Figure 4. IFN expression in cell lines following the induction of innate immunity by various stimuli. (**A**, **B**) The indicated cell lines were stimulated by exposure to LIC-pIC (1 μg/ml). (**A**) The mRNA levels of IFN-β- and IFN-λ1-encoding genes (*IFNB1* and *IFNL1* (*IL29*), respectively) before and after (12 h) stimulation, as determined by qRT-PCR. (**B**) The concentration of IFN-λ1 (IL-29) in the culture medium 24 h after stimulation, as determined by ELISA. (**C**, **D**) The indicated cell lines were stimulated by viral infection with NDV or VSV. (**C**) The mRNA levels of IFN-β- and IFN-λ1-encoding genes (*IFNB1* and *IFNL1* (*IL29*), respectively) in HepG2 cells and MRC-5 cells in the absence or presence (12 h) of NDV or VSV, as determined by qRT-PCR. (**D**) The concentration of IFN-λ1 protein in the culture medium at 24 h post-infection, as determined by ELISA. (**E**, **F**) Expression in HepG2 cells of genes encoding MAVS, TICAM-1, and IFN-λ1 (*MAVS, TICAM1,* and *IFNL1* (*IL29*) respectively), as determined by qRT-PCR, after 12 h stimulation with LIC-pIC (1 μg/ml). Cells were pre-treated for 48 h with siRNAs against *MAVS*, *TICAM-1*, or a non-target control. Bars indicate SD ($n = 3$ per group). (**G**) Schematic outline of the proposed mechanism of the antiviral response induced by LIC-pIC.
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remained upregulated through at least five daily LIC-pIC administrations to chimeric mice infected with HCV. Consistent with this bias in IFN production, human hepatocytes were expected to be more receptive to stimulation by IFN-λs than their mouse counterparts.

We demonstrated the robust induction (by LIC-pIC) of IFN-λ rather than IFN-α or IFN-β in human hepatocytes. In several human cell lines derived from other tissues, IFN-λ1 induction by LIC-pIC stimulation was reduced in comparison to that seen in human hepatocytes, indicating that induction of IFN-based innate immunity differs among tissues and that human hepatocytes are high producers of IFN-λs. We also demonstrated that NDV and VSV, ssRNA viruses that are recognized by cytoplasmic sensors (RIG-I-like receptors) [36], could induce the expression of IFN-λs in several human cell lines. However, in contrast to LIC-pIC stimulation, the expression level of IFN-λ1 induced by these viruses was as low in HepG2 hepatocyte cells as in MRC-5 fibroblast cells. These results motivated us to investigate which sensors recognize LIC-pIC resulting in robust IFN-λ production. The presence of two distinct viral sensing innate immune pathways (TLR3 and RIG-I-like receptors) in human hepatocytes has been reported [37,38]. We showed here that the effects of LIC-pIC were mediated by both MAVS, a signaling adaptor molecule of RIG-I-like receptors, and TICAM-1, a TLR adaptor molecule. Given that both NDV and VSV effects are mediated by MAVS, we speculate that the strong induction of IFN-λs by LIC-pIC reflects dual recognition of LIC-pIC by RIG-I-like receptors and TLR3. In this context, we note that both MAVS and TICAM-1 are direct targets for proteolytic degradation by the HCV-encoded NS3/4A protease [39,40]. Related reports [39,40,41] suggest that the inhibitory effect of this viral protease may affect the induction of IFN-λs, particularly in HCV-infected hepatocytes. However, we observed significantly higher levels of IFN-λ expression in response to treatment with LIC-pIC, regardless of *in vivo* HCV infection status (Fig. 3 and data not shown), suggesting that the *in vivo* induction of IFN- λs by LIC-pIC treatment may not be affected by this viral protease in our experimental system. Further analyses will be needed to clarify the balance between viral evasion and activation of the innate immune response.

Our results demonstrate that the dominant IFN systems exhibit tissue- and animal-specific induction patterns, and that IFN-λs plays a critical role in innate immune response in human hepatocytes. These findings expand our understanding of the role of IFN-λs in the human immune system.

Supporting Information

Figure S1 Hepatotropic property of LIC. Biodistribution of RNA following administration of a complex of LIC and ^{3}H-labeled short dsRNA to normal mice.
(EPS)

Figure S2 Time course studies in 3 mice inoculated with HCV genotype 1b or HBV genotype C. (**A**) HCV RNA levels in chimeric mouse serum after inoculation. (**B**) HBV DNA in chimeric mouse serum after inoculation.
(EPS)

Figure S3 Hepatotoxicity of the LIC-pIC complex. (**A**, **B**) Chimeric mice infected with HCV genotype 1a were treated daily for 8 days (Days 0–7) with LIC-pIC at 0.01, 0.03, or 0.1 mg/kg. The sera of chimeric mice were collected on the indicated days following the initial administration of LIC-pIC and the concentration of human albumin (**A**) or alanine transferase (ALT) (**B**) was determined ($n = 3$–5 per group).
(EPS)

Figure S4 Expression of IFN-α, IFN-β, and IFN-γ in HBV-infected chimeric mice with humanized livers following treatment with LIC-pIC. (**A**) Schedule of the LIC-pIC treatments and serum or liver sampling in the HBV-infected chimeric mice. (**B**) Serum concentrations of human and mouse IFN-β and IFN-α, as determined by ELISA. (**C**) The liver mRNA levels of genes encoding IFN-β, IFN-α2 and IFN-γ (human genes: *IFNB1*, *IFNA2* and *IFNG*, respectively; mouse genes: *Ifnb1*, *Ifna2* and *Ifng*, respectively), as determined by qRT-PCR. For (**B**) and (**C**), each point indicates the value for a single chimeric mouse; groups consisted of 1–4 mice each.
(EPS)

Figure S5 Expression of genes encoding human IFN-β (*IFNB1*) and mouse IFN-β (*Ifnb1*) in HCV-infected or uninfected chimeric mice with humanized livers following treatment with LIC-pIC, as determined by qRT-PCR. The data points indicate the mRNA levels in individual chimera; the horizontal bars indicate the mean transcript concentrations for the human (red) and mouse (green) genes ($n = 3$ per group).
(EPS)

Figure S6 Mouse NK and NKT cells are not involved in the anti-HCV effects of LIC-pIC. Chimeric mice with humanized livers were infected with HCV genotype 1b and treated for 14 days (Days 0–13) with one of the following: saline, daily; 1 μg/kg α-galactosylceramide (αGalCer, a specific activator of NKT cells), weekly (Days 0 and 7); or 0.1 mg/kg LIC-pIC, daily. Half of the chimeras receiving the LIC-pIC treatment were pre-treated with a TM-β1 antibody in order to deplete NK and NKT cells. The other half were left untreated (ctrl). The serum HCV genomic RNA levels were determined using qRT-PCR on the indicated days after the initial treatment was administered. Data points indicate mean values and the bars in---dicate SD ($n = 4$–5 per group). *$P<0.05$ and **$P<0.01$, treatments vs. the saline control by Dunnett's multiple comparison test.
(EPS)

Figure S7 The anti-HCV response elicited by IFN-λ in HCV replicon cells. FLR3-1 HCV replicon cells were treated with the indicated recombinant human IFN-λs or with saline control for 72 h, and HCV replication (diamonds) and cell viability (triangles) of the cells then were assessed. Values are

expressed as a percentage of the untreated control ($n = 4$ per group). Data are mean values and the bars indicate SD. $^{**}P<0.01$, IFN treatments vs. the vehicle by Dunnett's multiple comparison test.
(EPS)

Figure S8 The anti-HBV response elicited by IFN-λ. Serum HBV genomic DNA levels were assayed in chimeric mice infected with HBV and administered daily treatments of recombinant human IFN-λ2 or LIC-pIC. HBV levels were assayed on the indicated days after the initial treatments. Values are expressed relative to the baseline value. Data points indicate mean values and the bars indicate SD ($n = 3$–5 per group). $^{**}P<0.01$, treatments vs. the saline control by Dunnett's multiple comparison test.
(EPS)

Figure S9 Neutralizing activity of anti-human IFN-λ antibodies. FLR 3-1 HCV replicon cells were pre-treated with 1 μg/ml or 10 μg/ml of anti-human IFN-λ1 or anti-human IFN-λ2 antibodies before the addition of recombinant human IFN-λ1, IFN-λ2, or IFN-λ3 to the culture medium. The replication of HCV replicons was determined by luciferase assays (red bars), and cell viability was determined using a WST-8 assay (gray bars). Values are expressed as a percentage of the untreated control. Data points are mean values and the error bars indicate SD ($n = 4$ per group).
(EPS)

Figure S10 Induction of IFN-λs by LIC-pIC and antiviral effect in human hepatocytes with genetic variants near the *IFNL3* (*IL28B*) gene. Chimeric mice were transplanted with human hepatocytes from donors bearing a T- or G-allele of the rs8099917 (*IFNL3*-proximal) SNP. Animals were infected with HCV genotype 1b and treated daily with LIC-pIC. (**A**) The liver mRNA levels of human IFN-λ-encoding genes (*IFNL1*, *IFNL2*, and *IFNL3* (also known as *IL29*, *IL28A*, and *IL28B*, respectively)) were assayed by qRT-PCR. (**B**) The serum HCV genomic RNA levels were assayed by qRT-PCR. Values are expressed relative to the baseline value. Data points are presented as mean values with SD ($n = 1$–6 per group). $^{**}P<0.01$, treatments vs. the saline control by Dunnett's multiple comparison test.
(EPS)

Figure S11 The expression of mouse IFN genes and corresponding receptors in chimeric mice with humanized livers. (**A**) The liver mRNA levels of mouse IFN-encoding genes (*Ifnl2* (*Il28a*), *Ifnl3* (*Il28b*), and *Ifnb1*) ($n = 3$–5 per group). (**B**, **C**, **D**) The liver mRNA levels of human and mouse IFN receptor-encoding genes (mouse/human genes are *IFNAR1*/*Ifnar1*, *IFNAR2*/*Ifnar2*, and *IFNLR1*/*Ifnlr1* (*IL28RA*/*Il28ra*), respectively) ($n = 8$–9 per group) in uninfected chimeric mice (HCV-) and in chimeric mice infected with HCV genotype 1a (HCV+). Bars indicate SD. *P*-values were determined using unpaired *t*-tests.
(EPS)

Text S1 Supporting materials, methods, and references. Methods for "Biodistribution of nucleic acids complexed with LIC", "Measurement of human serum albumin", "Measurement of alanine aminotransferase", "Enzyme-linked immunosorbent assay", "Luciferase and WST-8 assays of HCV replicon cells", "Depletion of NK and natural killer T cells", "Activation of NKT cells", "Neutralizing activity of the anti-human IFN-λ antibody", and "SNP genotyping of *IFNL3* (*IL28B*)", along with supporting references, are provided in the Supporting Materials section.
(DOC)

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Author Contributions

Performed the experiments that investigated the antiviral effects of the LIC-pIC complex and the molecules that it induced: SN. Performed the analyses of type-III IFNs, both in vitro and in vivo: YH. Established the system for analysis of the type-III IFNs: Y. Tokunaga. Performed Microarray analysis: YN. Performed the in vitro analyses: TK AT. Conceived and designed the experiments: MK. Analyzed the data: MK. Contributed reagents/materials/analysis tools: KH JY TO CT Y. Tanaka MM KT-K KI MY. Wrote the paper: SN YH.

