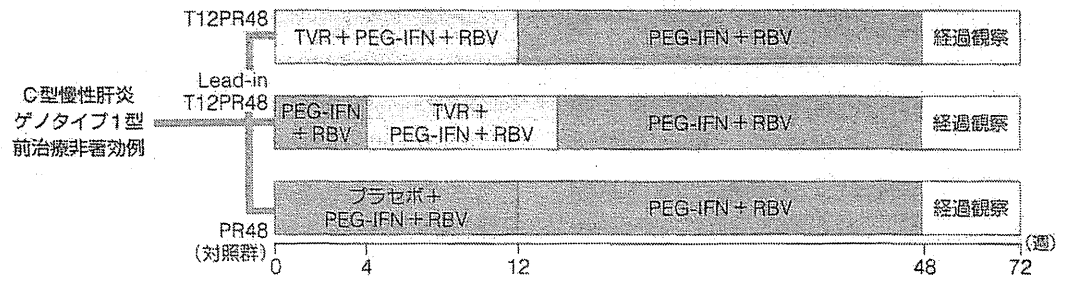
パビリン3剤併用に先だって行う群(Lead-in T12PR48)，およびPEG-IFN/リバビリン48週治療の対照群(PR48)である(図2a)．その結果，テラプレビル/PEG-IFN/リバビリン3剤併用治療はPEG-IFN/リバビリン2剤治療に比べ，前治療再燃，無効例いずれの群においても高い治療効果が得られた(図2b)．本研究では，前治療無効群をさらに，治療中に血中HCV-RNAが2-log以上低下した群(partial response)としなかった群(no response)に分け治療効果を解析したところ，2-log以上のウイルス量低下が得られなかった症例では3剤併用48週治療を施行しても最終効果が29%および33%と低く，とくに難治であることが明らかとなった．また，PEG-IFN/リバビリン2剤併用4週間のLead-inを加えることでは治療効果の向上を得られなかった．海外で使われているboceprevir併用IFN療法では，このLead-in治療の優位性が示されており，製剤間の特性の差異によるものと思われる[11,12]．治療中止に至った有害事象は，テラプレビル併用2群でそれぞれ15%および11%に発生し，PR48対象群の3%に比べ有意に多く，おもに貧血，皮疹・瘙痒感などの皮膚症状が発症した．

Response-guideプロトコルの有用性検証試験(ILLUMINATE Study)

本研究は，ゲノタイプ1型C型肝炎未治療例540例に対して，テラプレビル/PEG-IFN/リバビリン3剤併用12週投与のあと，PEG-IFN/リバビリン2剤を継続投与するものであるが，治療開始4週かつ12週時点におけるHCV-RNA陰性化(eRVR，表1)が得られた症

a REALIZE Study：プロトコル



b REALIZE Study：前治療の効果別成績

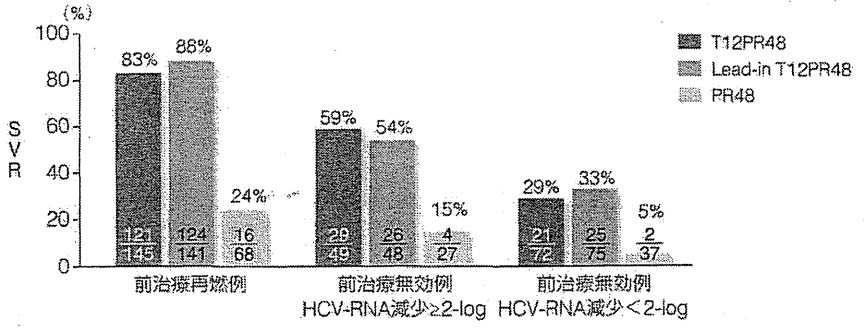


図2 REALIZE Study

(文献10)より引用)

例を，PEG-IFN/リバビリン12週投与(総治療期間24週)と36週投与(総治療期間48週)に無作為割り付けを行った[13]．eRVRが得られなかった症例は，全例PEG-IFN/リバビリン36週投与を行った．この結果，65%(352例)がeRVRを達成した．eRVR達成例での24週，48週治療の持続性ウイルス陰性化(SVR)はそれぞれ，92%，88%であるのに対し，eRVR非達成例での48週治療効果は64%であった(表2)．このことより，eRVR達成例では24週治療が妥当であることが示された．

国内開発臨床試験

わが国においても，ゲノタイプ1型C型慢性肝炎に対するテラプレビル/PEG-IFN/リバビリン3剤併用治療の第Ⅲ相開発臨床試験が行われている[14]．初回投与例では，対照をPEG-IFN/リバビリン48週投与群(PR48)として，テラプレビル/PEG-IFN/リバビリン3剤併用12週投与後にPEG-IFN/リバビリン12週投与の計24週治療(T12PR24)の2群に無作為割り付けして効果を比較し，過去の抗ウイルス療法での再燃例(109例)，無効例(32例)ではテラプレビル併用24週療法1群での検討が成された．この結果，初回投与例でのSVRはPR48群で49%に対し，T12PR24群で73%と有意に高率であった．前治療再燃例のT12PR24治療では88%のSVRが得られた．一方，前治療無効例においてはSVRが34%と難治であった．貧血，皮疹などの重篤な有害事象は，PR48で9.5%，T12PR24では11.9～9.4%に認められた．

わが国におけるテラプレビル開発治験は，海外のそれと使用薬剤，患者背景に差異があり，結果の解釈に注意が必要である．第1に，海外では併用薬剤としてPEG-IFNα2a(ペガシス®)が使用されているが，わが国ではPEG-IFNα2b(ペグイントロン®)が使用される．両薬剤は付加されているPEG(ポリエチレングリコール)の分子量が異なるため，薬物血中動態および用量・用法が異なる．第2に，インターフェロン治療効果にIL28B遺伝子の多型が大きく影響を与えるが，アジア人は欧米人種・アフリカ系人種に比して治療抵抗性の多型が少ない．これらの問題は市販後コホート研究で明らかにすべき課題である．

おわりに

現在，ゲノタイプ1型に対し標準治療となったテラプレビル/PEG-IFN/リバビリン併用療法は，従来治療に比べ格段に高い治療効果が得られることがこれまでに行われた国内外の臨床試験で明らかになった．一方で，重篤な皮疹や貧血などの副作用も報告されており，世界に際立って感染者の高齢化が進行している日本においては，個々の症例の治療適応について慎重な決定が求められる．今後登場する次世代治療薬の臨床試験の動向を視野に入

表2 Response-guideプロトコルにおける治療成績(ILLUMINATE Study)

	全症例	eRVR達成		eRVR非達成例	20週前中断例
		T12PR24	T12PR48	T12PR48	
症例数	540	162	160	118	100
SVR	72%	92%	88%	64%	23%

(文献13)より引用)

れながら，安全性に配慮し，より適切な投与法で治療を進めるのが肝要である．

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消化器と免疫
No.48 2011

HCV-NS4B 蛋白と IFN 発現系シグナル分子との分子間相互作用の解析

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要 旨：C型肝炎の慢性化にはウイルスによる自然免疫応答の阻害が関与していると考えられている。今回IFN発現系のシグナル分子であるCardif, STINGに対するNS4Bの作用について解析を行った。NS4BによりCardifおよびSTING誘導性IFN活性が抑制された。免疫沈降, BiFC assayではSTINGとNS4Bの直接結合が示唆された。免疫染色ではSTINGとNS4Bの共局在が観察された。以上よりNS4BがSTINGに直接結合し機能を抑制することによってIFN-β活性を抑制していると考えられた。

《消化器と免疫 48：174–177, 2011》

Keywords：HCV-NS4B ； STING ； IFN signaling

背 景

ウイルス感染時には，宿主の自然免疫機構が働き interferon (IFN) が産生されることが知られているが，hepatitis C virus (HCV) 感染では，約7割でウイルスが排除されず感染が持続し高率に慢性化することから，この要因の一つとしてHCVによる自然免疫応答の阻害が関与していると考えられている[1]。自然免疫機構の一つであるRIG-I依存系IFN発現経路ではHCV感染後，細胞内レセプターであるRIG-IがウイルスRNAを認識し，CardifやTBK1などの下流分子へシグナル伝達を介しIFNを誘導する[2]。

HCV-NS3/4Aプロテアーゼは Cardif を切断することによりRIG-I/Cardif依存性のIFN活性化経路を抑制することが知られており，多くの研究がなされている[3,4]。一方，我々は以前にNS4BがRIG-I依存性のIFN活性を抑制することを報告した[5]。また近年，RIG-I

