transfected into HEK293/IL28B-luc cells and the cells were treated with IFNα for 24h next day. HEK293/IL28B-luc cells were co-transfected with IFNλ4 plasmids and IRF7, p50: p65 or control plasmids and incubated for 24h. MTS viability and single luciferase assays were conducted by 1420 Multilabel Counter (ARVO MX, PerkinElmer, Boston, MA) using a CellTiter 96 AQueous One Solution System (Promega, Madison, WI) and a Bright-Glo Luciferase Assay System (Promega), as previously described [25, 26].

Statistical analyses. The data were analyzed using the Welch's *t* test for continuous variables and the chi-square test for categorical data. p values < 0.05 were considered statistically significant.

Results

Genotype of *IL28B* SNP and expression of *IL29*, *IL28A*, and *IL28B* mRNA in PBMC. Three SNPs near the *IL28B* gene (rs8099917, rs12979860, and ss469415590) were genotyped. The number of patients with each genotype is shown in Table 1. In agreement with a recent

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report from the HapMap Project in Asia [21], the genotype of ss469415590 was completely

correlated with that of rs12979860 in this study, while 3 of 50 patients have different

genotype between ss469415590 and rs8099917. Baseline mRNA expression levels of *IL29*,

IL28A, and *IL28B* were not influenced by the rs12979860 genotype (Fig. 1A). However,

when PBMCs were stimulated with IFNα and poly(I:C), the induction of *IL28B* expression

was significantly lower in patients with the *IL28B*-unfavorable genotype (rs12979860 CT/TT)

than in those without (rs12979860 CC) (p = 0.049) (Fig. 1B).

Relationship of therapy response with *IL29*, *IL28A*, and *IL28B* mRNA levels in

PBMC. We assessed the relationship between the expression level of the *IFNλ*s and the

virological response to PEG-IFNα/RBV therapy. At baseline, there was no significant

difference in *IFNλ*s expression between the SVR, relapser, and NR patients (data not shown).

On the other hand, the induction of *IL28B* expression by IFNα and poly(I:C) decreased with

the patients' response to therapy (Fig. 2A). The mRNA levels of NR patients were

significantly lower than those for relapsers (p = 0.04) as well as VR (p=0.005). The induction

of *IL29* expression of NR patients were lower than those for VR (p=0.048). In contrast, the

induction of *IL28A* did not reveal any association between mRNA levels and treatment

response.

When the *IL28B* induction levels of VR and NR patients were further stratified by

genotype, it was significantly lower in NR than in VR patients in both rs12979860 CC and

CT/TT subgroups (p = 0.01 and 0.02, respectively) (Fig. 2B).

Furthermore, 11 of 32 non-SVR patients were re-treated with NS3 protease inhibitor (TVR

or SMV) plus PEG-IFNα/RBV triple therapy; 2 of them were IL28B-favorable and 9 were

unfavorable. Even treated with NS3 protease inhibitor, it should be noted that IL28B

inductions in non-SVR of the triple therapy were significantly lower than those in SVR of the

PEG-IFNα/RBV therapy or triple therapy (p=0.017). IL28B inductions in non-SVR were also

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lower than those in SVR of triple therapy (3.5 vs 12.1 fold induction). IL28A inductions in non-SVR of the triple therapy were also significantly lower than those in SVR (p=0.042) (Fig. 2C).

Impact of *IL28B* genotype and induction on *IFNλ4* mRNA expression. We measured the expression level of *IFNλ4* in PBMCs derived from CHC patients. Because we could not detect *IFNλ4* mRNA in PBMCs with RNA sequencing nor the previously reported TaqMan real-time quantitative RT-PCR system [21], we designed a new highly sensitive RT-PCR system using 4 sets of primers. The detection threshold was as low as 1–10 copies/assay (Supplementary Table 1; Supplementary Fig. 1A). This RT-PCR assay allowed us to confirm the full length mRNA sequence of *IFNλ4* in poly(I:C)-treated HepG2, HeLa, HEK293T cells, and BLC from ss469415590-ΔG/ Δ G patients by amplicon sequencing (Supplementary Figs. 1B,C).