References

1. Kumar H, Kawai T, Akira S (2011) Pathogen recognition by the innate immune system. Int Rev Immunol 30: 16–34.
2. Akira S (2011) Innate immunity and adjuvants. Philos Trans R Soc Lond B Biol Sci 366: 2748–2755.
3. Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, et al. (2002) Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med 347: 975–982.
4. Yuen MF, Hui CK, Cheng CC, Wu CH, Lai YP, et al. (2001) Long-term follow-up of interferon alfa treatment in Chinese patients with chronic hepatitis B infection: The effect on hepatitis B e antigen seroconversion and the development of cirrhosis-related complications. Hepatology 34: 139–145.
5. Marukian S, Andrus L, Sheahan TP, Jones CT, Charles ED, et al. (2011) Hepatitis C virus induces interferon-lambda and interferon-stimulated genes in primary liver cultures. Hepatology 54: 1913–1923.
6. Thomas E, Gonzalez VD, Li Q, Modi AA, Chen W, et al. (2012) HCV Infection Induces a Unique Hepatic Innate Immune Response Associated with Robust Production of Type III Interferons. Gastroenterology 142: 978–988.
7. Ge D, Fellay J, Thompson AJ, Simon JS, Shianna KV, et al. (2009) Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature 461: 399–401.
8. Rauch A, Kutalik Z, Descombes P, Cai T, Di Iulio J, et al. (2010) Genetic variation in IL28B is associated with chronic hepatitis C and treatment failure: a genome-wide association study. Gastroenterology 138: 1338–1345, 1345 e1331–1337.
9. Suppiah V, Moldovan M, Ahlenstiel G, Berg T, Weltman M, et al. (2009) IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. Nat Genet 41: 1100–1104.
10. Tanaka Y, Nishida N, Sugiyama M, Kurosaki M, Matsuura K, et al. (2009) Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. Nat Genet 41: 1105–1109.
11. Hirabayashi K, Yano J, Inoue T, Yamaguchi T, Tanigawara K, et al. (1999) Inhibition of cancer cell growth by polyinosinic-polycytidylic acid/cationic liposome complex: a new biological activity. Cancer Res 59: 4325–4333.
12. Yano J, Hirabayashi K, Nakagawa S, Yamaguchi T, Nogawa M, et al. (2004) Antitumor activity of small interfering RNA/cationic liposome complex in mouse models of cancer. Clin Cancer Res 10: 7721–7726.
13. Alexopoulou L, Holt AC, Medzhitov R, Flavell RA (2001) Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature 413: 732–738.
14. Yoneyama M, Fujita T (2009) RNA recognition and signal transduction by RIG-I-like receptors. Immunol Rev 227: 54–65.
15. Dandri M, Burda MR, Torok E, Pollok JM, Iwanska A, et al. (2001) Repopulation of mouse liver with human hepatocytes and in vivo infection with hepatitis B virus. Hepatology 33: 981–988.
16. Mercer DF, Schiller DE, Elliott JF, Douglas DN, Hao C, et al. (2001) Hepatitis C virus replication in mice with chimeric human livers. Nat Med 7: 927–933.
17. Nakagawa S, Umehara T, Matsuda C, Kuge S, Sudoh M, et al. (2007) Hsp90 inhibitors suppress HCV replication in replicon cells and humanized liver mice. Biochem Biophys Res Commun 353: 882–888.
18. Sugiyama M, Tanaka Y, Kato T, Orito E, Ito K, et al. (2006) Influence of hepatitis B virus genotypes on the intra- and extracellular expression of viral DNA and antigens. Hepatology 44: 915–924.
19. Tateno C, Yoshizane Y, Saito N, Kataoka M, Utoh R, et al. (2004) Near completely humanized liver in mice shows human-type metabolic responses to drugs. Am J Pathol 165: 901–912.

20. Takeuchi T, Katsume A, Tanaka T, Abe A, Inoue K, et al. (1999) Real-time detection system for quantification of hepatitis C virus genome. Gastroenterology 116: 636–642.
21. Tanaka T, Inoue K, Hayashi Y, Abe A, Tsukiyama-Kohara K, et al. (2004) Virological significance of low-level hepatitis B virus infection in patients with hepatitis C virus associated liver disease. J Med Virol 72: 223–229.
22. Kashiwakuma T, Hasegawa A, Kajita T, Takata A, Mori H, et al. (1996) Detection of hepatitis C virus specific core protein in serum of patients by a sensitive fluorescence enzyme immunoassay (FEIA). J Immunol Methods 190: 79–89.
23. Takaoka A, Wang Z, Choi MK, Yanai H, Negishi H, et al. (2007) DAI (DLM-1/ZBP1) is a cytosolic DNA sensor and an activator of innate immune response. Nature 448: 501–505.
24. Sakamoto H, Okamoto K, Aoki M, Kato H, Katsume A, et al. (2005) Host sphingolipid biosynthesis as a target for hepatitis C virus therapy. Nat Chem Biol 1: 333–337.
25. Watanabe T, Sudoh M, Miyagishi M, Akashi H, Arai M, et al. (2006) Intracellular-diced dsRNA has enhanced efficacy for silencing HCV RNA and overcomes variation in the viral genotype. Gene Ther 13: 883–892.
26. Minakuchi Y, Takeshita F, Kosaka N, Sasaki H, Yamamoto Y, et al. (2004) Atelocollagen-mediated synthetic small interfering RNA delivery for effective gene silencing in vitro and in vivo. Nucleic Acids Res 32: e109.
27. Gidlund M, Orn A, Wigzell H, Senik A, Gresser I (1978) Enhanced NK cell activity in mice injected with interferon and interferon inducers. Nature 273: 759–761.
28. Djeu JY, Heinbaugh JA, Holden HT, Herberman RB (1979) Role of macrophages in the augementation of mouse natural killer cell activity by poly I:C and interferon. J Immunol 122: 182–188.
29. Kotenko SV, Gallagher G, Baurin VV, Lewis-Antes A, Shen M, et al. (2003) IFN-lambdas mediate antiviral protection through a distinct class II cytokine receptor complex. Nat Immunol 4: 69–77.
30. Sheppard P, Kindsvogel W, Xu W, Henderson K, Schlutsmeyer S, et al. (2003) IL-28, IL-29 and their class II cytokine receptor IL-28R. Nat Immunol 4: 63–68.
31. Prokunina-Olsson L, Muchmore B, Tang W, Pfeiffer RM, Park H, et al. (2013) A variant upstream of IFNL3 (IL28B) creating a new interferon gene IFNL4 is associated with impaired clearance of hepatitis C virus. Nat Genet 45: 164–171.
32. Pott J, Mahlakoiv T, Mordstein M, Duerr CU, Michiels T, et al. (2011) IFN-lambda determines the intestinal epithelial antiviral host defense. Proc Natl Acad Sci U S A 108: 7944–7949.
33. Iversen MB, Ank N, Melchjorsen J, Paludan SR (2010) Expression of type III interferon (IFN) in the vaginal mucosa is mediated primarily by dendritic cells and displays stronger dependence on NF-kappaB than type I IFNs. J Virol 84: 4579–4586.
34. Mordstein M, Neugebauer E, Ditt V, Jessen B, Rieger T, et al. (2010) Lambda interferon renders epithelial cells of the respiratory and gastrointestinal tracts resistant to viral infections. J Virol 84: 5670–5677.
35. Mordstein M, Kochs G, Dumoutier L, Renauld JC, Paludan SR, et al. (2008) Interferon-lambda contributes to innate immunity of mice against influenza A virus but not against hepatotropic viruses. PLoS Pathog 4: e1000151.
36. Kumar H, Kawai T, Kato H, Sato S, Takahashi K, et al. (2006) Essential role of IPS-1 in innate immune responses against RNA viruses. J Exp Med 203: 1795–1803.
37. Li K, Chen Z, Kato N, Gale M Jr, Lemon SM (2005) Distinct poly(I-C) and virus-activated signaling pathways leading to interferon-beta production in hepatocytes. J Biol Chem 280: 16739–16747.
38. Wang N, Liang Y, Devaraj S, Wang J, Lemon SM, et al. (2009) Toll-like receptor 3 mediates establishment of an antiviral state against hepatitis C virus in hepatoma cells. J Virol 83: 9824–9834.
39. Li K, Foy E, Ferreon JC, Nakamura M, Ferreon AC, et al. (2005) Immune evasion by hepatitis C virus NS3/4A protease-mediated cleavage of the Toll-like receptor 3 adaptor protein TRIF. Proc Natl Acad Sci U S A 102: 2992–2997.
40. Meylan E, Curran J, Hofmann K, Moradpour D, Binder M, et al. (2005) Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. Nature 437: 1167–1172.
41. Bellecave P, Sarasin-Filipowicz M, Donze O, Kennel A, Gouttenoire J, et al. (2010) Cleavage of mitochondrial antiviral signaling protein in the liver of patients with chronic hepatitis C correlates with a reduced activation of the endogenous interferon system. Hepatology 51: 1127–1136.

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ORIGINAL ARTICLE

Hepatitis C virus kinetics by administration of pegylated interferon-α in human and chimeric mice carrying human hepatocytes with variants of the *IL28B* gene

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ABSTRACT

Objective Recent studies have demonstrated that genetic polymorphisms near the *IL28B* gene are associated with the clinical outcome of pegylated interferon α (peg-IFN-α) plus ribavirin therapy for patients with chronic hepatitis C virus (HCV). However, it is unclear whether genetic variations near the *IL28B* gene influence hepatic interferon (IFN)-stimulated gene (ISG) induction or cellular immune responses, lead to the viral reduction during IFN treatment.

Design Changes in HCV-RNA levels before therapy, at day 1 and weeks 1, 2, 4, 8 and 12 after administering peg-IFN-α plus ribavirin were measured in 54 patients infected with HCV genotype 1. Furthermore, we prepared four lines of chimeric mice having four different lots of human hepatocytes containing various single nucleotide polymorphisms (SNP) around the *IL28B* gene. HCV infecting chimeric mice were subcutaneously administered with peg-IFN-α for 2 weeks.

Results There were significant differences in the reduction of HCV-RNA levels after peg-IFN-α plus ribavirin therapy based on the *IL28B* SNP rs8099917 between TT (favourable) and TG/GG (unfavourable) genotypes in patients; the first-phase viral decline slope per day and second-phase slope per week in TT genotype were significantly higher than in TG/GG genotype. On peg-IFN-α administration to chimeric mice, however, no significant difference in the median reduction of HCV-RNA levels and the induction of antiviral ISG was observed between favourable and unfavourable human hepatocyte genotypes.

Conclusions As chimeric mice have the characteristic of immunodeficiency, the response to peg-IFN-α associated with the variation in *IL28B* alleles in chronic HCV patients would be composed of the intact immune system.

Significance of this study

What is already known on this subject?

- Genetic polymorphisms near the *IL28B* gene are associated with a chronic HCV treatment response.
- HCV-infected patients with the *IL28B* homozygous favourable allele had a more rapid decline in HCV kinetics in the first and second phases by peg-IFN-α-based therapy.
- During the acute phase of HCV infection, a strong immune response among patients with the *IL28B* favourable genotype could induce more frequent spontaneous clearance of HCV.

What are the new findings?

- In chronically HCV genotype 1b-infected chimeric mice that have the characteristic of immunodeficiency, no significant difference in the reduction in serum HCV-RNA levels and the induction of antiviral hepatic ISG by the administration of peg-IFN-α was observed between favourable and unfavourable human hepatocyte *IL28B* genotypes.
- By comparison of serum HCV kinetics between human and chimeric mice, the viral decline in both the first and second phases by peg-IFN-α treatment was affected by the variation in *IL28B* genotypes only in chronic hepatitis C patients.

How might it impact on clinical practice in the foreseeable future?

- The immune response according to *IL28B* genetic variants could contribute to the first and second phases of HCV-RNA decline and might be critical for HCV clearance by peg-IFN-α-based therapy.

INTRODUCTION

Hepatitis C is a global health problem that affects a significant portion of the world's population. The WHO estimated that, in 1999, 170 million hepatitis C virus (HCV)-infected patients were present worldwide, with 3–4 million new cases appearing per year.[1]

The standard therapy for hepatitis C still consists of pegylated interferon-α (peg-IFN-α), administered once weekly, plus daily oral ribavirin for 24–48 weeks in countries where protease inhibitors are not available.[2] This combination therapy is quite successful in patients with HCV genotype 2 or 3

infection, leading to a sustained virological response (SVR) in approximately 80–90% of patients treated; however, in patients infected with HCV genotype 1 or 4, only approximately half of all treated individuals achieved a SVR.[3 4]

Host factors were shown to be associated with the outcome of the therapy, including age, sex, race, liver fibrosis and obesity.[5] Genome-wide association studies have demonstrated that genetic variations in the region near the interleukin-28B (*IL28B*) gene, which encodes interferon (IFN)-λ3, are associated with a chronic HCV treatment response.[6–10] Furthermore, it was demonstrated that genetic variations in the *IL28B* gene region are also associated with spontaneous HCV clearance.[11–12]

Interestingly, a recent report showed the effect of genetic polymorphisms near the *IL28B* gene on the dynamics of HCV during peg-IFN-α plus ribavirin therapy in Caucasian, African American and Hispanic individuals;[13] HCV-infected patients with the *IL28B* homozygous favourable allele had a more rapid decline of HCV in the first phase, which is associated with the inhibition of viral replication as well as the second phase associated with immuno-destruction of viral-infected hepatocytes.[14] However, it is unknown how a direct effect by the *IL28B* genetic variation, such as the induction of IFN-stimulated genes (ISG) or cellular immune responses, would influence the viral kinetics during IFN treatment. Over recent periods, engineered severe combined immunodeficient (SCID) mice transgenic for urokinase-type plasminogen activator (uPA) received human hepatocyte transplants (hereafter referred to as chimeric mice)[15–17] and are suitable for experiments with hepatitis viruses in vivo.[18 19] We have also reported that these chimeric mice carrying human hepatocytes are a robust animal model to evaluate the efficacy of IFN and other anti-HCV agents.[20 21]

The purpose of this study was to reveal the association between genetic variations in the *IL28B* gene region and viral decline during peg-IFN-α treatment in patients with HCV, and to clarify the association between different *IL28B* alleles of human hepatocytes in chimeric mice and the response to peg-IFN-α without immune response. These studies will elucidate whether the immune response by the *IL28B* genetic variation affects the viral kinetics during peg-IFN-α treatment.