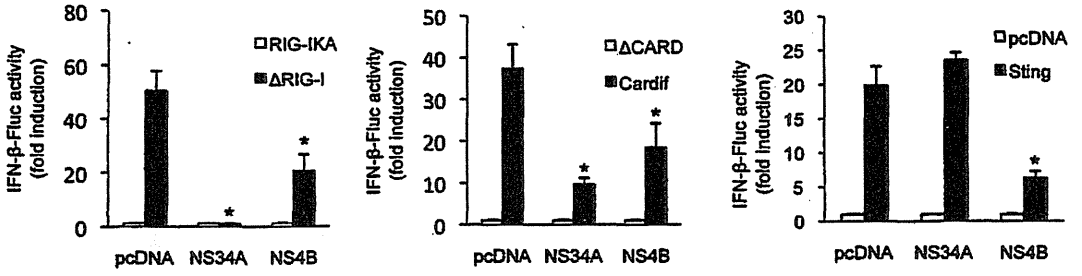

図1

293T細胞にΔRIG-I, Cardif, STINGそれぞれをNS3/4Aもしくは NS4Bと共に導入し，24時間後にIFN-β-promoterを用いてレポーターアッセイを行い，IFN-β-promoter活性の変化について比較検討した。

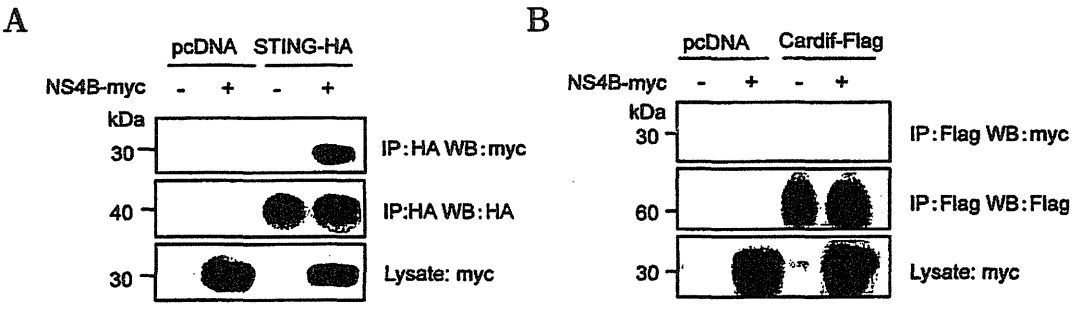

図2

293T細胞にNS4BとCardif (A) あるいはSTING (B) を導入し，24時間後に細胞を回収し蛋白を抽出して免疫沈降を行った。

依存性IFN産生経路に関わるシグナル分子の一つとして stimulator of interferon genes (STING) が同定され，CardifおよびTBK1と結合し機能すると考えられている[6,7]。STINGは種々のフラビウイルスのNS4Bと一部相同性を示し，黄熱病ウイルスのNS4BによりSTING誘導性IFN-β活性が抑制されることが報告された[8]。以上より，HCV-NS4BがSTINGとの相互作用を介してIFN抑制効果を示している可能性が考えられる。

今回我々はHCV-NS4B蛋白とCardifあるいはSTINGとの分子間相互作用の解析を行いNS4BによるIFN抑制作用の分子機構を明らかにした。

結　果

はじめにreporter assayを用いて解析を行った。ΔRIG-I, CardifあるいはSTINGをIFN-β reporterと共に293T細胞にトランスフェクションし，ルシフェラーゼ活性を定量した。ΔRIG-I, Cardifそれぞれの強制発現によりIFN-β-promoter活性が上昇したが，それらはNS3/4Aあるいは NS4Bの共発現下で強く抑制された。一方STINGの強制発現によってもIFN-β-promoter活性の誘導が確認されたが，誘導されたIFN-β-promoter活性はNS3/4Aの共発現によっては抑制されず，NS4Bの共発現によって強く抑制された（図1）。

RIG-I依存性IFN発現経路では，下流のシグナル分子が活性化され最終的にTBK1やIKKεによってIRF3がリン酸化され核内に移行することによりIFN-βが誘導される。そこで，IFN発現経路の活性化を示すリン酸化IRF3の発現についてウェスタンブロット法にて検出し，更にNS4Bの発現による影響について評価した。ΔRIG-I, Cardif, STINGをそ

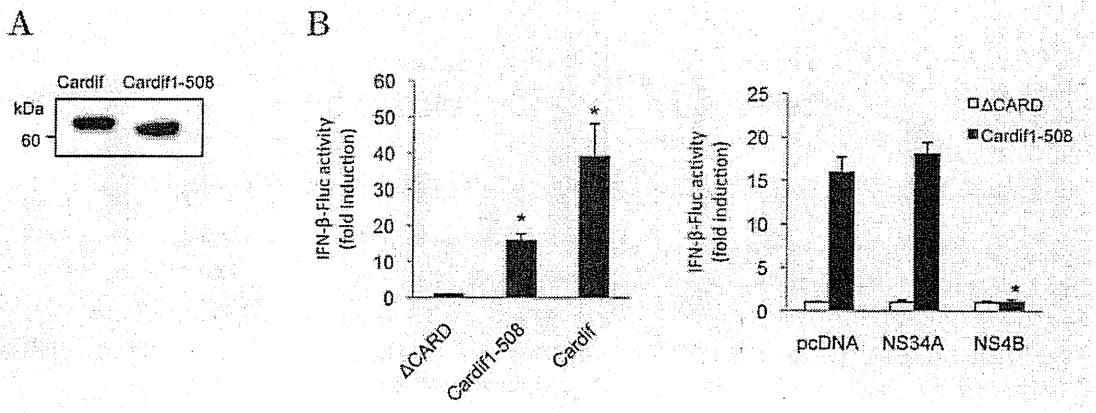

図3

CardifのC末端を欠失したプラスミド(Cardif1-508)を作成した。作成したプラスミドの蛋白発現を示す（A)。Cardif1-508を293T細胞に導入し、24時間後にIFN-β-promoterを用いてレポーターアッセイを行った。更に，NS3/4AあるいはNS4Bを共発現させた時のIFN-β-promoter活性についてもレポーターアッセイを行い比較検討した（B)。

れぞれ293T細胞にトランスフェクションし24時間後にウェスタンブロットを行うと，リン酸化IRF3の発現を認めた。更に，NS4Bを共発現させた場合とさせなかった場合でのリン酸化IRF3の発現の差に関して同様にウェスタンブロット法で検出し比較した。NS4Bを共発現させると，リン酸化IRF3の発現は抑制された。以上より，NS4BはRIG-I/STING依存性IFN発現経路を抑制していると考えられた。一方で，NS3/4AはSTING誘導性のIFN-β-promoter活性を抑制せず，両者は異なる機序でIFN発現経路を抑制していると考えられた。

Cardif，STINGおよびNS4Bの細胞内局在に関して共焦点顕微鏡を用いて観察を行った。CardifとNS4Bは一部で共局在を示した。STINGとNS4Bはほぼ完全に局在が一致しており，ERで共局在しているものと考えられた。

CardifあるいはSTINGとNS4Bとの相互作用を解析するために，免疫沈降およびBimolecular fluorescence complementation (BiFC) assay[9]を行った。免疫沈降ではSTINGとNS4Bは共沈を示したが，CardifとNS4Bとの共沈は明らかではなかった（図2)。BiFC assayでは，STINGとNS4Bの解析において全ての組み合わせで蛍光発現細胞の割合が有意に増加した。一方CardifとNS4Bとの解析においては一部の組合せで蛍光発現細胞の割合の増加を認めたが，殆どの組合せでは蛍光発現細胞を認めなかった。以上より，NS4BはSTINGと直接結合し，IFN発現系を抑制していると考えられた。また，CardifとNS4Bとは弱く結合あるいは間接的に結合している可能性が考えられた。

HCV感染においてはNS3/4AによりCardifが切断されIFN発現経路が抑制される。この状況下において，Cardifより下流のシグナル分子と考えられるSTINGの機能がNS4Bによって阻害されることが，IFN-β活性の抑制にどれだけ影響を与えているかということについても検討を行った。NS3/4Aにより切断され産生されるアミノ酸1-508から成るtruncated Cardifを作成し，IFN-β-promoterを用いたreporter assayを行った。truncated Cardifを強制発現させると，弱いIFN-β-promoter活性が誘導された。そこで，更にNS3/4AあるいはNS4Bを共発現させ検討した。誘導された弱いIFN-β-promoter活性はNS3/4Aの共発現によっては全く抑制を認めなかったが，NS4Bの共発現によりほぼ完全に抑制された

(図3)。以上より，NS4B は NS3/4A による Cardif 切断後に誘導される弱い IFN-β 活性を抑制すると考えられた。

結　論

HCV-NS4B 蛋白は STING と直接結合することによりその機能を阻害し，NS3/4A protease とは両者は独立，かつ協調的に IFN 発現経路を抑制していると考えられた。

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Ex vivo induction of IFN-λ3 by a TLR7 agonist determines response to Peg-IFN/Ribavirin therapy in chronic hepatitis C patients