Using this system, we tested PBMCs from 47 CHC patients for the presence of *IFNλ4* mRNA. Among the 23 patients with *IL28B*-unfavorable rs12979860 [T] and ss469415590 [ΔG]-allele, *IFNλ4* mRNA was detected in 12 patients (7 in non-stimulated PBMCs and 8 in IFN-poly(I:C)-stimulated PBMCs). In marked contrast, *IFNλ4* mRNA was not detected in any of the *IL28B*-favorable patients (Supplementary Fig. 2). There was no significant difference in baseline expression of *IFNλ*s between patients with or without detectable *IFNλ4* expression (Fig. 3A). However, the induction of *IL28B* expression by IFN-poly(I:C) was significantly lower in patients with *IFNλ4* mRNA than those without detectable *IFNλ4* (p = 0.008) (Fig. 3B). Even among *IL28B*-unfavorable patients (rs12979860 CT/TT), *IL28B* induction levels were significantly lower in *IFNλ4*-positive patients (p = 0.04) (Fig. 3B). Although induction of *IL28A* was lower in *IFNλ4*-positive patients than *IFNλ4*-negative patients (p = 0.04), there was no significant relation between *IFNλ4* expression and the induction of *IL28A* and *IL29* among *IL28B*-favorable patients.

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Association between *IFNλ4* expression and clinical response to antiviral therapy. The rate of virological non-response was significantly higher in patients with *IFNλ4* mRNA than in all those without detectable *IFNλ4* (p = 0.003; Fig. 3C). Among the *IL28B*-unfavorable patients (rs12979860 CT/TT), the virological non-response rate also tended to be higher in patients expressing *IFNλ4* (p = 0.08).

Suppression of *IL28B* induction by *IFNλ4* in vitro. The mechanism behind the lower induction of *IL28B* mRNA in CHC patients expressing *IFNλ4* was investigated by testing whether the expression of *IL28B* is influenced by overexpression of *IFNλ4* in vitro. When IFNλ4 was overexpressed, baseline expression of IL28B was significantly increased in HEK293, BLC (Supplementary Fig. 3). However, as shown in Fig. 4A, IL28B expression was increased by IFNα (1.8 fold induction, p=0.012) but that induction was suppressed in the presence of IFNλ4 (1.2 fold induction, p=0.28) in BLC. As *IL28B* promoter is known to be activated by the transcription factors such as IRF7 and NFκB [11, 31], we next evaluated IL28B induction by IRF7. *IL28B* mRNA was induced by IRF7 in dose dependent manner and the induction levels were suppressed by *IFNλ4* overexpression significantly (Fig. 4B). *IL28B* promoter activities induced by IFNα, IRF7 and p50:p65 were also inhibited by *IFNλ4* overexpression (Fig. 4C-E).

Discussion

The present study shows that the inducibility of *IL28B* expression is associated with virological responsiveness to IFNα in CHC patients, and it is also related to the *IL28B* genotype. Furthermore, we detected *IFNλ4* mRNA in PBMCs using an original sensitive RT-PCR system. *IFNλ4* suppressed *IL28B* induction and associated with virological non-responses to IFNα-based antiviral therapy.

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Earlier studies reported the lower production of *IL28B* in blood cells of *IL28B*-unfavorable CHC patients [5, 27]. However, the relationship between *IL28B* genotype and expression level remained controversial, probably due to the very low expression level of *IL28B*. In the present study, there was no significant difference in baseline expression level between the *IL28B* genotypes. However, stimulation to PBMCs with IFNα and poly(I:C) raised *IL28B* expression, and this induction was significantly lower in *IL28B*-unfavorable CHC patients. More importantly, the degree of *IL28B* induction was positively correlated to the responsiveness to PEG-IFNα/RBV therapy.