MATERIALS AND METHODS

Patients

Fifty-four Japanese patients with chronic HCV genotype 1 infection at Nagasaki Medical Center and Nagoya City University were enrolled in this study (table 1). Patients received peg-IFN-α2a (180 μg) or 2b (1.5 μg/kg) subcutaneously every week and were administered a weight-adjusted dose of ribavirin (600 mg for <60 kg, 800 mg for 60–80 kg, and 1000 mg for >80 kg daily), which is the recommended dosage in Japan. Patients with other hepatitis virus infection or HIV coinfection were not included in the study. The study protocol conformed to the ethics guidelines of the 1975 Declaration of Helsinki as reflected by earlier approval by the institutions' human research committees.

Table 1 Characteristics of 54 patients infected HCV genotype 1

	IL28B SNP rs8099917		
	TT (n=34)	TG (n=19) + GG (n=1)	p Value
Age (years)	55.6±10.1	54.7±11.3	0.746
Gender (male %)	70	50	0.199
Body mass index (kg/m^2)	24.6±3.1	24.7±3.3	0.870
Viral load at therapy (log IU/ml)	6.0±0.7	5.8±0.8	0.357
SVR rate (%)	50	11	0.012
Serum ALT level (IU/l)	100.3±80.8	79.3±45.0	0.226
Platelet count (×10^4/μl)	17.1±9.0	16.5±5.8	0.771
Fibrosis (F3+4 %)	42	40	0.877

HCV, hepatitis C virus; SNP, single nucleotide polymorphism; SVR, sustained virological response.

Laboratory tests

Blood samples were obtained before therapy, as well as on day 1 and at weeks 1, 2, 4, 8 and 12 after the start of therapy and were analysed for the HCV-RNA level by the commercial Abbott Real-Time HCV test with a lower limit of detection of 12 IU/ml (Abbott Molecular Inc., Des Plains, Illinois, USA). Genetic polymorphism in the *IL28B* gene (rs8099917), a single nucleotide polymorphism (SNP) recently identified to be associated with treatment response,[6–8] was tested by the TaqMan SNP genotyping assay (Applied Biosystems, Foster City, California, USA).

HCV infection of chimeric mice with the liver repopulated for human hepatocytes

SCID mice carrying the uPA transgene controlled by an albumin promoter were injected with 5.0–7.5×10^5 viable hepatocytes through a small left-flank incision into the inferior splenic pole, thereafter chimeric mice were generated. The chimeric mice were purchased from PhoenixBio Co, Ltd (Hiroshima, Japan).[17] Human hepatocytes with the *IL28B* homozygous favourable allele, heterozygous allele or homozygous unfavourable allele were imported from BD Biosciences (San Jose, California, USA) (table 2). Murine serum levels of human albumin and the body weight were not significantly different among four chimeric mice groups, providing a reliable comparison for anti-HCV agents.[22] Three different serum samples were obtained from three chronic HCV patients (genotype 1b).[21 22] Each mouse was intravenously infected with serum sample containing 10^5 copies of HCV genotype 1b. Administration of peg-IFN-α2a (Pegasys; Chugai Pharmaceutical Co., Ltd., Tokyo, Japan) at the dose formulation (30 μg/kg) was consecutively applied to each mouse on days 0, 3, 7 and 10 (table 3).

HCV-RNA quantification

HCV-RNA in mice sera (days 0, 1, 3, 7 and 14) was quantified by an in-house real-time detection PCR assay with a lower quantitative limit of detection of 10 copies/assay, as previously reported.[21]

Quantification of IFN-stimulated gene-expression levels

For analysis of endogenous ISG levels, total RNA was isolated from the liver using the RNeasy RNA extraction kit (Qiagen, Valencia, California, USA) and complementary DNA synthesis was performed using 2.0 μg of total RNA (High Capacity RNA-to-cDNA kit; Applied Biosystems). Fluorescence real-time PCR analysis was performed using an ABI 7500 instrument (Applied Biosystems) and TaqMan Fast Advanced gene expression assay (Applied Biosystems). TaqMan Gene Expression Assay primer and probe sets (Applied Biosystems) are shown in the supplementary information (available online only). Relative amounts of messenger RNA, determined using a FAM-Labeled TaqMan probe, were normalised to the endogenous RNA levels of the housekeeping reference gene, glyceraldehyde-3-phosphate dehydrogenase. The delta Ct method ($2^{-(\text{delta Ct})}$) was used for quantitation of relative mRNA levels and fold induction.[23 24]

Table 2 Four lines of uPA/SCID mice from four different lots of human hepatocytes (donor) containing various SNP around the *IL28B* gene

uPA/SCID mice	Donor	Race	Age	Gender	rs8103142	rs12979860	rs8099917
PXB mice	A	African American	5 Years	Male	CC	TT	TG
	B	Caucasian	10 Years	Female	CC	TT	TG
	C	Hispanic	2 Years	Female	TT	CC	TT
	D	Caucasian	2 Years	Male	TT	CC	TT

PXB mice; urokinase-type plasminogen activator/severe combined immunodeficiency (uPA/SCID) mice repopulated with approximately 80% human hepatocytes.
SCID, severe combined immunodeficient; SNP, single nucleotide polymorphism.

Statistical analyses

Statistical differences were evaluated by Fisher's exact test or the χ^2 test with the Yates correction. Mice serum HCV-RNA and intrahepatic ISG expression levels were compared using the Mann–Whitney U test. Differences were considered significant if p values were less than 0.05.

RESULTS

Characteristics of the study patients

Genotypes (rs8099917) TT, TG and GG were detected in 34, 19 and one patient infected with HCV genotype 1, respectively. SVR rates were significantly higher in HCV patients with genotype TT than in those with genotype TG/GG (50% vs 11%, p=0.012). The initial HCV serum load was comparable between genotypes TT and TG/GG (6.0±0.7 vs 5.8±0.8 log IU/ml). There were no significant differences in sex (male%, 70% vs 50%), age (55.6±10.1 vs 54.7±11.3 years), serum alanine aminotransferase level (100.3±80.8 vs 79.3±45.0 IU/L), platelet count (17.1±9.0 vs 16.5±5.8×10^4/μl) and fibrosis stages (F3/4%, 42% vs 40%) between HCV patients with the favourable (rs8099917 TT) and unfavourable (rs8099917 TG/GG) *IL28B* genotypes (table 1).

Changes in serum HCV-RNA levels in patients treated by peg-IFN-α plus ribavirin

Figure 1 shows the initial change in the serum HCV-RNA level for 14 days after peg-IFN-α plus ribavirin therapy in patients infected with HCV genotype 1 based on the genetic polymorphism near the *IL28B* gene. The immediate antiviral response (viral drop 24 h after the first IFN injection) was significantly higher in HCV patients with genotype TT than genotype TG/GG (−1.08 vs −0.39 log IU/ml, p<0.001). Figure 2 also shows the subsequent change in the serum HCV-RNA reduction after peg-IFN-α plus ribavirin therapy in patients infected with HCV genotype 1. Similarly, during peg-IFN-α plus ribavirin therapy, a statistically significant difference in the median reduction in serum HCV-RNA levels was noted according to the genotype (TT vs TG/GG). The median reduction in the serum HCV-RNA levels (log IU/ml) at 1, 2, 4, 8 and 12 weeks between genotypes TT and TG/GG was as follows: −1.58 vs −0.62, p<0.001; −2.35 vs −0.91, p<0.001; −3.48 vs −1.56, p<0.001; −4.53 vs −2.37, p<0.01; −4.93 vs −2.86, p<0.001. Furthermore, the initial first-phase viral decline slope per day (Ph1/day) and subsequent second-phase viral decline slope per week (Ph2/week) in TT genotype were significantly higher than in genotype TG/GG (Ph1/day 0.94±0.83 vs 0.38±0.40 log IU/ml, p<0.001; Ph2/week 0.08±0.06 vs 0.04±0.03 log IU/ml, p<0.001) (figure 3).

Changes in serum HCV-RNA levels in chimeric mice treated by peg-IFN-α

In order to clarify the association between *IL28B* alleles of human hepatocytes and the response to peg-IFN-α, we prepared four lines of uPA/SCID mice and four different lots of human hepatocytes containing various rs8099917, rs8103142 and rs12979860 SNPs around the *IL28B* gene (table 2). The chimeric mice were inoculated with serum samples from each HCV-1b patient, and then HCV-RNA levels had increased and reached more than 10^6 copies/ml in all chimeric mice sera at 2 weeks after inoculation. After confirming the peak of HCV-RNA in all chimeric mice, they were subcutaneously administered with four times injections of the bolus dose of peg-IFN-α2a for 2 weeks (table 3). Figure 4 shows the change in the serum HCV-RNA levels for 14 days during IFN injection into chimeric mice transplanted with *IL28B* favourable or unfavourable human hepatocyte genotypes. On peg-IFN-α administration, no significant difference in the median reduction in HCV-RNA levels in the serum A-infected[22] chimeric mice sera was observed between favourable (n=7) and unfavourable

Table 3 Dosage and time schedule of pegIFN-α2a* treatment for HCV genotype 1b infected chimeric mice

				Dose			
Donor hepatocytes†	No of chimeric mice	Inoculum	Test compound	Level (μg/kg)	Concentrtion (μg/ml)	Volume (ml/kg)	Frequency
A	3	Serum A	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
B	4	Serum A	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
C	3	Serum A	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
D	3	Serum A	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
A	2	Serum B	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
C	2	Serum B	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
A	2	Serum C	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
C	2	Serum C	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10

*Pegasys; Chugai Pharmaceutical Co., Ltd., Tokyo, Japan.
†The *IL28B* genetic variation of the donor hepatocytes was indicated in table 2.
HCV, hepatitis C virus; peg-IFN-α, pegylated interferon α.

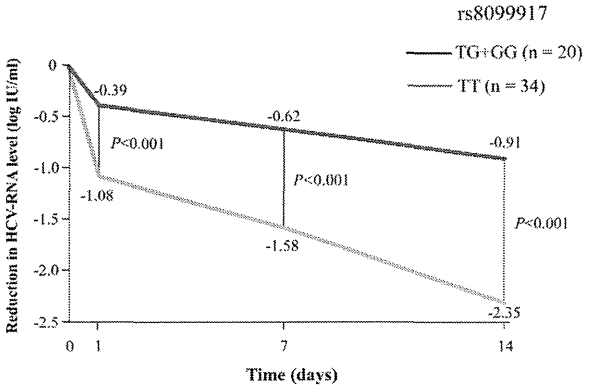

Figure 1 Rapid reduction of median hepatitis C virus (HCV)-RNA levels (log IU/ml) at 1, 7 and 14 days between *IL28B* single nucleotide polymorphisms rs8099917 genotype TT (n=34) and TG/GG (n=20) in HCV genotype 1-infected patients treated with peg-IFN-α plus ribavirin.

(n=6) *IL28B* genotypes on days 1, 3, 7 and 14 (−1.2 vs −1.3, −1.4 vs −1.4, −1.8 vs −1.7, and −2.3 vs −1.9 log copies/ml) (figure 4A). Moreover, we prepared two additional serum samples from the other HCV-1b patients (serum B and C)[21] to confirm the influence of *IL28B* genotype in early viral kinetics during IFN treatment. After establishing persistent infection with new HCV-1b strains in all chimeric mice, they were also administered four times injections of the bolus dose of peg-IFN-α2a for 2 weeks (figure 4B,C). In a similar fashion, no significant difference in HCV-RNA reduction in chimeric mice sera was observed between favourable and unfavourable *IL28B* genotypes.

Expression levels of ISG in chimeric mice livers

Because chimeric mice have the characteristic of severe combined immunodeficiency, the viral kinetics in chimeric mice sera during IFN treatment could be contributed by the innate immune response of HCV-infected human hepatocytes. Therefore, ISG expression levels in mice livers transplanted with human hepatocytes were compared between favourable and unfavourable *IL28B* genotypes (figure 5).

As shown in figure 5A, ISG expression levels in mice livers were measured at 8 h and 24 h after IFN treatment. The levels

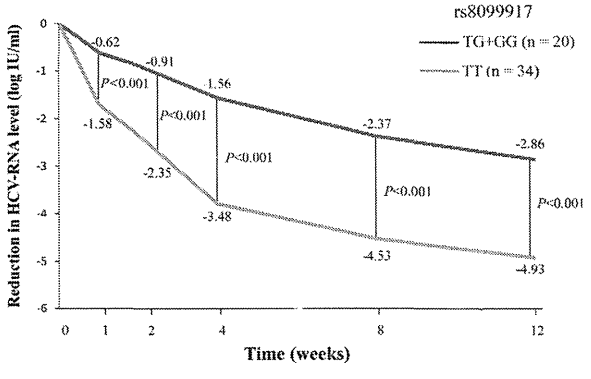

Figure 2 Weekly reduction of median hepatitis C virus (HCV)-RNA levels (log IU/ml) at 1, 2, 4, 8 and 12 weeks between *IL28B* single nucleotide polymorphisms rs8099917 genotype TT (n=34) and TG/GG (n=20) in HCV genotype 1-infected patients treated with pegylated interferon α plus ribavirin.

of representative antiviral ISG (eg, myxovirus resistance protein A, oligoadenylate synthetase 1, RNA-dependent protein kinase) and other ISG for promoting antiviral signalling (eg, Toll-like receptor 3, retinoic acid-inducible gene 1) were significantly induced at least 8 h after treatment, and prolonged at 24 h. No significant difference in ISG expression levels in HCV-infected livers was observed between favourable and unfavourable *IL28B* genotypes. The other inoculum for persistent infection of HCV-1b also demonstrated no significant difference in ISG expression levels between favourable and unfavourable *IL28B* genotypes (figure 5B,C). Interestingly, IFN-λ expression levels by treatment of peg-IFN-α were significantly induced in HCV-infected human hepatocytes harbouring the favourable *IL28B* genotype (figure 5 A–C).