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Abstract
Background Genetic variation around interleukin-28B (*IL28B*), encoding IFN-λ3, predict non-responders to pegylated interferon-α/ribavirin (Peg-IFN/RBV) therapy in chronic hepatitis C (CHC). However, it remains unclear the expression and the role of *IL28B* itself. The aim of this study is to develop easy and useful methods for the prediction of treatment outcomes.
Methods The mRNA and protein levels of IFN-λ3 induced by ex vivo stimulation of peripheral blood mononuclear cells (PBMC) or magnetically selected dendritic cells (DCs) with toll-like receptor agonists (TLR3; poly I:C, TLR7; R-837) were measured by the quantitative real-time polymerase chain reaction and our newly developed chemiluminescence enzyme immunoassays, respectively, and compared with the clinical data.
Results We found that BDCA-4^+ plasmacytoid and BDCA-3^+ myeloid DCs were the main producers of IFN-λs when stimulated with R-837 and poly I:C, respectively. Detectable levels of IFN-λs were inducible even in a small amount of PBMC, and IFN-λ3 was more robustly up-regulated by R-837 in PBMC of CHC patients with favorable genotype for the response to Peg-IFN/RBV (TT in *rs8099917*) than those with TG/GG. Importantly, the protein levels of IFN-λ3 induced by R-837 clearly differentiated the response to Peg-IFN/RBV treatment ($p = 1.0 \times 10^{-10}$), including cases that *IL28B* genotyping failed to predict the treatment response. The measurement of IFN-λ3 protein more accurately predicted treatment efficacies (95.7 %) than that of *IL28B* genotyping (65.2 %).
Conclusions Genetic variations around *IL28B* basically affect IFN-λ3 production, but different amounts of IFN-λ3 protein determines the outcomes of Peg-IFN/RBV treatment. This study, for the first time, presents compelling evidence that *IL28B* confer a functional phenotype.

Keywords Chronic hepatitis C · IL28B · IFN-λ3 · Peg-IFN/RBV

Abbreviations

ARFI	Acoustic radiation force impulses
CHC	Chronic hepatitis C
GWAS	Genome-wide association study
IL28B	Interleukin-28B
Peg-IFN/RBV	Pegylated interferon-α/ribavirin
PBMC	Peripheral blood mononuclear cells
SNP	Single nucleotide polymorphisms
SVR	Sustained viral response
TLR	Toll-like receptor
TVR	Transient viral response
VR	Viral response

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Introduction

Recently, we and others independently identified single nucleotide polymorphisms (SNPs) on chromosome 19 associated with the interleukin-28B gene (*IL28B*), encoding IFN-λ3, that were strongly associated with the response to pegylated interferon-α/ribavirin (Peg-IFN/RBV) in chronic hepatitis C (CHC) patients, through a genome-wide association study (GWAS) [1–3]. According to our results, about 80 % of CHC patients with the TT genotype (*rs8099917*) showed viral virologic response (VR), including SVR (sustained virologic response) or TVR (transient virologic response), whereas only about 20 % of HCV patients with the TG/GG genotype showed VR [1]. Thus, by genotyping of *IL28B*, we can predict the efficacy of Peg-IFN/RBV before beginning treatment, avoiding unnecessary side effects and the high cost of Peg-IFN/RBV treatment. However, it is still unknown whether genetic variation of *IL28B* is a functional phenotype for Peg-IFN/RBV treatment. In addition, genotyping of *IL28B* alone failed to predict about 20 % of the response [1], which would be reasonable because final products of the genes are affected by DNA methylation or chromatin modifications as well as genetic variations [4].

Type III IFNs, consisting of IFN-λ1, λ2, and λ3 (also known as *IL29*, *IL28A* and *IL28B*, respectively), have recently been characterized [5, 6]. IFN-λs up-regulate IFN-stimulated genes (ISGs) via Janus kinase/signal transducer and activator of transcription (Jak/STAT) intracellular signaling, inhibiting hepatitis B virus (HBV) or hepatitis C virus (HCV) replication [7]. Antiviral responses evoked by toll-like receptor (TLR)3 or TLR9 agonists are attenuated in IL28RA$^{-/-}$ mice [8], indicating the central role of IFN-λs in antiviral protection. Clinically, early virologic response by Peg-IFN/RBV is associated with a high probability of SVR in HCV patients [9]. Genetic variations of *IL28B* influence spontaneous clearance of HCV [10], or on-treatment viral kinetics [11]. These results suggest a mechanistic link between innate immunity and genetic variations of *IL28B*.

To recognize viruses and trigger innate antiviral responses, mammals have 2 independent receptors, retinoic acid-induced gene-I (RIG-I)-like receptors (RLRs) and TLRs, distinct families of pattern recognition receptors that sense nucleic acids derived from viruses [12]. RIG-I is a double-stranded RNA-binding DExD/H box RNA helicase that is essential for initiating the intracellular response to RNA viral infection [13]. However, NS3/4A, the major serine protease expressed by HCV, disrupts the RIG-I pathway through proteolysis of essential signaling components of IFN regulatory factor 3 (IRF-3) activation [13, 14], reducing immune response. Alternatively, the TLR-families play an important role in innate immune responses in mammals [15]. Among them, TLR3 recognizes viral double-stranded RNA, whereas TLR7 recognize single-stranded RNA. Because some TLRs ligands induce IFN-λ in human macrophages [16], contributing to antiviral defense and HCV is a single-stranded RNA [8], we hypothesized that IFN-λ3 induced via the TLR pathway might contribute to early antiviral response against HCV, which could lead to accurate prediction of treatment efficacy. Therefore, we investigated IFN-λs production in peripheral blood mononuclear cells (PBMC) in healthy volunteers or CHC patients by ex vivo stimulation with TLR agonists, and analyzed whether this method could predict the responses to Peg-IFN/RBV treatment in clinical practice.

Patients, materials, and methods

Study population

Blood samples were obtained from 12 healthy volunteers and 100 consecutive Japanese outpatients with CHC (genotype 1b and high viral load) who visited our hospital between April 2011 and March 2012. The study protocol was conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the ethical committee of our institutes (NCGM-G-001023-01). Written informed consent was obtained from all volunteers and patients. All subjects were negative for HBV and human immunodeficiency virus, and did not have hepatocellular carcinoma. IFN treatment was not being given to any patient at the time blood samples were taken. The subjects were all evaluated for SNP near *IL28B* (*rs8099917*, *rs12979860*) using the InvaderPlus assay (Invader Chemistry, Madison, WI, USA) as previously reported [17].

Definition of treatment responses

Non-virologic response (NVR) was defined as less than a 2-log-unit decline in the serum level of HCV RNA from the pre-treatment baseline value within the first 12 weeks, and detectable viremia 24 weeks after initiation of treatment. VR was defined as achieving SVR or TVR. SVR was defined as undetectable HCV RNA in the serum 6 months after the end of treatment, whereas TVR was defined as reappearance of HCV RNA in the serum during or after completion of treatment.

Preparation of PBMC and selection of plasmacytoid or myeloid dendritic cells (DCs)

Whole blood anti-coagulated with EDTA was obtained from healthy volunteers and CHC patients. PBMC were isolated by Ficoll-Hypaque (Mediatech, Herndon, VA, USA) density

gradient centrifugation. BDCA-1, 3, 4^+DCs were negatively or positively selected by BDCA-1^+DC isolation kit, BDCA-3 MicroBead kit and BDCA-4/Neuropilin-1 MicroBead kit, respectively (Miltenyi Biotec, Auburn, CA, USA) according to the manufacturer's instructions.

Ex vivo induction of IFN-λ1, IFN-λ2, and IFN-λ3

After pre-treatment with or without 100 U/ml of IFN-α (Hayashibara Co. Ltd., Okayama, Japan) in 200 μl of Roswell Park Memorial Institutes (RPMI) medium supplemented with 10 % fetal bovine serum for 16 h, 100,000 of mononuclear cells were stimulated with 30 μg/ml of poly I:C (TLR3 agonist; Imgenex, San Diego, CA, USA), or 5 μg/ml of imiquimod (R-837; TLR7 agonist, Imgenex) as previously reported [16]. For chemiluminescence enzyme immunoassays (CLEIA), 200,000 cells were subjected to the same stimulation protocol.

RNA isolation and cDNA synthesis

After stimulation with TRL-agonists for 4 h, the PBMC were lysed with ISOGEN-II (Nippon Gene, Tokyo, Japan). In some experiments, PBMC were harvested at each indicated time point. The lysate was supplemented with chloroform, incubated for 15 min on ice, and centrifuged at 22,000 g for 15 min. The aqueous layer was removed and precipitated with isopropanol. The RNA was pelleted by centrifugation, washed with ethanol, and dissolved in 20 μl of water. Reverse transcription was performed using the SuperScript III first-strand synthesis system (Invitrogen, Carlsbad, CA, USA).