Our findings are consistent with a previous study showing ex vivo induction of *IL28B* by TLR7 agonists [28], and we further confirmed *IL28B* inducibility using IFNα and poly(I:C), which mimic exogenous IFNα administration in HCV patients. Because IFNλ is an essential element of innate anti-HCV responses [16, 29, 30], our data suggest that inadequate induction of IL28B is primarily responsible for virological non-response to IFNα-based therapy.

To elucidate the mechanisms responsible for the genotype-specific inducibility of *IL28B*, we focused on *IFNλ4*. We report, for the first time, the presence of *IFNλ4* mRNA in PBMCs derived from CHC patients with the *IL28B*-unfavorable allele. We could not detect *IFNλ4* mRNA with the previously reported TaqMan real-time RT-PCR system [21]. *IFNλ4* expression was confirmed with a highly sensitive RT-PCR system we designed for this study, which could detect even a single copy of *IFNλ4* mRNA per assay. Although *IFNλ4* mRNA was not detected in 16 of the 23 unstimulated PBMC samples of CHC patients with the *IL28B*-unfavorable genotype, we cannot exclude the presence of *IFNλ4* mRNA under the detection limit of this RT-PCR system in these patients. However, it is important to mention that detectable level of *IFNλ4* expression was associated with NR and more severe impairment of *IL28B* induction. These data suggest that the baseline expression of *IFNλ4* in

PBMCs is responsible for the non-response to IFNα treatment through suppression of *IL28B* induction.

Our in vitro experiments in cell lines demonstrated that *IL28B* induction by IFNα, IRF7 or NFκB was suppressed by IFNλ4 overexpression. These data are consistent with the relationship between *IFNλ4* and *ISG* induction [20-22]. Our finding of base line *IL28B* induction by *IFNλ4* is also reasonable because *IFNλ* promoters contain IFN-stimulated response element (ISRE) sites [11, 31] that could be activated by IFNλ4 through STAT1 and STAT2 phosphorylation [21]. IFNλ4 may pre-activate IL28B promoter through ISRE activation, and moreover, it may influence NFκB-induced promoter activity by unknown mechanism. Our in vitro data support our observation in the clinical samples, and suggest that the expression of *IFNλ4* in immune cells of *IL28B*-unfavorable CHC patients may weakly induce basal *IL28B* expression, which may be insufficient for HCV eradication [32]. But it may prevent additional induction of *IL28B* by exogenous IFNα treatment through impairment of *IL28B* promoter activity. The molecular mechanism by which *IFNλ4* suppresses *IL28B* mRNA induction and promoter activation should be further investigated, although *IFNλ4* may also have important functions affecting IFN regulation [20, 33, 34].

The lower induction of *IL28B* might be caused by the decrease of the frequency of IFNλs producing cells. However, in the present study, because we measured the expression of *IFNλ*s in all PBMCs, we could not specify the subset of IFNλ4 producer cells. A recent study demonstrated that blood dendritic cell antigen 3 (BDCA3)[+] dendritic cells (DCs) produce IFNλ3 and expression levels of *IL28B* from BDCA3+ DCs were significantly higher in subjects with *IL28B* major than those with minor type in response to HCV infection [35]. In their experiment, large volumes of blood samples (i.e., 400 ml) were required to sort very small populations of BDCA3+DC (0.054% of all PBMCs), but obtaining such a large amount

of blood per patient was ethically impossible in our study. We also considered that *IFNλ4* mRNA levels might be higher when analyzed in those specific IFNλ producer cells.

In conclusion, the induction of *IL28B* mRNA expression by ex vivo stimulation with IFNα and poly(I:C) in PBMCs was significantly associated with virological responsiveness in CHC patients treated with IFNα-based therapy. The impaired induction of *IL28B* was associated with the expression of *IFNλ4,* generated by unfavorable dinucleotide polymorphisms near the *IL28B* gene. These data improve our understanding of IFN resistance and may lead to the development of new antiviral therapies targeting the IFNλ induction system.

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