DISCUSSION

Several recent studies have demonstrated a marked association between the chronic hepatitis C treatment response[6–9] and SNP (rs8099917, rs8103142 and rs12979860) near or within the region of the *IL28B* gene, which affected the viral dynamics during peg-IFN-α plus ribavirin therapy in Caucasian, African American and Hispanic individuals.[13]

It has been reported that when patients with chronic hepatitis C are treated by IFN-α or peg-IFN-α plus ribavirin, HCV-RNA generally declines after a 7–10 h delay.[25] The typical decline is biphasic and consists of a rapid first phase lasting for approximately 1–2 days during which HCV-RNA may fall 1–2 logs in patients infected with genotype 1, and subsequently a slower second phase of HCV-RNA decline.[26] The viral kinetics had a predictive value in evaluating antiviral efficacy.[14] In this study, biphasic decline of the HCV-RNA level during peg-IFN-α treatment was observed in both patients and chimeric mice infected with HCV genotype 1; however, in the first and second phases of viral kinetics, a difference between *IL28B* genotypes was observed only in HCV-infected patients; a more rapid decline in serum HCV-RNA levels after administering peg-IFN-α plus ribavirin was confirmed in patients with the TT genotype of rs8099917 compared to those with the TG/GG genotype.

On the other hand, in-vivo data using the chimeric mouse model showed no significant difference in the reduction of HCV-RNA titers in mouse serum among four different lots of human hepatocytes containing *IL28B* favourable (rs8099917 TT) or unfavourable (rs8099917 TG) genotypes, which was confirmed by the inoculation of two additional HCV strains. These results indicated that variants of the *IL28B* gene in donor hepatocytes had no influence on the response to peg-IFN-α under immunosuppressive conditions, suggesting that the immune response according to *IL28B* genetic variants could contribute to the first and second phases of HCV-RNA decline and might be critical for HCV clearance by peg-IFN-α-based therapy.

Two recent studies indeed revealed an association between the *IL28B* genotype and the expression level of hepatic ISG in human studies.[27 28] Quiescent hepatic ISG before treatment among patients with the *IL28B* favourable genotype have been associated with sensitivity to exogenous IFN treatment and viral eradication; however, it is difficult to establish whether the hepatic ISG expression level contributes to viral clearance independently or appears as a direct consequence of the *IL28B* genotype. Another recent study addressed this question and the results suggested that there is no absolute correlation with the *IL28B* genotype and hepatic expression of ISG.[29] Our results on the hepatic ISG expression level in immunodeficient chimeric mice also suggested that no significant difference in ISG expression levels was observed between favourable and unfavourable

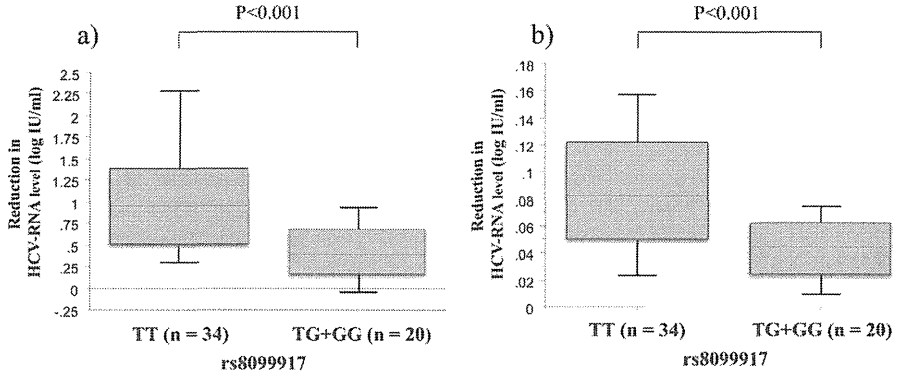

Figure 3 (A) The first-phase viral decline slope per day (Ph1/day) and (B) second-phase viral decline slope per week (Ph2/week) in hepatitis C virus (HCV) genotype 1-infected patients treated with pegylated interferon α plus ribavirin. The lines across the boxes indicate the median values. The hash marks above and below the boxes indicate the 90th and 10th percentiles for each group, respectively.

IL28B genotypes. However, these results were not consistent with a previous report using chimeric mice that the favourable *IL28B* genotype was associated with an early reduction in HCV-RNA by ISG induction.[30] The reasons for the discrepancy might depend on the dose and type of IFN treatment, as well as the time point when ISG expression was examined in the liver. In addition, although IFN-λ transcript levels measured in peripheral blood mononuclear cells or liver revealed inconsistent results in the context of an association with the *IL28B* genotype,[7 8] our preliminary assay on the IL28A, IL28B and IL29 transcripts in the liver first indicated that the induction of IFN-λ on peg-IFN-α administration could be associated with the *IL28B* genotype. Therefore, the induction of IFN-λ followed by immune response might contribute to different viral kinetics and treatment outcomes in HCV-infected patients, because no difference was found in chimeric mice without immune response.

It has also been reported that the mechanism of the association of genetic variations in the *IL28B* gene and spontaneous clearance of HCV may be related to the host innate immune response.[11] Interestingly, participants with seroconversion illness with jaundice were more frequently rs8099917 homozygous favourable allele (TT) than other genotypes (32% vs 5%, p=0.047). This suggests that a stronger immune response during the acute phase of HCV infection among patients with the *IL28B* favourable genotype would induce more frequent spontaneous clearance of HCV.

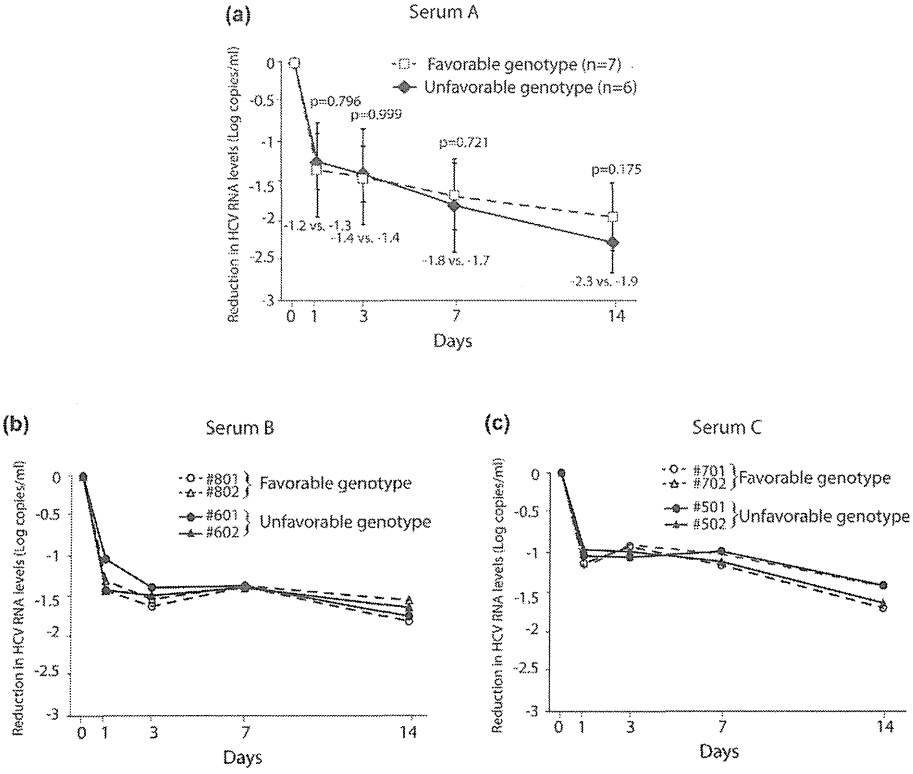

Figure 4 Median reduction of hepatitis C virus (HCV)-RNA levels (log copies/ml) after administering pegylated interferon α to chimeric mice having human hepatocytes containing various single nucleotide polymorphisms around the *IL28B* gene as favourable (rs8099917 TT) and unfavourable (rs8099917 TG) genotypes. Data are represented as mean+SD. Chimeric mice infected with a) serum A (n=7; favourable genotype, n=6; unfavourable genotype), (B) serum B (n=2, each genotype), and (C) serum C (n=2, each genotype). All serum samples were obtained from HCV-1b patients.

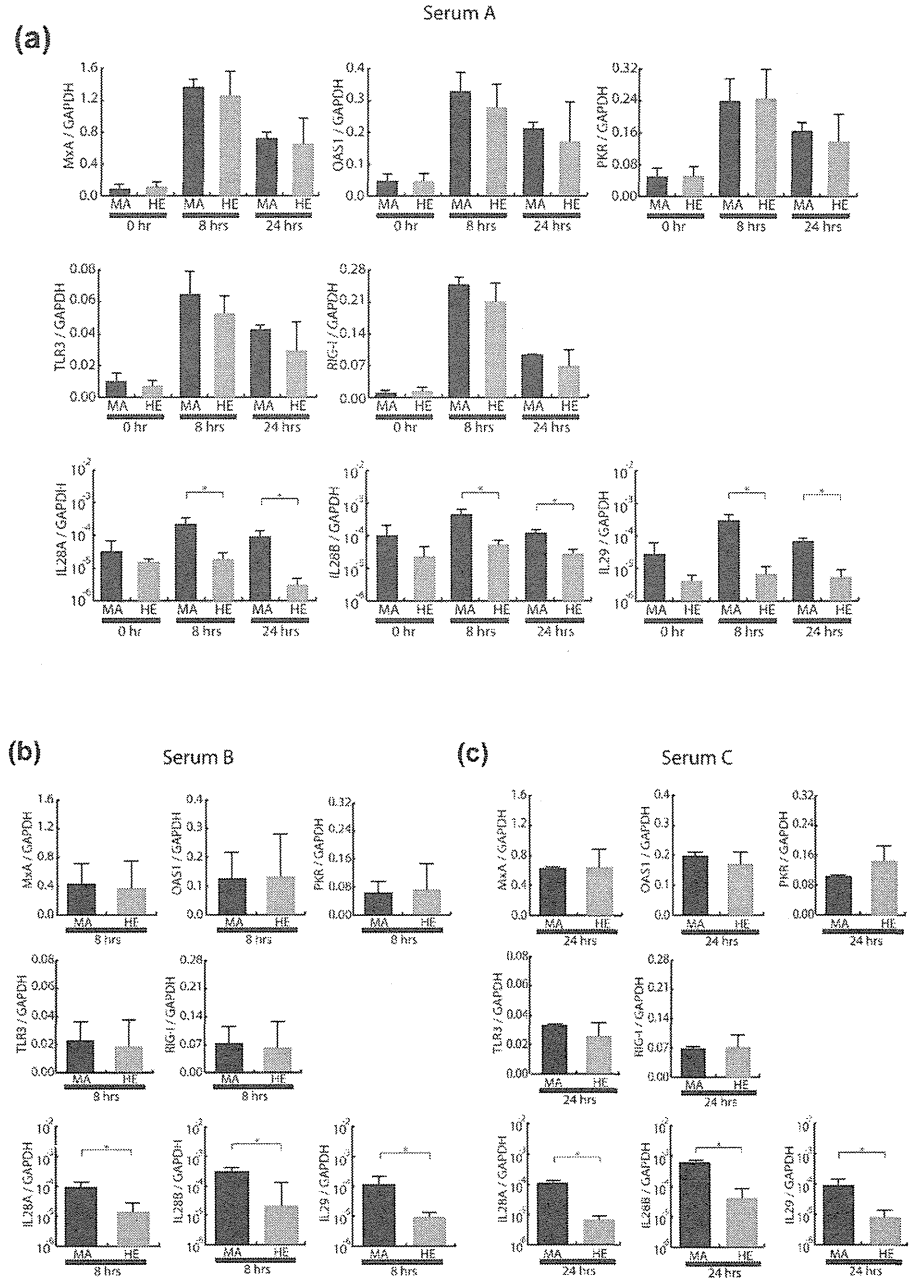

Figure 5 Intrahepatic interferon (IFN)-stimulated gene (ISG) expression levels in the pegylated interferon α (peg-IFN-α)-treated chimeric mice having human hepatocytes containing homozygous favourable allele (rs8099917 TT; MA) and heterozygous unfavourable allele (rs8099917 TG; HE) were measured and expressed relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) messenger RNA. Data are represented as mean +SD. (A) Time kinetics of ISG after administration of the peg-IFN-α in serum A-infected chimeric mice (n=3, each genotype). Comparison of ISG expression levels at (B) 8 h in serum B-infected mice and (C) 24 h in serum C-infected mice after administering peg-IFN-α (n=3, each genotype). Predesigned real-time PCR assay of IL28B transcript purchased from Applied Biosystems can be cross-reactive to IL28A transcript. *p<0.05. MxA, myxovirus resistance protein A; OAS1, oligoadenylate synthetase 1; PKR, RNA-dependent protein kinase; RIG-1, retinoic acid-inducible gene 1; TLR3, Toll-like receptor 3.