Real-Time quantitative polymerase chain reaction (PCR)

Quantitative real-time PCR was performed to estimate IFN-λ1, IFN-λ2, and IFN-λ3 mRNA expression based on SYBR green fluorescence (Roche Diagnostics Japan), using TaqMan Universal PCR master mix (Roche Diagnostics Japan), according to the manufacturer's protocol. Relative gene expression was calculated as a fold induction. Data were analyzed using the $2\text{-}\Delta\Delta C(t)$ method with Sequence Detector version 1.7 software (Applied Biosystems, Carlsbad, CA, USA) and were normalized using human hypoxanthine phosphoribosyltransferase (HPRT). A standard curve was prepared by serial 10-fold dilutions of human cDNA. The curve was linear over 7 log units with a 0.998 correlation coefficient. Quantitative mRNA expression was determined by triplicate real-time PCR.

Chemiluminescence enzyme immunoassays

We recently developed a CLEIA system for IFN-λ3 that showed a wide detection range of 0.1–10,000 pg/ml with little or no cross-reactivity to IFN-λ1 or IFN-λ2 [18]. In addition, this CLEIA system can correctly detect IFN-λ3 from different *IL28B* genotypes.

Acoustic radiation force impulse (ARFI) elastography

For non-invasive evaluation of liver fibrosis, ARFI elastography was performed using a Siemens Acuson S2000™ ultrasound system (Mochida Siemens Medical System Co, Ltd, Tokyo, Japan) as previously reported [19]. We performed 5 measurements for each patient, and a median value was calculated. Liver stiffness was expressed as the shear wave velocity (m/s) and has been reported to be well correlated with histological liver fibrosis [19].

Statistical analyses

Continuous variables between groups were compared using the Mann–Whitney *U* test, and categorical data were compared using the Chi square test or Fisher's exact test. Correlations between continuous variables were searched using the Pearson correlation test. Values of $p < 0.05$ were considered significant.

Results

Genetic variation in *IL28B*

In CHC patients ($n = 100$), only 1 patient showed discrepancy between *rs8099917* and *rs12979860* with the same prediction for the treatment response by genotyping (TG in *rs8099917*, and TT in *rs12979860*). In addition, we recently reported that *rs8099917* has the greatest accuracy in determining the outcome of Peg-IFN/RBV treatment in Japanese patients [17]. Therefore, *rs8099917* is used in the following analyses. The major homologous (TT) in *rs8099917* is considered a predictive factor for a favorable response to Peg-IFN/RBV treatment, while having minor alleles (TG or GG) is considered predictive for non-responders. Seven of 12 healthy volunteers had the TT genotype of *IL28B* and 5 had TG genotype. In CHC patients, 59 patients had the TT genotype, 36 had TG, and 5 had GG in *rs8099917*.

BDCA-4^+ plasmacytoid DCs are the major producers of IFN-λs in response to R-837

Since Lauterbach et al. [20] found that human DCs expressing BDCA3 (CD141) in myeloid DC subsets were the primary producers of IFN-λs, we sought which cell types are the main producers of IFN-λs when stimulated with R-837. Because DCs from the different *IL28B*

genotype are supposed to produce different amounts of IFN-λs, we used DCs from healthy volunteers with TT genotype. After negative or positive magnetic selection of BDCA-1, 3, 4^+DCs using 100 ml of peripheral blood, each collection was stimulated with IFN-α, following poly I:C or R-837 as previously reported [16], and evaluated the mRNA of IFN-λs or the protein levels of IFN-λ3. We confirmed that BDCA-3^+DCs were the main producers of IFN-λs when stimulated with poly I:C as previously reported (Fig. 1a) [20]. Interestingly, when stimulated with R-837, positive selection of BDCA-4^+DCs (plasmacytoid DCs), not BDCA-3^+DCs, produced IFN-λs whereas depletion of BDCA-4^+DCs showed marked reduction of IFN-λs (Fig. 1b). Therefore, BDCA-4^+DCs were the main producers of IFN-λs when stimulated with R-837. Thus, different stimulation targeted different DC subsets to induce IFN-λs.

Induction of IFN-λs (IFN-λ1, IFN-λ2, and IFN-λ3) in PBMC from healthy volunteers

We confirmed that the main producers of IFN-λs were DCs. However, analyses of IFN-λs using DC subsets need a lot of blood, which cannot apply to patients, because

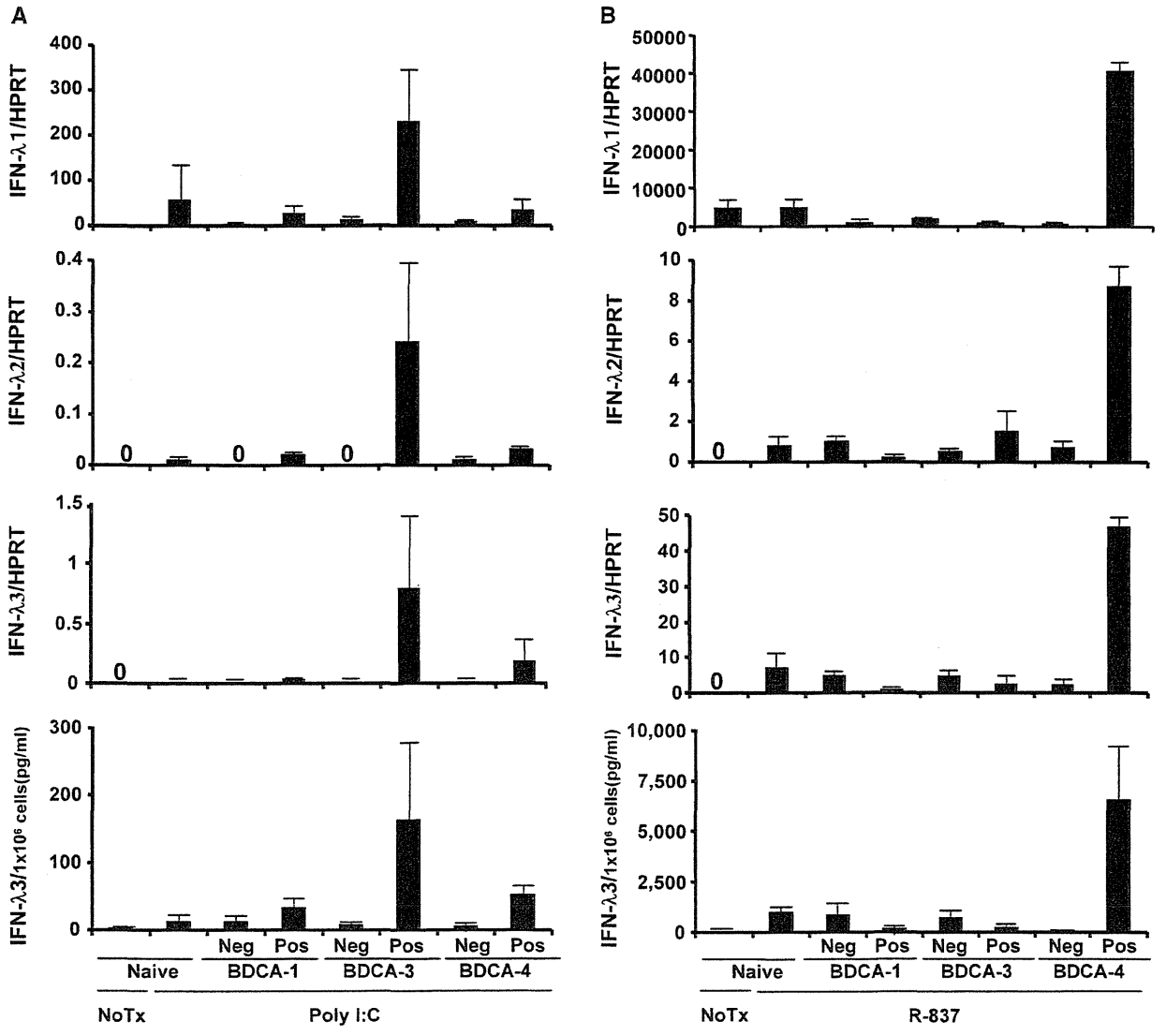

Fig. 1 IFN-λs were produced from different subsets of dendritic cells (DCs) when stimulated with different TLR agonists. BDCA-3^+ or BDCA-4^+DCs was negatively or positively selected using peripheral blood mononuclear cells (PBMC) from healthy volunteers ($n = 5$). PBMC or DCs were stimulated with IFN-α, following poly I:C (**a**) or R-837 (**b**). The mRNA and the protein levels of IFN-λs were determined by real-time PCR and CLEIA, respectively. *Neg* negative selection, *Pos* positive selection of each DCs