Taking into account both the above results in acute HCV infection and our results conducted on chimeric mice that have the characteristic of immunodeficiency, it is suggested that the response to peg-IFN-α associated with the variation in *IL28B* alleles in chronic hepatitis C patients would be composed of the intact immune system.

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Contributors YT and MM conceived the study. TW and FS and YT conducted the study equally. TW and FS coordinated the analysis and manuscript preparation. All the authors had input into the study design, patient recruitment and management or mouse management and critical revision of the manuscript for intellectual content. TW, FS and YT contributed equally.

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Competing interests None.

Patient consent Obtained.

Ethics approval This study was conducted with the approval of each ethics committee at the Nagoya City University and Nagasaki Medical Center (see supplementary information, available online only).

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REFERENCES

1 Ray Kim W. Global epidemiology and burden of hepatitis C. *Microbes Infect* 2002;4:1219–25.

2 Foster GR. Past, present, and future hepatitis C treatments. *Semin Liver Dis* 2004;24(Suppl. 2):97–104.
3 Fried MW, Shiffman ML, Reddy KR, *et al*. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002;347:975–82.
4 Manns MP, McHutchison JG, Gordon SC, *et al*. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 2001;358:958–65.
5 Mihm U, Herrmann E, Sarrazin C, *et al*. Review article: predicting response in hepatitis C virus therapy. *Aliment Pharmacol Ther* 2006;23:1043–54.
6 Ge D, Fellay J, Thompson AJ, *et al*. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. *Nature* 2009;461:399–401.
7 Suppiah V, Moldovan M, Ahlenstiel G, *et al*. IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. *Nat Genet* 2009;41:1100–4.
8 Tanaka Y, Nishida N, Sugiyama M,, *et al* Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. *Nat Genet* 2009;41:1105–9.
9 Rauch A, Kutalik Z, Descombes P, *et al*. Genetic variation in IL28B is associated with chronic hepatitis C and treatment failure: a genome-wide association study. *Gastroenterology* 2010;138:1338–45.
10 Tanaka Y, Nishida N, Sugiyama M, *et al*. lambda-Interferons and the single nucleotide polymorphisms: a milestone to tailor-made therapy for chronic hepatitis C. *Hepatol Res* 2010;40:449–60.
11 Thomas DL, Thio CL, Martin MP, *et al*. Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. *Nature* 2009;461:798–801.
12 Grebely J, Petoumenos K, Hellard M, *et al*. Potential role for interleukin-28B genotype in treatment decision-making in recent hepatitis C virus infection. *Hepatology* 2010;52:1216–24.
13 Thompson AJ, Muir AJ, Sulkowski MS, *et al*. Interleukin-28B polymorphism improves viral kinetics and is the strongest pretreatment predictor of sustained virologic response in genotype 1 hepatitis C virus. *Gastroenterology* 2010;139:120–29.
14 Layden-Almer JE, Layden TJ. Viral kinetics in hepatitis C virus: special patient populations. *Semin Liver Dis* 2003;23(Suppl. 1):29–33.
15 Heckel JL, Sandgren EP, Degen JL, *et al*. Neonatal bleeding in transgenic mice expressing urokinase-type plasminogen activator. *Cell* 1990;62:447–56.
16 Rhim JA, Sandgren EP, Degen JL, *et al*. Replacement of diseased mouse liver by hepatic cell transplantation. *Science* 1994;263:1149–52.
17 Tateno C, Yoshizane Y, Saito N, *et al*. Near completely humanized liver in mice shows human-type metabolic responses to drugs. *Am J Pathol* 2004;165:901–12.
18 Mercer DF, Schiller DE, Elliott JF, *et al*. Hepatitis C virus replication in mice with chimeric human livers. *Nat Med* 2001;7:927–33.
19 Tsuge M, Hiraga N, Takaishi H, *et al*. Infection of human hepatocyte chimeric mouse with genetically engineered hepatitis B virus. *Hepatology* 2005;42:1046–54.
20 Kurbanov F, Tanaka Y, Chub E, *et al*. Molecular epidemiology and interferon susceptibility of the natural recombinant hepatitis C virus strain RF1_2k/1b. *J Infect Dis* 2008;198:1448–56.
21 Kurbanov F, Tanaka Y, Matsuura K, *et al*. Positive selection of core 70Q variant genotype 1b hepatitis C virus strains induced by pegylated interferon and ribavirin. *J Infect Dis* 2010;201:1663–71.
22 Inoue K, Umehara T, Ruegg UT, *et al*. Evaluation of a cyclophilin inhibitor in hepatitis C virus-infected chimeric mice in vivo. *Hepatology* 2007;45:921–8.
23 Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 2001;25:402–8.
24 Silver N, Best S, Jiang J, *et al*. Selection of housekeeping genes for gene expression studies in human reticulocytes using real-time PCR. *BMC Mol Biol* 2006;7:33.
25 Dahari H, Layden-Almer JE, Perelson AS, *et al*. Hepatitis C viral kinetics in special populations. *Curr Hepat Rep* 2008;7:97–105.
26 Neumann AU, Lam NP, Dahari H, *et al*. Hepatitis C viral dynamics in vivo and the antiviral efficacy of interferon-alpha therapy. *Science* 1998;282:103–7.
27 Honda M, Sakai A, Yamashita T, *et al*. Hepatic ISG expression is associated with genetic variation in interleukin 28B and the outcome of IFN therapy for chronic hepatitis C. *Gastroenterology* 2010;139:499–509.
28 Urban TJ, Thompson AJ, Bradrick SS, *et al*. IL28B genotype is associated with differential expression of intrahepatic interferon-stimulated genes in patients with chronic hepatitis C. *Hepatology* 2010;52:1888–96.
29 Dill MT, Duong FH, Vogt JE, *et al*. Interferon-induced gene expression is a stronger predictor of treatment response than IL28B genotype in patients with hepatitis C. *Gastroenterology* 2011;140:1021–31.
30 Hiraga N, Abe H, Imamura M, *et al*. Impact of viral amino acid substitutions and host interleukin-28b polymorphism on replication and susceptibility to interferon of hepatitis C virus. *Hepatology* 2011;54:764–71.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Kinetics of peripheral hepatitis B virus-specific CD8$^+$ T cells in patients with onset of viral reactivation

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Abstract

Background Patients with resolved hepatitis B virus (HBV) infection undergoing chemotherapy or immunosuppressive therapy are potentially at risk of HBV reactivation. However, it remains unclear how liver disease develops after HBV reactivation. To compare the host immune response against HBV, we performed immunological analyses of six HBV reactivation patients.

Methods The numbers of peripheral HBV-specific CD8$^+$ T cells were investigated longitudinally in six HLA-A2- and/or A24-positive patients with HBV reactivation. In addition, 34 patients with resolved HBV, 17 patients with inactive chronic hepatitis B (ICHB), 17 patients with chronic hepatitis B (CHB) and 12 healthy controls were analyzed. The number and function of HBV-specific CD8$^+$ T cells were assessed by flow cytometry using tetramer staining and intracellular IFN-γ production. Furthermore, the numbers of CD4$^+$CD25$^+$ or CD4$^+$Foxp3$^+$ T cells and serum inflammatory cytokine levels were analyzed.

Results The frequency of HBV-specific CD8$^+$ T cells was significantly increased in HBV reactivation patients compared with ICHB and CHB patients. In addition, the number of HBV-specific CD8$^+$ T cells was increased in resolved HBV patients compared with ICHB patients. PD-1 expression was decreased in HBV reactivation patients compared with ICHB and CHB patients. The numbers of HBV-specific CD8$^+$ T cells and CD4$^+$CD25$^+$ or CD4$^+$Foxp3$^+$ T cells were negatively correlated following onset of HBV reactivation.

Conclusions During HBV reactivation, the frequency of HBV-specific CD8$^+$ T cells increased even though the administration of immunosuppressive drugs and interactions with CD4$^+$ regulatory T cells may be important for the onset of liver disease.

Keywords Hepatitis B virus reactivation · Cytotoxic T lymphocyte · Regulatory T cell · PD-1

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Abbreviations

HBV	Hepatitis B virus
CTL	Cytotoxic T lymphocyte
sALT	Serum alanine aminotransferase
TNF	Tumor necrosis factor
IFN	Interferon
MIP	Macrophage inflammatory protein
MCP	Monocyte chemotactic protein

Introduction

More than 300 million people worldwide suffer from persistent hepatitis B virus (HBV) infection, making the virus a common cause of morbidity and mortality [1, 2]. Each year, an estimated one million people die of complications associated with chronic HBV infection, including cirrhosis, end-stage liver disease and hepatocellular carcinoma [3, 4]. Viral reactivation in hepatitis B surface antigen (HBsAg) carriers undergoing immunosuppressive therapy is well documented, as some immunosuppressive therapies can enhance HBV replication in hepatocytes at the same time as they curb host immune responses, resulting in detectable viremia followed by clinical hepatitis [1]. In general, although the development of surface and core antibodies and loss of surface antigen following HBV infection are thought to represent clearance of the virus, evidence exists to support the possibility that the virus may remain latent within the liver [5, 6]. The course and outcome of HBV infection are modulated by the host immune response [7, 8], and the loss of immune surveillance can cause reactivation of viral replication and exacerbation of disease activity. HBV reactivation is a well-characterized syndrome marked by the abrupt reappearance or elevation of HBV DNA in the serum of a patient with previously inactive or resolved HBV infection [1, 9]. Although the mechanisms of reactivation and associated liver damage remain unclear, they may include a rebound increase in the lymphocyte number following cessation of immunosuppressive and myelosuppressive chemotherapy, leading to a rapid destruction of infected hepatocytes with subsequent severe hepatitis. As one of the viral factors, it has been reported that the fulminant outcome of HBV reactivation can be associated with genotype Bj, which exhibits high replication owing to the A1896 mutation [10]. Therefore, although there are increasing reports regarding viral factors for the mechanism of HBV reactivation, it is still premature to conclude how the host immune response affects the liver injury and viral load.

It has been demonstrated that chronic persistent HBV infection is manifested by cytotoxic T lymphocytes (CTLs) that are functionally impaired or exhausted [7]. Recent reports have indicated that PD-1 is markedly upregulated on the surface of exhausted virus-specific $CD8^+$ T cells in mice with lymphocytic choriomeningitis virus infection [11] and humans with human immunodeficiency virus (HIV) infection [12, 13] or hepatitis C virus (HCV) infection [14]. Based on these observations, we evaluated the hypothesis that HBV-specific $CD8^+$ T cells can restore their function during HBV reactivation, by analyzing PD-1 expression on HBV-specific $CD8^+$ T cells. In a previous report, we demonstrated that fulminant hepatitis in HBV patients was responsible for high amounts of interferon (IFN)-γ production by $CD8^+$ T cells from peripheral blood mononuclear cells (PBMCs) [15]. Thus, HBV-specific $CD8^+$ T cells may be responsible for the liver disease at the onset of HBV reactivation, although a causal relationship among these events has not been defined.

In this study, we found that the frequency of HBV-specific $CD8^+$ T cells was increased and the interactions between these $CD8^+$ T cells and $CD4^+$ regulatory T cells (Tregs) showed a negative correlation at the onset of HBV reactivation.

Patients and methods

Patients

Six patients with HBV reactivation from resolved HBV infection before chemotherapy or immunosuppressive therapy, 34 patients with resolved HBV, 17 patients with inactive chronic hepatitis B (ICHB), 17 patients with chronic hepatitis B (CHB) and 12 healthy controls gave informed consent to participate in the study. The study was performed at Tokyo Metropolitan Komagome Hospital after receiving institutional review board approval (approved ID number: 714). We defined resolved HBV infection as serum HBsAg (−), anti-HBcAb (+) and/or anti-HBsAb (+), ICHB as serum HBsAg (+), HBV DNA (+) and normal serum alanine aminotransferase (sALT; 5–40 IU/mL), and CHB as serum HBsAg (+), HBV DNA (+) and continuous serum ALT elevation (>2 × normal level). The study protocol and procedures were conducted in accordance with the ethical guidelines of the Declaration of Helsinki. The patient characteristics are summarized in Table 1. The healthy controls comprised six males and six females, who ranged in age from 32 to 63 years. There were no significant differences in age between the HBV reactivation patients and the other groups. The patients were human leukocyte antigen (HLA)-A2- or A24-positive and negative for HCV and HIV-1/2.

Sample preparation

PBMCs were isolated from whole blood using Lymphoprep™ (Axis-Shield, Oslo, Norway). The isolated cells were washed twice in phosphate-buffered saline (Gibco, Auckland, NZ) and used immediately. The cells were cultured in RPMI 1640 medium supplemented with 10 % heat-inactivated fetal calf serum, 100 U/mL penicillin, 100 mg/mL streptomycin, 50 μg/mL gentamicin and 2 mM L-glutamine (all from Invitrogen, Carlsbad, CA) at 37 °C in a humidified 5 % CO_2 incubator, as previously described [15]. Plasma preparation tubes (BD Biosciences, San Jose, CA) were used to isolate plasma from whole

Table 1 Characteristics of the respective patient groups

	Number	Sex (M/F)	Age	ALT (U/L)	HBV DNA (log/mL)
Healthy volunteer	12	6/6	57 ± 11	25 ± 11	–
Resolved HBV	34	18/16	59 ± 15	33 ± 20	–
ICHB	17	8/9	55 ± 14	33 ± 14	3.9 ± 1.8
CHB	17	8/9	53 ± 11	75 ± 19	5.5 ± 1.9
Reactivation	6	3/3	66 ± 12	82 ± 66	5.8 ± 3.3

Results are shown as mean ± SD

ALT alanine transaminase, *ICHB* inactive chronic hepatitis B, *CHB* chronic hepatitis B, *HBV* hepatitis B virus, *M* male, *F* female

blood. The plasma samples were frozen and subsequently thawed for viral load and genotype testing.

Synthetic peptides

Three HBV peptides; HLA-A*0201 core 18–27 (FLPSDFFPSV), envelope 183–191 (FLLTRILTI), polymerase 575–583 (FLLSLGIHL), HLA-A*2402 core 117–125 (EYLVSFGVW), polymerase 756–764 (KYTSFPWLL) and were synthesized by Sigma Aldrich (Hokkaido, Japan).

Major histocompatibility complex (MHC) class I tetramer staining

Patients expressing HLA-A2 or A24 were assessed for antigen-specific responses to HBV by tetramer staining. For the staining, phycoerythrin (PE)-conjugated HLA-A*0201-restricted HBV core (FLPSDFFPSV) and HLA-A*2402 HBV core (EYLVSFGVW) HBV polymerase (KYTSFPWLL) were purchased from MBL (Nagoya, Japan).

FACS analysis

HLA typing was performed by staining PBMCs with fluorescein isothiocyanate (FITC)-conjugated anti-HLA-A2 and PE-conjugated anti-HLA-A24 antibodies (MBL) according to the manufacturer's instructions. PBMCs were surface-stained on ice for 20 min with the following monoclonal antibodies: FITC-conjugated anti-human CD62L, anti-PD-1 and anti-CD25 (BD Biosciences); PE-conjugated anti-human Foxp3 (eBioscience, San Diego, CA); and PE-Cy5-conjugated anti-human CD4 and anti-human CD8 (BD Biosciences). For intracellular staining, isolated PBMCs were incubated with peptides (10 μl/mL) in the presence of human recombinant IL-2 (50 U/mL) and 1 μl/mL of BD GolgiPlug protein transport inhibitor (BD Bioscience) for 4 h. After incubation (37 °C, 5 % CO_2), cells from each well were stained with PE-Cy5-conjugated anti-human CD8 (BD Biosciences). Prior to staining with intracellular antibodies against PE-conjugated anti-human IFN-γ, cells were fixed and permeabilized by adding Cytofix-Cytoperm (BD Pharmingen). Cells were acquired by FACS scan (BD Biosciences), and the data was analyzed using FlowJo software (Tree Star, Ashland, OR).

Cytokine and chemokine profiles

Bio-Plex Cytokine Assay Kits (Bio-Rad Laboratories, Hercules, CA) were used to measure the amounts of cytokines and chemokines in sera in accordance with the manufacturer's instructions. Specifically, we used the Bio-Plex Human Cytokine 17-Plex Panel. The resulting samples were analyzed in a 96-well plate reader using a Bio-Plex Suspension Array System and Bio-Plex Manager software (all from Bio-Rad Laboratories).

Serum HBV assay

The serum HBV DNA concentrations were quantified using the COBAS AmpliPrep/COBAS TaqMan HBV Test (Roche Diagnostics, Basel, Switzerland). The four major HBV genotypes (A–D) were determined by enzyme-linked immunosorbent assay with monoclonal antibodies directed against distinct epitopes on the preS2-region products using commercial kits (HBV GENOTYPE EIA; Institute of Immunology Co. Ltd., Tokyo, Japan). HBV DNA sequences bearing the core promoter and precore or core regions were amplified by PCR with hemi-nested primers. The PCR products were directly sequenced by the di-deoxy-chain termination method using a Big Dye Terminator Kit and an ABI PRISM 3100-Avant Analyzer (both from Applied Biosystems, Foster City, CA).

Statistical analysis

Data are shown as mean ± SD. The data were analyzed by the nonparametric Mann–Whitney or Kruskal–Wallis tests or ANOVA using Prism 5 for Macintosh software (GraphPad, San Diego, CA). Values of $P < 0.05$ were considered to indicate statistical significance.

Table 2 Characteristics of HBV reactivation patients

Patient	Sex	Disease	HBsAg	HBsAb	HBcAb	Chemotherpy/ Immunosuppressant	HBV genotype	HBVcore/precore mutation
#1	Female	ALL, BMT	−	+	+	PSL, FK	C	−
#2	Male	AML, BMT	−	+	+	PSL, CsA	C	−
#3	Male	Eso.ca	−	+	+	5FU + CDDP	C	−
#4	Male	B cell lymphoma	−	+	+	R-CHOP	B	−
#5	Male	B cell lymphoma	−	+	+	CHOP	B	−
#6	Female	T-LBL, BMT	−	+	+	Anthracycline + AraC	B	−

ALL acute lymphoblastic leukemia, *BMT* bone marrow transplantation, *PSL* prednisolone, *FK* tacrolimus, *CsA* ciclosporin A, *CDDP* cisplatin, *5FU* 5-fluorouracil, *R-CHOP* rituximab, cyclophosphamide, doxorubicin hydrochloride (hydroxydaunorubicin), vincristine (oncovin), and prednisone, *AraC* cytarabine

Results

HBV reactivation patient profiles

We identified six patients with HBV reactivation (Table 2). Patients with malignant lymphoma and bone marrow transplantation for leukemia were observed, and treatment with rituximab, which is a well-known inducer of HBV reactivation, was encountered in the case of one patient (patient #4). There was no sex difference regarding the incidence of HBV reactivation. All patients were negative for HBsAg and positive for anti-HBc and anti-HBs antibodies as HBV-related markers. In addition, three patients were HBV genotype C and one patient was genotype B. No core and precore promoter mutations were detected in the patients. All patients received entecavir (0.5 mg/day) when serum HBV DNA was initially detected or sALT elevation was observed.

Serum inflammatory cytokine and chemokine levels

To determine whether the serum cytokine and chemokine levels correlated with the development of HBV reactivation, we examined the concentrations of various cytokines and chemokines. We measured serum cytokine/chemokine levels at the time of diagnosis of HBV reactivation. In the groups, serum was isolated prior to chemotherapy or antiviral therapy.

The data for the serum cytokine and chemokine levels are shown in Fig. 1. Serum IL-1β was significantly higher in resolved HBV patients (median, 0.32 pg/mL; range 0.10–0.75 pg/mL) than in healthy controls (median, 0.17 pg/mL; range 0.04–0.43 pg/mL) or ICHB patients (median, 0.17 pg/mL; range 0.09–0.24 pg/mL). Serum IL-7 was elevated in resolved HBV patients (median, 6.4 pg/mL; range 0.54–28.12 pg/mL) compared with ICHB patients (median, 3.2 pg/mL; range 0.54–12.6 pg/mL) or CHB patients (median, 3.13 pg/mL; range 0.29–5.16 pg/mL). There were no significant differences in serum IL-6 among resolved HBV, ICHB, CHB and HBV reactivation patients. Serum IL-8 and MCP-1 were significantly increased in resolved HBV patients (IL-8: median, 12.5 pg/mL; range 5.15–34.2 pg/mL; MCP-1: median, 75.2 pg/mL; range 0.7–300.23 pg/mL) compared with healthy controls (IL-8: median, 5.24 pg/mL; range not detected–17.46 pg/mL; MCP-1: median, 23.3 pg/mL; range not detected–48.51 pg/mL) and CHB patients (IL-8: median, 7.25 pg/mL; range 1.13–23.84 pg/mL; MCP-1: median, 21.8 pg/mL; range 3.49–58.66 pg/mL). Although circulating IFN-γ was detected at very low levels in all samples, ICHB patients exhibited significant suppression of serum IFN-γ. Regarding HBV reactivation, serum G-CSF was slightly increased compared with healthy controls.

Comparison of HBV-specific $CD8^+$ T cell frequencies

The development of hepatitis with HBV infection is mediated by antigen-specific CTLs [7]. To compare the frequencies and phenotypes of HBV-specific $CD8^+$ T cells from PBMCs, 16 HLA-A2- and 19 HLA-A24-positive resolved HBV patients, 9 HLA-A2- and 11 HLA-A24-positive ICHB patients, 11 HLA-A2- and 13 HLA-A24-positive CHB patients, and four HLA-A2- and four HLA-A24-positive HBV reactivation patients were examined using a panel of three MHC class I tetramers containing frequently detected HBV epitopes (A2 core, amino acids 18–27; A24 core, amino acids 117–125; and A24 polymerase, amino acids 756–764) (Fig. 2a). The frequency of peripheral HLA-A2 core-specific $CD8^+$ T cells was significantly higher in HBV reactivation patients than in ICHB and CHB patients (Fig. 2b, upper panels). Interestingly, the frequencies of HLA-A2 core-specific $CD8^+$ cells were also significantly higher in resolved HBV patients than in ICHB patients, indicating that HBV-specific $CD8^+$ T cells are circulating even though serum HBV DNA was not detected. Consistent with these data,

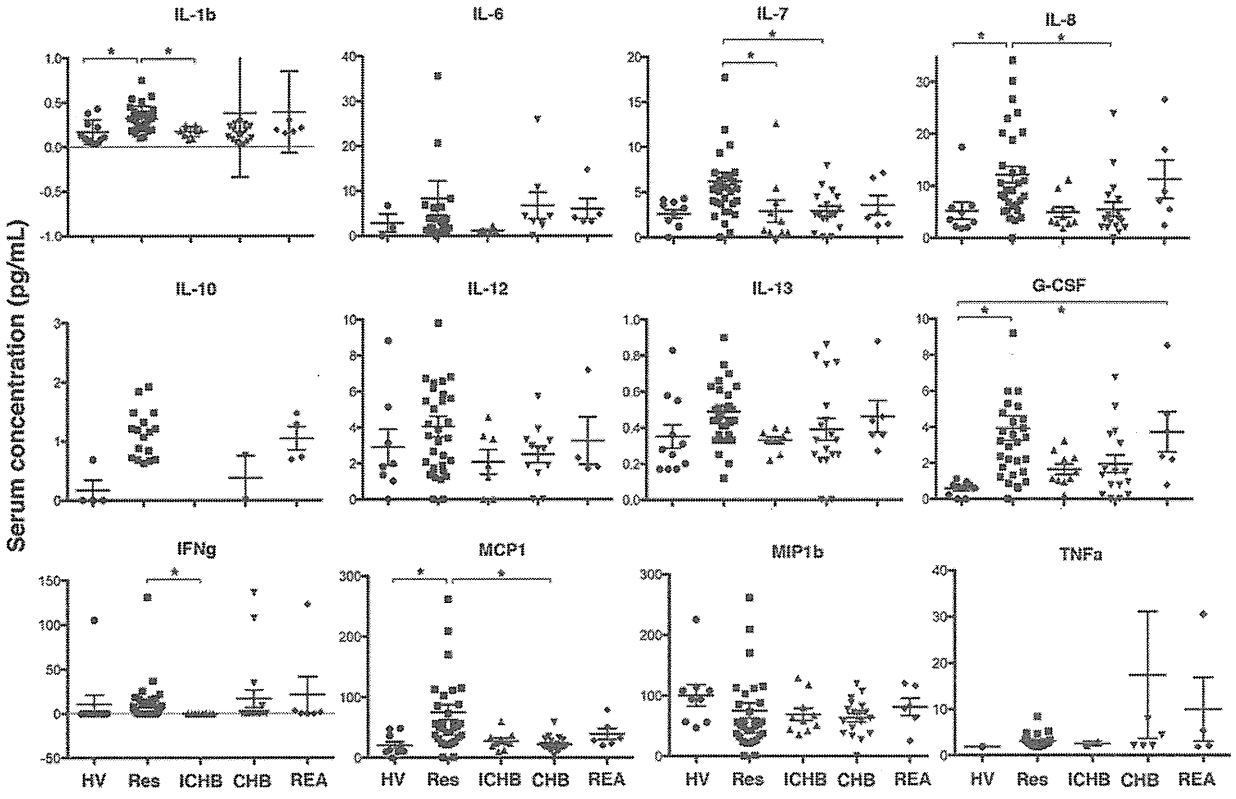


Fig. 1 Serum concentrations of cytokines and chemokines. Serum cytokines and chemokines were measured at the time of diagnosis in HBV reactivation patients and prior to chemotherapy or antiviral therapy in the other groups. The serum concentrations were compared among healthy controls, resolved HBV patients, ICHB patients, CHB patients and HBV reactivation patients. $*P < 0.05$, significant difference between the linked items

the frequencies of HLA-A24 core- and polymerase-specific $CD8^+$ T cells were significantly higher in HBV reactivation patients than in ICHB and CHB patients.