BDCA-3$^+$ or 4$^+$DCs are very minor subsets in peripheral blood (0.03, 0.5 %, respectively) [21]. Therefore, we examined if a small amount of PBMC, using 2–3 ml of whole blood from healthy volunteers with negative anti-HCV (Supplementary Table 1), still induced detectable levels of IFN-λs. We confirmed that, even with a small amount of PBMC, detectable levels of IFN-λs were induced by R-837 and the levels of IFN-λ3 were different between *IL28B* genotypes (Fig. 2a, b). Therefore, whole PBMC from healthy volunteers with the TT genotype were

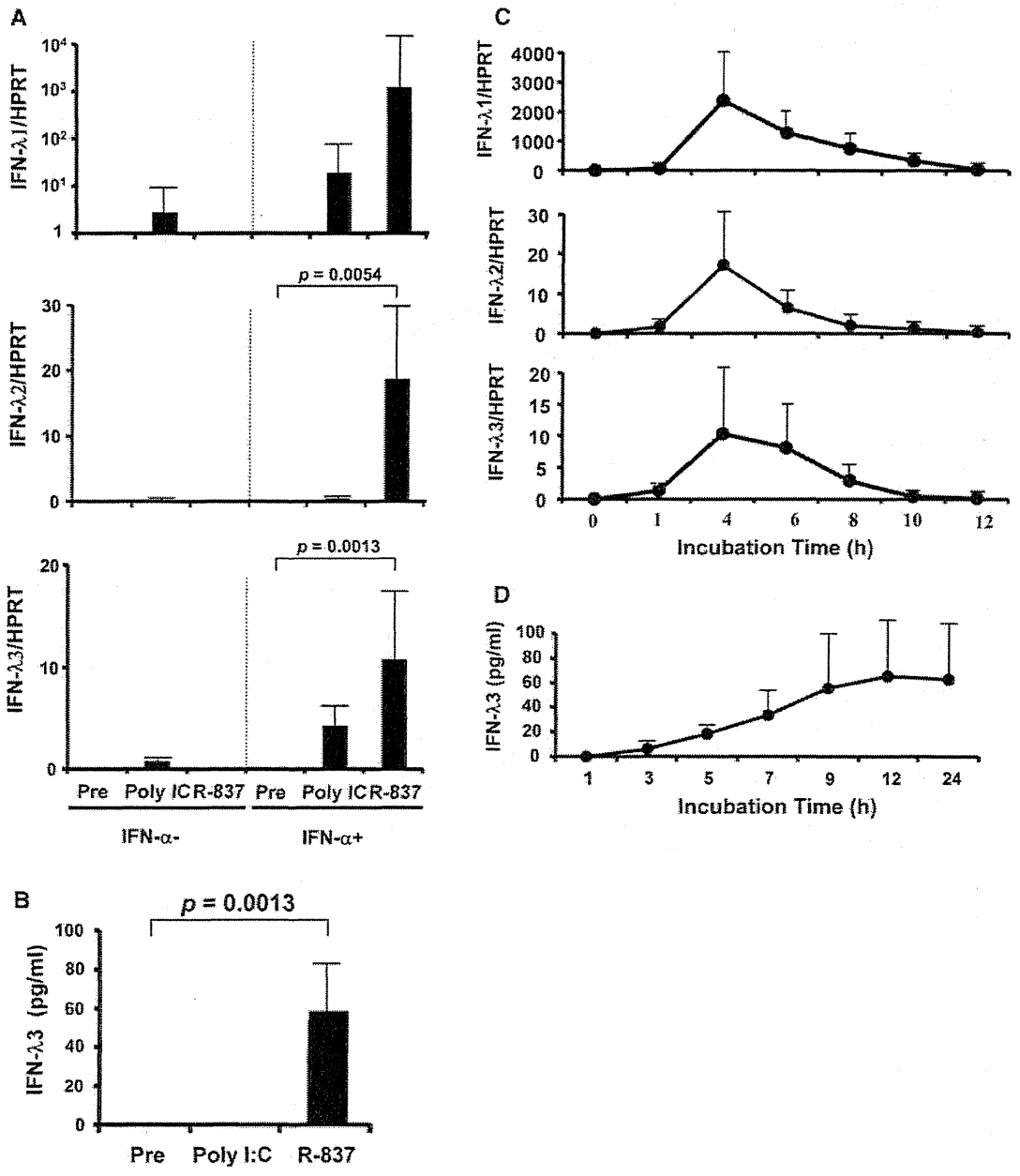

Fig. 2 Ex vivo induction of IFN-λs in PBMC from healthy volunteers. **a** mRNA expression levels of IFN-λs by real-time quantitative PCR. After pre-treatment with or without 100 U/ml of IFN-α for 16 h, 100,000 of mononuclear cells were stimulated with 30 μg/ml of poly I:C (a TLR3 agonist) or 5 μg/ml of R-837 (a TLR7 agonist). After stimulation with TRL-agonists for 4 h, the PBMC were harvested. **b** Protein levels of IFN-λs. After pre-treatment with 100 U/ml of IFN-α, 200,000 of mononuclear cells were stimulated with 30 μg/ml of poly I:C (a TLR3 agonist) or 5 μg/ml of R-837 (a TLR7 agonist), and the supernatant was harvested 24 h after stimulation with TLR agonists. **c** Kinetics of IFN-λs mRNA levels. After pre-incubation with IFN-α for 16 h, PBMC was stimulated with 5 μg/ml of R-837. Real-time quantitative PCR was conducted at each time point. **d** CLEIA results for IFN-λ3 protein in the supernatant at each time point

used for the initial experiments (Fig. 2a–d). IFN-α or TLR-agonists alone failed to induce significant amounts of IFN-λs. All IFN-λs mRNA were strongly induced by R-837 along with IFN-α (Fig. 2a). Poly I:C also induced detectable levels of IFN-λs, but those were not prominent in our setting. Protein levels of IFN-λ3 were detectable only by R-837 (Fig. 2b). Therefore, we focused on R-837 in the following experiments. Next, we sought to confirm the kinetics of IFN-λs after stimulation with R-837. When PBMC from volunteers with the TT genotype ($n = 7$) were stimulated with R-837, an IFN-λ mRNA peak was observed 4 h after incubation, rapidly decreasing to undetectable levels at 12 h (Fig. 2c). In the CLEIA results, IFN-λ3 was detected in the supernatant beginning 3 h after stimulation with R-837, peaking at 9–12 h, then plateauing (Fig. 2d). From these observations, mRNA levels of IFN-λs and protein levels of IFN-λ3 induced by R-837 were measured at 4 and 24 h after R-837, respectively.

Patients' characteristics

Consecutive 100 HCV-RNA positive CHC patients with genotype 1b (35 male, 65 female) were enrolled in this study (Supplementary Table 2). Our patients with the TG/GG genotype were more prevalent (41.0 %, 41/100) than in the normal population (about 20 %), which was expected given that patients who had previously failed to benefit from HCV treatment tended to visit our center. Each patient who had histories of Peg-IFN/RBV ($n = 38$) was treated with Peg-IFN-α2b (1.5 μg per kg body weight (μg/kg) subcutaneously once a week) or PEG-IFN- α2a (180 μg once a week) plus RBV (600–1,000 mg daily depending on body weight) (Fig. 3). Since a reduction in the dose of PEG-IFN-α and RBV can contribute to less SVR [22], 2 patients with an adherence of <80 % dose for either drugs during the first 12 weeks who had shown NVR were excluded from "the known treatment-response" group. Patients who had been treated with IFN monotherapy ($n = 5$) were also excluded because *IL28B* was identified in patients having had Peg-IFN/RBV treatment, but not IFN monotherapy, through the GWAS. Among them ($n = 36$), 19 had shown TVR whereas 17 had shown NVR. After enrollment to this study, 10 treatment-naïve patients started Peg-IFN/RBV therapy. One patient showed undetectable HCV-RNA during therapy, and the therapy is ongoing (this patient was categorized in VR and/or TVR). Seven patients achieved SVR (categorized in VR and/or SVR), whereas 2 patients showed NVR (categorized in NVR). Therefore, 47 CHC patients were treatment-naïve and 46 CHC patients had the known response to Peg-IFN/RBV treatment. There were no differences in the characteristic backgrounds between patients with the TT and TG/GG genotypes in treatment-naïve CHC patients ($n = 47$) except for their serum γ-GTP level (32 ± 19 and 59 ± 53, respectively, $p = 0.017$) (Table 1), which were consistent with our recent report [23].

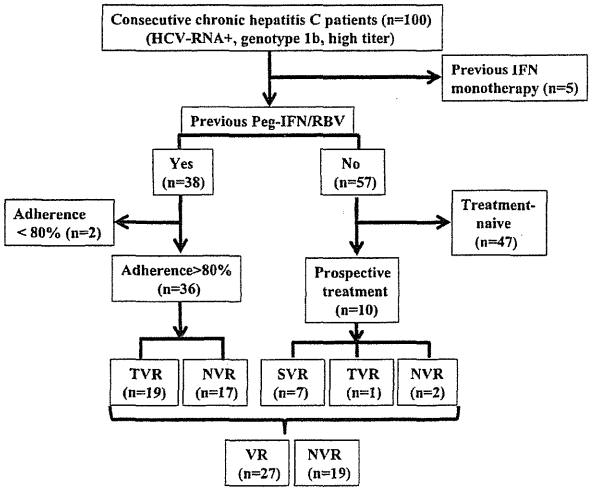

Fig. 3 Enrolled chronic hepatitis C patients with or without histories of treatment against HCV

Association of IFN-λ3 induction with genetic variations around *IL28B* in healthy volunteers or treatment-naïve CHC patients

R-837 induced higher levels of IFN-λ2 or IFN-λ3 mRNA in healthy volunteers with the TT genotype than in those with the TG/GG genotype whereas no differences were observed in IFN-λ1 (Fig. 4a). However, no statistical differences were observed in the protein levels of IFN-λ3

Table 1 Patients' characteristics of HCV treatment-naïve patients in TT or TG/GG genotype ($n = 47$)

	TT ($n = 28$)	TG/GG ($n = 19$)	p value
Age	66 ± 9	62 ± 14	ns
M:F	12:16	6:13	ns
WBC	4,493 ± 1,240	4,337 ± 1,096	ns
Hb	13.7 ± 1.7	13.7 ± 1.3	ns
Plt	15.5 ± 5.5	16.8 ± 6.0	ns
TP	7.7 ± 0.5	7.8 ± 0.5	ns
Alb	4.2 ± 0.5	4.4 ± 0.4	ns
AST	52 ± 30	44 ± 22	ns
ALT	56 ± 35	49 ± 29	ns
γ-GTP	32 ± 19	59 ± 53	0.017
ChE	280 ± 100	315 ± 87	ns
T-cho	170 ± 26	177 ± 37	ns
LDL	89 ± 23	97 ± 31	ns
HCV RNA	6.5 ± 0.6	6.3 ± 0.8	ns
ARFI[a]	1.50 ± 0.51	1.33 ± 0.37	ns

[a] ARFI (acoustic radiation force impulse) represents shear wave velocity (m/s)

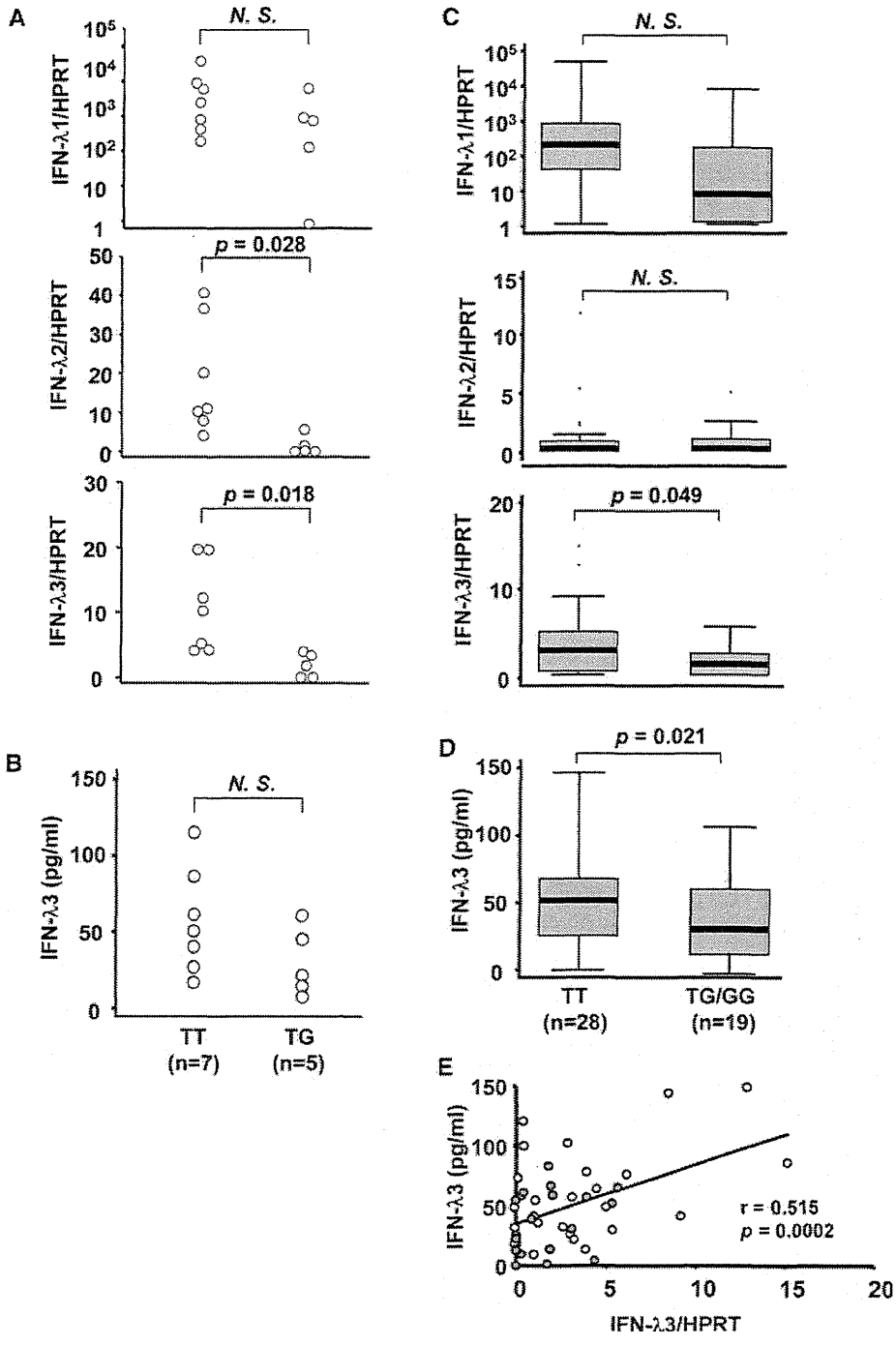

Fig. 4 Ex vivo induction of IFN-λs in PBMC from healthy volunteers and treatment-naïve CHC patients. **a** Differences of IFN-λs mRNA levels between each *IL28B* genotype in healthy volunteers ($n = 12$). After pre-treatment with 100 U/ml of IFN-α for 16 h, 100,000 of mononuclear cells were stimulated with 5 μg/ml of R-837. After stimulation with R-837 for 4 h, the PBMC were harvested. **b** Differences of IFN-λ3 protein levels between each IL28B genotype in healthy volunteers ($n = 12$) with or without R-837. **c** Differences of IFN-λs mRNA levels between each *IL28B* genotype in treatment-naïve CHC patients ($n = 47$). **d** Differences of IFN-λ3 protein levels between each *IL28B* genotypes in treatment-naïve CHC patients ($n = 47$) with or without R-837. **e** Correlation between mRNA and protein levels of IFN-λ3 in treatment-naïve CHC patients ($n = 47$). Each *open circle* represents TT genotype whereas each *closed circle* represents TG/GG genotype

between TT and TG genotype. The protein levels of IFN-λ3 were robustly induced by R-837 (Fig. 4b). These findings were similarly observed when using PBMC from treatment-naïve CHC patients (Fig. 4c, d). The protein levels of IFN-λ3 in PBMC from treatment-naïve CHC patients with TT genotype were significantly higher than those with TG/GG genotype. The IFN-λ3 mRNA levels were well correlated with those protein levels (Fig. 4e). Similar findings were obtained in all CHC patients ($n = 100$) (Supplementary Fig. 1).