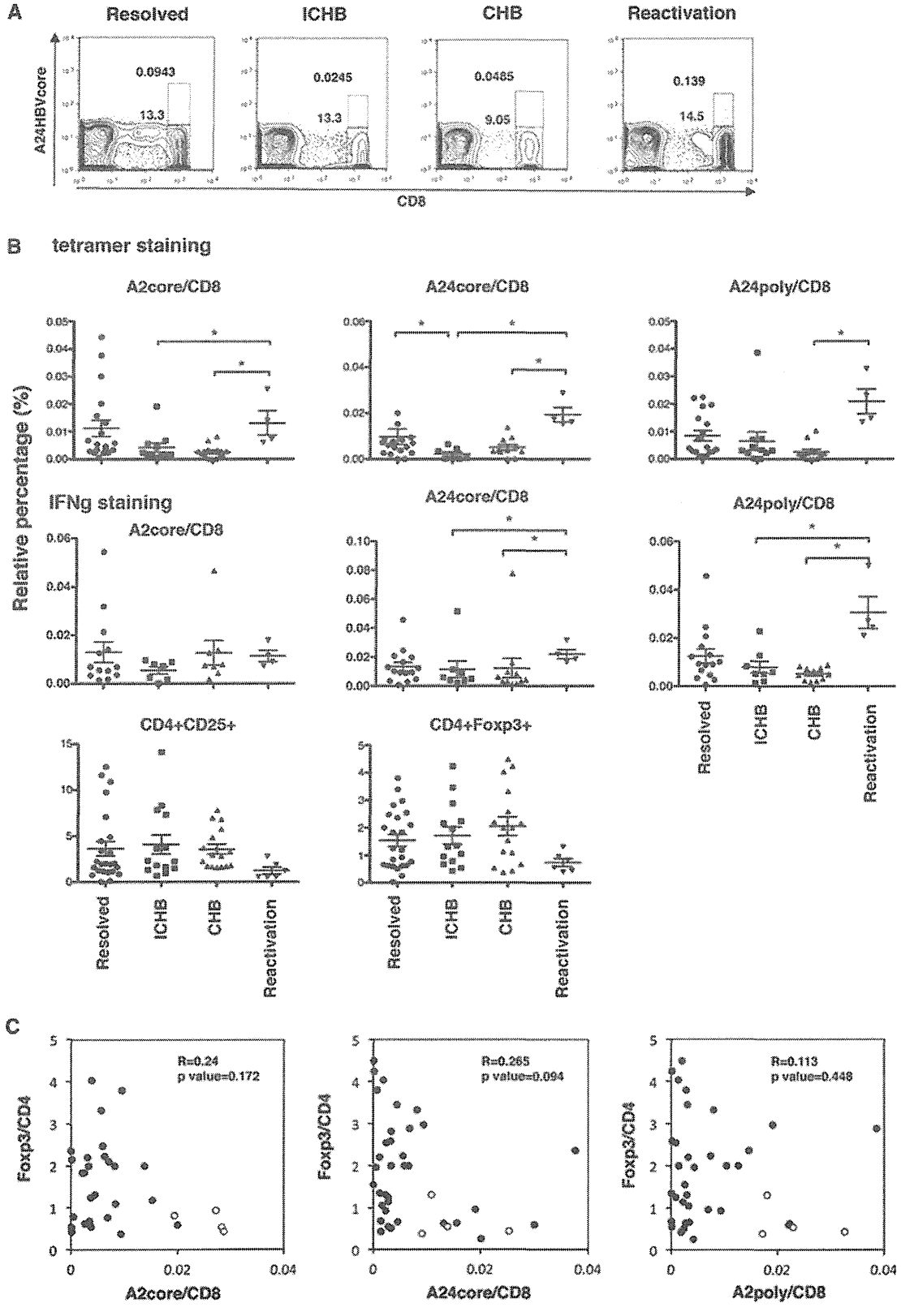
In addition, to evaluate the functional profile of HBV-specific $CD8^+$ T cells we analyzed intracellular IFN-γ production following stimulation with five peptides. Although we did not observe a significant difference in the numbers of IFN-γ producing cells in HBV reactivation patients by HLA-A2 core peptide stimulation, similar results were obtained by tetramer staining following HLA-A24 core and polymerase stimulation (Fig. 2b). We also observed that the number of IFN-γ producing cells in HBV reactivation patients increased by HLA-A2 envelope peptide stimulation (supplementary Fig. 1). These results demonstrated that the frequency of functional HBV-specific $CD8^+$ T cells increased in HBV reactivation patients compared with ICHB and CHB patients.

It was demonstrated that the frequency of circulating $CD4^+CD25^+$ Tregs significantly correlated with the serum viral load in severe CHB patients [16]. To determine whether $CD4^+CD25^+$ and $CD4^+Foxp3^+$ Tregs contribute to liver injury during HBV reactivation, we monitored their numbers in PBMCs. The numbers of $CD4^+CD25^+$ and $CD4^+Foxp3^+$ Tregs had a tendency to be low in HBV reactivation patients compared with the other groups, although the differences were not significant (Fig. 2b, lower panels).

To confirm the inverse correlation between the percentages of HBV-specific $CD8^+$ T cells and the percentages of $CD4^+Foxp3^+$ T cells, we assessed these cells in all patients. As shown in Fig. 2c, a significant inverse correlation was not detected in all patients (white circles), whereas an inverse correlation was noted in HBV reactivation patients (black dots).

PD-1 and CD62L expression on HBV-specific and total $CD8^+$ T cells

The function of antigen-specific $CD8^+$ T cells is impaired, termed "exhaustion," during persistent chronic infection diseases like HBV [17], and exhausted antigen-specific $CD8^+$ T cells express high levels of PD-1 and low levels of CD62L [18]. Based on these findings, we evaluated the expression of PD-1 and CD62L on total $CD8^+$ T cells and


A
Resolved
ICHB
CHB
Reactivation
A24HBVcore
CD8
0.0943
13.3
0.0245
13.3
0.0465
9.05
0.139
14.5
B
tetramer staining
A2core/CD8
A24core/CD8
A24poly/CD8
Relative percentage (%)
IFNg staining
A2core/CD8
A24core/CD8
A24poly/CD8
CD4+CD25+
CD4+Foxp3+
Resolved
ICHB
CHB
Reactivation
C
Foxp3/CD4
R=0.24
p value=0.172
R=0.265
p value=0.094
R=0.113
p value=0.448
A2core/CD8
A24core/CD8
A2poly/CD8

◀ **Fig. 2** **a** FACS analysis using tetramer staining. To detect HBV specific CTLs in the PBMCs, we isolated PBMCs from 4 groups. The samples were stained with PE-conjugated anti-human HLA-A24 HBV core antibody and a PE-Cy5-conjugated anti-human CD8 antibody. **b** Frequencies of HBV-specific $CD8^+$ T cells and $CD4^+$ Tregs. The numbers of HBV-specific $CD8^+$ T cells and Tregs were analyzed by FACS at the time of diagnosis in HBV reactivation patients and prior to chemotherapy or antiviral therapy in the other groups. The *upper panels* show the percentages of HBV-specific $CD8^+$ T cells, among which the *left panel* shows the A2 core, the *center panel* shows the A24 core and the *right panel* shows A24 poly-specific $CD8^+$ T cells. The *middle panels* shows the percentage of IFN-γ producing $CD8^+$ T cells stimulated by peptides for A2 core, A24 core, and A24 poly, respectively. The *lower panels* show the percentages of Tregs, of which the *left panel* shows $CD4^+CD25^+$ Tregs and the *right panel* shows $CD4^+Foxp3^+$ Tregs. $*P < 0.05$, significant difference between the linked items. **c** Relationships between the frequencies of HBV-specific $CD8^+$ T cells and Tregs. The *scatter diagrams* show HBV reactivation patients (*white circle*) and other patients (*black dot*), respectively. The *left panel* shows the negative correlation between $CD4^+Foxp3^+$ Tregs and A2 core-specific $CD8^+$ T cells. The *center panel* shows the negative correlation between $CD4^+Foxp3^+$ Tregs and A24 core-specific $CD8^+$ T cells. The *right panel* shows the negative relationship between $CD4^+Foxp3^+$ Tregs and A24 poly-specific $CD8^+$ T cells

HBV-specific $CD8^+$ T cells to determine whether the $CD8^+$ T cell function in HBV reactivation patients was different from that in the other groups. Although PD-1 expression was higher on circulating HBV-specific $CD8^+$ T cells in ICHB and CHB patients, it was significantly lower on HBV-specific $CD8^+$ cells in HBV reactivation patients (Fig. 3). Curiously, PD-1 expression on HBV-specific $CD8^+$ T cells was low in resolved HBV patients compared with ICHB and CHB patients, indicating that HBV-specific $CD8^+$ T cells in resolved HBV patients may function in a similar manner to those in HBV reactivation patients.

It was also demonstrated that primary CD62L high expressing $CD8^+$ T cells were better at clearing LCMV infection compared with primary CD62L low expressing cells. In addition, CD62L high memory cells underwent robust expansion, and were efficient in preventing chronic LCMV infection [18]. Thus, to address the memory phenotype of cells we examined the expression of CD62L on HBV-specific $CD8^+$ T cells. However, we did not detect any significant differences in the expression of CD62L on $CD8^+$ T cells among the groups although CD62 expression in HBV reactivation patients had a tendency to be lower.

Longitudinal analysis of the frequencies of HBV-specific $CD8^+$ T cells and $CD4^+$ Tregs

To evaluate changes in the frequency of HBV-specific $CD8^+$ T cells during HBV reactivation, we monitored sALT levels, serum HBV DNA levels, percentages of HBV-specific $CD8^+$ T cells and numbers of $CD4^+CD25^+$ and $CD4^+Foxp3^+$ cells in the six HBV reactivation patients. As shown in Fig. 4, when serum HBV DNA was

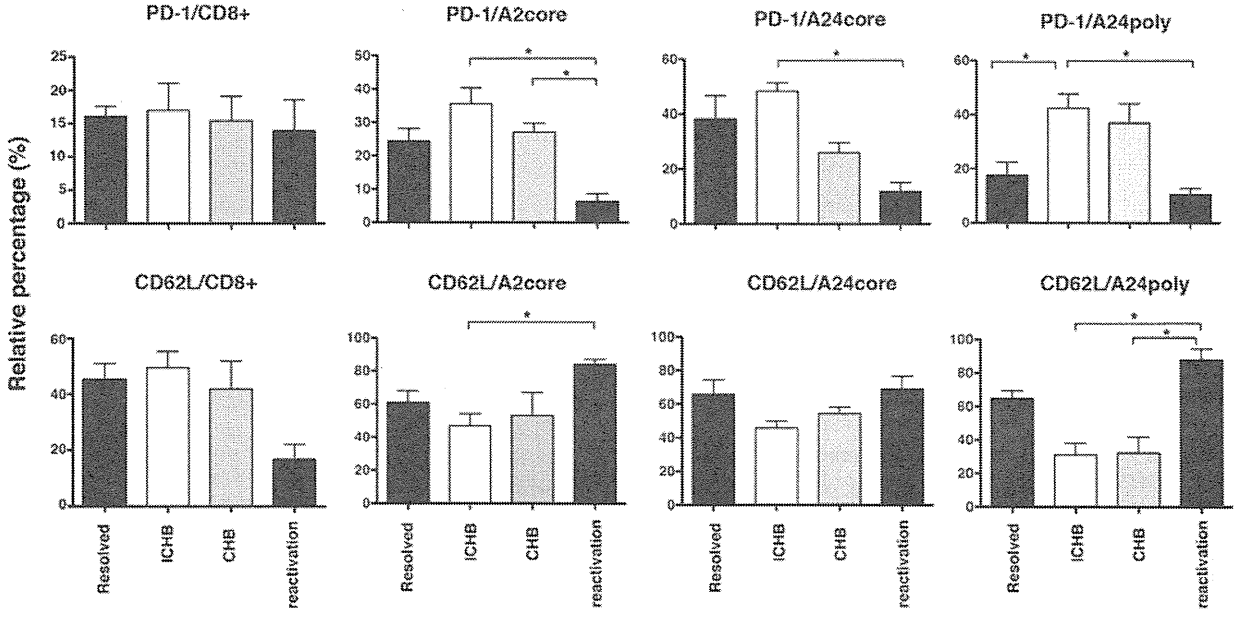


Fig. 3 PD-1 and CD62L expression in HBV-specific and total $CD8^+$ T cells. The PD-1 and CD62L expression levels in HBV-specific and total $CD8^+$ T cells were analyzed by FACS at the time of diagnosis in HBV reactivation patients and prior to chemotherapy or antiviral therapy in the other groups. The *left panel* shows the percentages of PD-1 or CD62L-positive cells in CD8 T^+ cells. The *three right panels* show PD-1 or CD62L-positive cells in HBV-specific $CD8^+$ T cells, respectively. $*P < 0.05$, significant difference between the linked items