Predictiveness of IFN-λ3 induction for response to Peg-IFN/RBV treatment

Among 46 HCV-RNA positive patients who had the known response to Peg-IFN/RBV, 27 patients showed VR and 19

Table 2 Patients' characteristics categorized in the response to treatment ($n = 46$)

	VR ($n = 27$)	NVR ($n = 19$)	p value
Age	63 ± 9	63 ± 9	ns
M:F	9:18	5:14	ns
TT:TG:GG	20:7:0	9:9:1	ns
WBC	4,181 ± 1,299	3,947 ± 1,127	ns
Hb	12.5 ± 2.0	13.1 ± 1.7	ns
Plt	18.1 ± 5.9	13.0 ± 3.6	0.002
TP	7.7 ± 0.6	7.8 ± 0.4	ns
Alb	4.4 ± 0.4	4.2 ± 0.3	ns
AST	46 ± 42	61 ± 35	ns
ALT	44 ± 44	73 ± 58	ns
γ-GTP	28 ± 22	51 ± 32	ns
ChE	293 ± 76	271 ± 75	ns
T-cho	178 ± 37	159 ± 23	ns
LDL	95 ± 22	88 ± 19	ns
HCV RNA	6.3 ± 0.6	6.3 ± 1.0	ns
Core 70 (W:M)	18:6	8:9	ns
ISDR (0:>0)	12:12	8:9	ns
ARFI[a]	1.20 ± 0.26	1.72 ± 0.34	<0.001

[a] ARFI (acoustic radiation force impulse) represents shear wave velocity (m/s)

showed NVR. Lower platelet counts and higher shear wave velocity were significantly observed in the NVR group (Table 2). The mRNA levels of IFN-λ3 were significantly higher in patients with VR than in those with NVR (Fig. 5c, $p = 0.0002$). The expression levels of IFN-λ1 (Fig. 5a) and IFN-λ2 (Fig. 5b) were also significantly higher in patients with VR although the statistical differences were markedly bigger than IFN-λ3. The protein levels of IFN-λ3 confirmed these results and more clearly differentiated between VR and NVR in patients with previous therapy ($n = 36$), in treatment-naïve patients with prospective therapy ($n = 10$), or combined ($n = 46$) (Fig. 5d, $p = 6.9 \times 10^{-9}$, $p = 0.014$, $p = 1.0 \times 10^{-10}$, respectively). Interestingly, 7 patients with the TG/GG genotype who showed VR demonstrated high IFN-λ3, whereas 8 patients with the TT genotype who showed NVR demonstrated low IFN-λ3 induction. Taken together, the response to Peg-IFN/RBV was mainly dependent on the capacity of IFN-λ3 production in the PBMC rather than on genetic variations in *IL28B*. Our method more accurately predicted treatment efficacies (44/46, 95.7 %) compared to *IL28B* genotyping (30/46, 65.2 %) when cut-off of IFN-λ3 protein levels was set at the median value (47.6 pg/ml) (Table 3).

Discussion

The present study demonstrated that the amount of endogenous IFN-λ3 in PBMC induced by the TLR7 agonist determines the outcome of Peg-IFN/RBV therapy, though its induction was basically dependent on the *IL28B* genotype. Since this method can evaluate the final gene products, the genetic factor or epigenetic factor is not necessary for consideration, which could provide more accurate prediction of the response to Peg-IFN/RBV therapy than *IL28B* genotyping. In addition, an annoying informed consent about gene handling is not necessary, and the cost is lower than genomic analyses. IFN-based treatment with novel drugs such as protease inhibitors or polymerase inhibitors could be still predictive by this method because genetic variations in *IL28B* are strongly associated with response to telaprevir/Peg-IFN/RBV treatment [24].

IFN-λs were shown to inhibit the replication of a number of viruses in vitro, including HBV or HCV [5–7], by up-regulation of ISGs. Several GWAS studies suggested that IFN-λ3 played a key role in the response to HCV [1–3, 10, 11]. Meanwhile, type III IFNs, produced by hepatocytes, in response to HCV infection, predominantly lead functional ISGs induction in comparison to type I IFNs [25]. The combination of *IL28B* genotype between recipients and donors determines the outcome of Peg-IFN/RBV therapy for recurrent hepatitis C after liver transplantation [26]. Therefore, a favorable response to anti-HCV treatment might be dependent on the amount of endogenous IFN-λ3 produced by both lymphocytes and hepatocytes.

Unsuccessful induction of IFN-λs by IFN-α or TLR-agonists alone (Fig. 1a) may explain controversial results of IFN-λ3 expression in either PBMC or intrahepatic lymphocytes from pre-treatment patients [1, 3, 23, 27]. IFN-α up-regulates the expression of TLR, TRIF, and MyD88, common adaptor molecules associated with TLR signaling [16]. UV-inactivated viruses, as well as infectious viruses, can induce IFN-λ, suggesting viruses can be sensed through a non-infectious route such as endocytosis of infectious or non-infectious viral particles [28]. TLR7 appears to play an important role in the induction of antiviral responses against single-stranded RNA viruses [29–31], and imidazoquinoline activates immune cells via the TLR7-MyD88-dependent signaling pathway [32]. Synthetic TLR7 agonists have recently been characterized with respect to their ability to induce cytokines [33]. Furthermore, in our study, IFN-λ3 induced by R-837 clearly differentiated the response to Peg-IFN/RBV treatment. Therefore, sequential stimulation with IFN-α following a TLR7 agonist (R-837) may mimic IFN therapy in CHC patients in terms of IFN-λs induction, and our sensitive CLEIA system may contribute to clear differentiation among the responses to Peg-IFN/RBV treatment in this study. It has recently been reported that expression levels and function of TLR7 were impaired in HCV-infected human hepatoma cells [34]. However, in the present study,

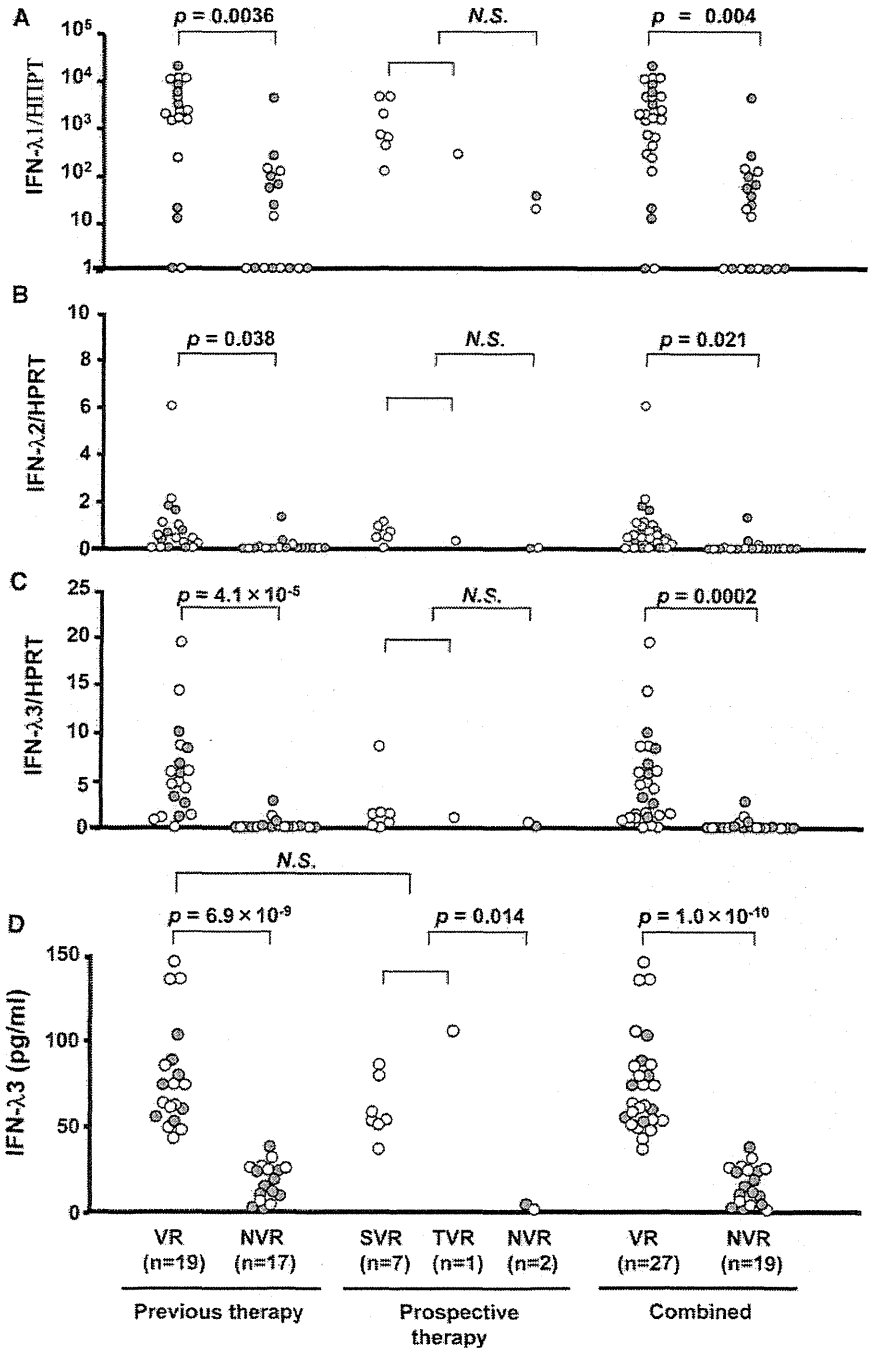

Fig. 5 The mRNA and protein levels of IFN-λ3 ex vivo induced by R-837 and treatment responses in CHC patients with "the known response to Peg-IFN/RBV". The mRNA levels of IFN-λ1 (**a**), IFN-λ2 (**b**), and IFN-λ3 (**c**) of virologic responders (VR) ($n = 27$) and non-virologic responders (NVR) ($n = 19$) were shown. (**d**) Protein levels of IFN-λ3 in CHC patients who had previously failed Peg-IFN/RBV therapy (previous therapy, $n = 36$) and who prospectively treated with Peg-IFN/RBV (prospective therapy, $n = 10$). Combined figures were also shown ($n = 46$). Each *open circle* represents TT genotype whereas each *closed circle* represents TG/GG genotype

a TLR7 agonist was able to induce IFN-λs in PBMC from HCV patients, although the response was slightly impaired in HCV patients compared with that in healthy volunteers. Therefore, impairment of expression or function of TLR7 in HCV patients may not be a critical factor for whole innate immunity.

IFN-λs display high sequence homology. In particular, IFN-λ2 and IFN-λ3 are virtually identical with 96 % amino acid homology [6]. However, the GWAS revealed that only SNPs near the *IL28B*, not *IL28A* or *IL29*, showed strong associations with response to Peg-IFN/RBV treatment [1–3]. In the current study, we found that the expression level of IFN-λ3 in the PBMCs was better correlated with genetic variations in *IL28B* or response to Peg-IFN/RBV treatment. Furthermore, the antiviral effect of recombinant IFN-λ3 was more potent than those of IFN-λ1 or IFN-λ2 [35].

Table 3 Correct prediction rate by genotyping of *IL28B* or IFN-λ3 value (cut-off 47.6 pg/ml)

	IL28B genotype				IFN-λ3		
	TT	TG/GG			High	Low	
VR	**20**	7	27	VR	**25**	2	27
NVR	9	**10**	19	NVR	0	**19**	19
	29	17	46		25	21	46

Bold values indicated correct prediction in each category

Collectively, these findings may support our GWAS data. Alternatively, there are significant differences in IFN-λ2 as well as IFN-λ3 between genotypes or treatment response. The specific primers and probe sets for the IFN-lambda family that we previously developed achieved approximately 10^7-fold specificity in each IFN-λ [18]. However, Osterlund et al. [36] reported that both IFN-λ2 and IFN-λ3 were regulated by a similar pathway of IRF7 resembling those of IFN-α gene expression. Therefore, it is possible that the upregulation of IFN-λ3 could affect that of IFN-λ2 because the homology of regulatory sequence between IFN-λ2 and IFN-λ3 is pretty high. On the other hand, IFN-λ1 and λ2, λ3 show rather low homology (81 %), which could explain the different responses among them.

About 20 % of the HCV patients with the TT genotype failed to respond to the treatment [1]. Indeed, in the current study, 8 of 28 (28.6 %) patients with favorable *IL28B* genotype (TT) showed NVR in Peg-IFN/RBV therapy. Interestingly, all these cases who showed NVR despite their favorable *IL28B* genotype demonstrated low IFN-λ3 production (Fig. 5a–d). Generally, poor response to HCV treatment is related to a number of factors that are unlikely to be solely due to the *IL28B* genotype, such as greater age, male gender, viral factors and liver fibrosis [37, 38]. Therefore, we have tried to calculate multivariate logistic regression to find the most predictive factors affecting the treatment response including clinical backgrounds and the current data. However, the calculations were impossible because of "complete separation" between the treatment-response groups on ELISA data. Liver fibrosis may be attributed to hyporesponsiveness to Peg-IFN/RBV because surrogate markers of liver fibrosis (low platelet counts and high shear wave velocity) were significantly observed in our NVR cases (Table 2). However, both of surrogate markers showed substantial overlap between VR and NVR (Supplementary Fig. 2). Other factors including genetic deficiency of molecules in the TLR7 signaling pathway [39] may affect IFN-λs production. Meanwhile, 7 of 18 (38.9 %) patients with the unfavorable *IL28B* genotype (TG/GG) showed VR in Peg-IFN/RBV therapy (Table 3) and all of these VR patients showed high IFN-λ3 production. Because recombinant IFN-λ from any *IL28B* genetic variant similarly represses HCV RNA [40], the amount of IFN-λ, whatever the genetic variations of *IL28B* are, would be important for antiviral effects. Enhanced transcriptional regulations of *IL28B* could partially be attributed to high IFN-λ3 production in patients with TG/GG [41]. Alternatively, the number of BDCA-4$^+$DCs was well correlated with IFN-λ3 production when PBMC from healthy volunteers was stimulated with R-837 (Supplementary Fig. 3), which suggest that the number of BDCA-4$^+$DC strongly affect IFN-λ3 production in our setting. However, the precise mechanisms of regulating IFN-λ3 production or the number of BDCA-4$^+$DCs in CHC patients should be addressed in the future. Importantly, our methods clearly predict the effectiveness of Peg-IFN/RBV including these exceptional cases.

In the present study, neither the mRNA nor the protein levels of IFN-λ3 were much different among genetic variations in *IL28B* (Fig. 4c, d). It is possible that the exceptional cases of IFN-λ3 levels (e.g., low IFN-λ3 levels in the TT genotype and high levels in the TG/GG genotype) affected the statistical differences. Substantial overlap in the mRNA levels of IFN-λ3 was observed between the different treatment response groups whereas the protein levels of IFN-λ3 clearly differentiated (Fig. 5a–d). One possibility would be that our newly developed CLEIA system [18] is more sensitive than real-time PCR. Moreover, IFN-λs mRNA levels were rapidly induced by R-837, and these effects were limited to several hours after stimulation (Fig. 2c) whereas protein levels of IFN-λ3 were stable from 9 to 24 h after R-837 stimulation (Fig. 2d). In the prospective study, there were no statistical differences in the mRNA levels of IFN-λs. However, the increase of the number of these patients would clarify the differences because the trend of these levels in the prospective study was similar to those in the previously treated group.

In conclusion, our findings suggest that genetic variations in *IL28B* basically affect IFN-λ3 production; however, the amount of endogenous IFN-λ3 determines the outcome of Peg-IFN/RBV therapy. This study, for the first time, presents compelling evidence that genetic variations in *IL28B* confer a functional phenotype and potentially explains our GWAS data. In addition, these results may explain discrepant cases related to *IL28B* genotyping in the response to Peg-IFN/RBV treatment. Thus, ex vivo induction of IFN-λ3 in PBMC by a TLR7-agonist may be a more accurate predictive method for determining the outcome of Peg-IFN/RBV therapy.

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Conflict of interest Tatsuji Kimura is an employee of the Institute of Immunology Co., Ltd. All other authors have nothing to declare.

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Tracing the Spread of Hepatitis C Virus in Turkey: A Phylogenetic Analysis

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Abstract
Background/Aims: Molecular epidemiology of hepatitis C virus (HCV) shows that HCV genotypes are unique with respect to their nucleotide sequence, geographical distribution and clinical relationship. ***Methods:*** In this study we enrolled 67 HCV-infected individuals with various stages of liver disease from four geographical regions of Turkey. A partial NS5B region of the HCV genome was sequenced and subjected to phylogenetic analysis to determine the circulating HCV genotypes and subtypes. ***Results:*** The results showed that HCV genotype 1 (subtype1b) is the main genetic variant of HCV in Turkey but did not reveal any Turkish indigenous phylogenetic cluster. Phylogenetic analysis showed that Turkish strains have their closest matches from both Asia (Japan) and Europe/USA. ***Conclusions:*** In view of Turkey's geographic position, HCV-1b transmission from Europe is not exceptional. This study could not establish a clear role of other HCV genotypes prevalent in neighboring Asian countries in Turkey's HCV transmission, which would need to be confirmed by further regional epidemiological studies.

The Study

Hepatitis C virus (HCV) infection causes acute and chronic hepatitis, cirrhosis and hepatocellular carcinoma and is responsible for approximately 250,000–350,000 deaths annually worldwide [1]. HCV is a genetically diverged virus and different genotypes of the virus have been associated with distinctive geographical and epidemiological features, as well as with different infection outcomes [2]. The most effective current standard of care in patients with chronic hepatitis C is a combination of pegylated interferon-alfa (PEG-IFN) with ribavirin (RBV). However, in the USA and Europe, only 42–52% of patients with HCV genotype 1 achieve a sustained virological response [3–5] and similar results have been reported in a relatively older Japanese population. Accumulated data has provided strong evidence that approximately 20% of patients with HCV genotype 1, and 4 and 5% of patients with genotype 2 or 3 have a null virological response to PEG-IFN/RBV.

Sequencing of an appropriate region of viral genome, such as the non-structural 5B region (NS5B), core, and E1, is sufficient for discriminating HCV types and subtypes [6]. Phylogenetic analysis of HCV sequences revealed over 70 different subtypes and six large groups of

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