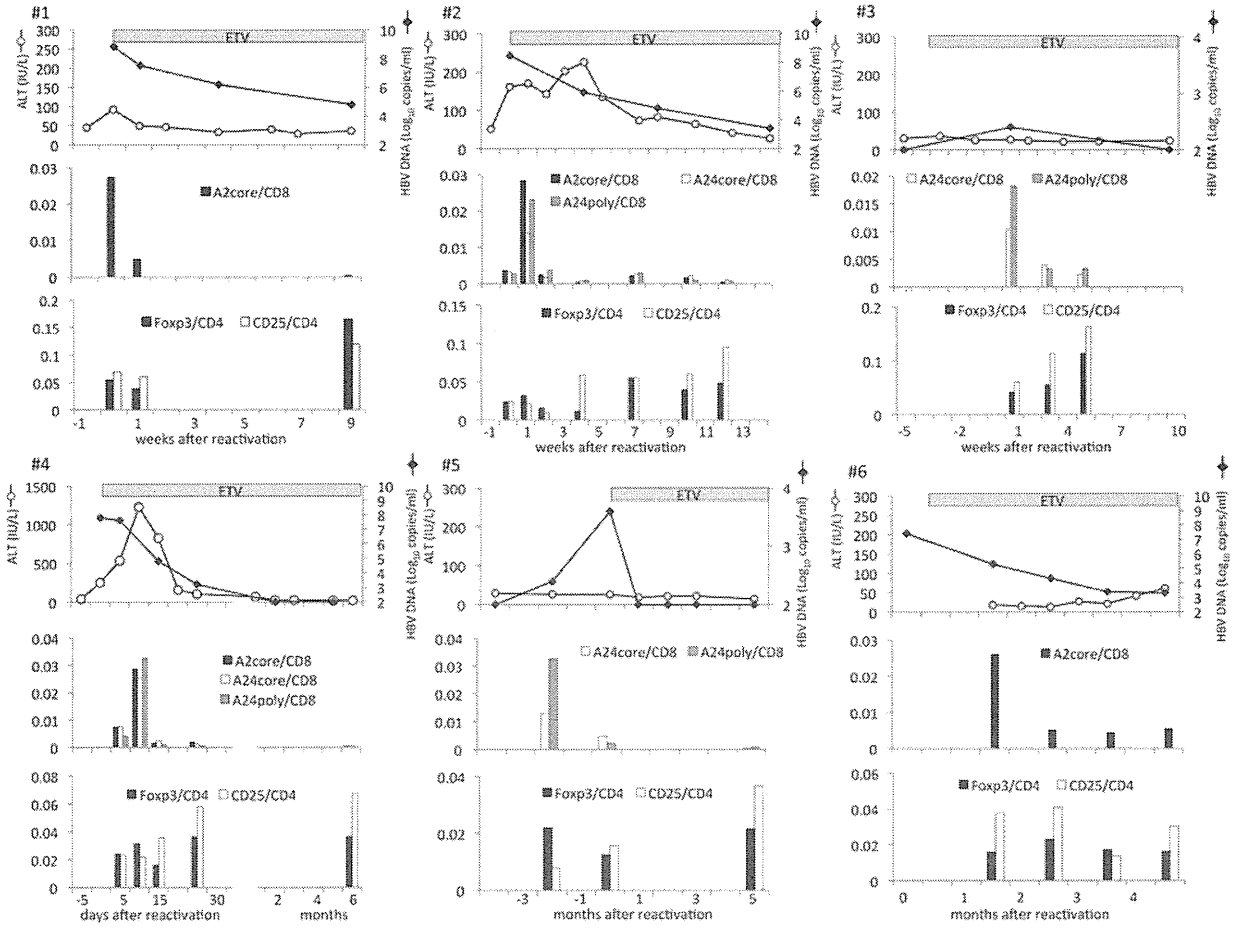


Fig. 4 Longitudinal analysis of the frequencies of HBV-specific $CD8^+$ T cells and Tregs. The *upper panels* show the kinetics of ALT (IU/L) (*white circle*) and HBV DNA (log_{10} copies/ml) (*black diamond*), the *middle panels* show the frequencies of HBV-specific $CD8^+$ T cells and the *lower panels* show the frequencies of Tregs, respectively. All patients were administered entecavir immediately following diagnosis

detected in resolved HBV patients, administration of entecavir was quickly started for all patients to prevent severe hepatitis. HBV-specific $CD8^+$ T cells were detected and reached their peak frequency levels at the onset of HBV reactivation in patients #1 to #6. Interestingly, a high percentage of HBV-specific $CD8^+$ T cells was observed in patient #4 compared with other patients and, consistent with this finding, the sALT level was markedly elevated to about 1200 IU/l, indicating that the number of HBV-specific $CD8^+$ T cells reflected the grade of liver damage, as previously reported [19]. Furthermore, when the numbers of HBV-specific $CD8^+$ T cells were maximal, the numbers of $CD4^+CD25^+$ and $CD4^+Foxp3^+$ T cells were minimal, indicating a negative correlation. Moreover, HBV-specific $CD8^+$ T cells decreased as the sALT level decreased, whereas $CD4^+CD25^+$ and $CD4^+Foxp3^+$ T cells showed tendencies to increase. However, the number of HBV-specific $CD8^+$ T cells in patient #6 did not decrease throughout the time course, although this patient showed tendencies for higher numbers of $CD4^+CD25^+$ and $CD4^+Foxp3^+$ T cells at the time of HBV reactivation. In this patient, the reduction in serum HBV DNA was slow and the sALT levels continued to be elevated. These findings suggest that $CD4^+$ Tregs may suppress an effective immune response against HBV.

Discussion

HBV reactivation is an almost universal event among patients with HBsAg undergoing bone marrow transplantation [20–22]. In retrospective analyses using sensitive serological and virological markers, a high proportion of people with anti-HBc antibodies without HBsAg in their serum also redevelop HBV DNA and HBsAg after bone marrow transplantation [23, 24]. In addition, the prolonged

impairment of immune-mediated control of intrahepatic HBV after extensive immunosuppression leads to reactivation of potential occult infection with HBsAg seroreversion [2]. Thus, although the risk of HBV reactivation during immunosuppression is well known, the mechanism for the induction of HBV reactivation is unclear.

In this study, we demonstrated that six patients with HBV reactivation showed increased numbers of HBV-specific $CD8^+$ T cells, similar to the case for self-limited acute hepatitis B, and that these T cells induced liver damage despite immunosuppression following treatment with an immunosuppressant and anti-cancer drug.

These findings are consistent with a previous report of a strong multifaceted CTL response in patients with acute hepatitis [7]. It is interesting to evaluate the function of antigen-specific $CD8^+$ T cells, including their proliferation and cytokine production, during immunosuppressive drug treatment, because a previous study showed that FK506 did not prevent the generation and proliferation of LCMV-specific T cells, but instead altered their differentiation so that these effector T cells lost their ability to control the virus [25]. Although we analyzed the role of $CD8^+$ T cells under the immunosuppressive status, it seems to be important to analyze macrophages, which produce TNF-α and IL-6 [25].

We found that the ratio of HBV-specific $CD8^+$ T cells was higher in resolved HBV patients than in ICHB and CHB patients, indicating that high viral loads suppress the frequency of these cells [26]. Furthermore, we showed that PD-1 expression on HBV-specific $CD8^+$ T cells was low in resolved HBV patients compared with ICHB and CHB patients, demonstrating that these cells can restore their function. These findings suggested that resolved HBV patients have numerous and functionally recovered HBV-specific $CD8^+$ T cells, and therefore, they may easily develop severe hepatitis once HBV reactivation is induced. This hypothesis was confirmed by a report that acute hepatitis in resolved HBV patients has a higher mortality rate than acute hepatitis in HBV-positive patients [23].

In addition, we found that the frequencies of HBV-specific $CD8^+$ T cells and $CD4^+Foxp3^+$ Tregs were reversible at the onset of HBV reactivation. These observations may imply that the reduction of $CD4^+Foxp3^+$ Tregs triggered the induction of antigen-specific CTLs. Although the effects of $CD4^+Foxp3^+$ Tregs are generally nonspecific or occur in a bystander manner, preferential inhibition of the antigen-specific T cell response has been observed in some cases, including human HBV infection [27]. In support of our results, Xu et al. [16] demonstrated that depletion of $CD4^+CD25^+$ Tregs led to an increase in HBV antigen-stimulated IFN-γ production and cellular proliferation of PBMCs in HBV-infected patients, and that coculture of $CD4^+CD25^+$ Tregs with effector cells significantly suppressed HBsAg-stimulated IFN-γ production and cellular proliferation. At the time of HBV reactivation in patient #6, when the number of $CD4^+Foxp3^+$ Tregs was increased, the reduction in serum HBV DNA was poor and liver damage was continuous. These findings suggest that a reduction in the number of $CD4^+Foxp3^+$ Tregs may induce an effective immune response.

We also observed that serum IL-7, IL-8 and MCP-1 were significantly higher in resolved HBV patients than in ICHB and CHB patients. However, the group of resolved HBV patients was quite miscellaneous and it remains unknown whether the differences among such cytokines and chemokines are responsible for HBV.

It has been demonstrated that IL-7 is required for T cell development and for maintaining and restoring the homeostasis of mature T cells. Administration of recombinant human IL-7 to patients resulted in widespread T cell proliferation, increased T cell numbers, modulation of peripheral T cell subsets and increased T cell receptor repertoire diversity [28]. Furthermore, IL-7 expression by hepatocytes directly controls T cell immune responses to Toll-like receptor signaling in vivo [29]. These observations suggest that IL-7 plays an important role for HBV-specific $CD8^+$ T cell proliferation and that a low level of IL-7 may be involved in the low frequencies of HBV-specific $CD8^+$ T cells in ICHB and CHB patients.

As previously reported, since rituximab therapy is a high risk factor for HBV reactivation, we examined a possible imbalance in serum Th1/Th2 cytokine secretion in HBV reactivation patients. As shown in supplementary Fig. 2, we analyzed the ratio of serum Th1/Th2 cytokines as follows: IFN-γ or IL-12 compared with IL-4 or IL-10. We observed a shift towards IL-10 compared with IFN-γ. However, when we compared IL-12 with IL-4 and IL-10, we observed a shift towards IL-12. Thus, we did not detect an obvious shift towards either Th1 or Th2 cytokines in the serum at the onset of HBV reactivation.

Finally, our study showed that HBV-specific $CD8^+$ T cells are increased at the onset of HBV reactivation despite an immunosuppressive status and declined following resolution of liver disease. In contrast, a reduced number of $CD4^+Foxp3^+$ Tregs was also observed and showed a negative correlation with the frequency of HBV-specific $CD8^+$ T cells. We plan to analyze additional resolved HBV patients prospectively and to clarify the relationships among $CD4^+Foxp3^+$ Tregs, HBV-specific $CD8^+$ T cells and liver damage.

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Conflict of interest The authors have no conflicts of interest to disclose.

References

1. Hoofnagle JH. Reactivation of hepatitis B. Hepatology. 2009;49:S156–65.
2. Raimondo G, Pollicino T, Cacciola I, Squadrito G. Occult hepatitis B virus infection. J Hepatol. 2007;46:160–70.
3. Yang JD, Roberts LR. Hepatocellular carcinoma: a global view. Nat Rev Gastroenterol Hepatol. 2010;7:448–58.
4. McMahon BJ. The natural history of chronic hepatitis B virus infection. Hepatology. 2009;49:S45–55.
5. Mason AL, Xu L, Guo L, Kuhns M, Perrillo RP. Molecular basis for persistent hepatitis B virus infection in the liver after clearance of serum hepatitis B surface antigen. Hepatology. 1998;27:1736–42.
6. Rehermann B, Ferrari C, Pasquinelli C, Chisari FV. The hepatitis B virus persists for decades after patients' recovery from acute viral hepatitis despite active maintenance of a cytotoxic T-lymphocyte response. Nat Med. 1996;2:1104–8.
7. Chisari FV, Ferrari C. Hepatitis B virus immunopathogenesis. Annu Rev Immunol. 1995;13:29–60.
8. Kimura K, Kakimi K, Wieland S, Guidotti LG, Chisari FV. Activated intrahepatic antigen-presenting cells inhibit hepatitis B virus replication in the liver of transgenic mice. J Immunol. 2002;169:5188–95.
9. Vento S, Cainelli F, Longhi MS. Reactivation of replication of hepatitis B and C viruses after immunosuppressive therapy: an unresolved issue. Lancet Oncol. 2002;3:333–40.
10. Sugauchi F, Tanaka Y, Kusumoto S, Matsuura K, Sugiyama M, Kurbanov F, et al. Virological and clinical characteristics on reactivation of occult hepatitis B in patients with hematological malignancy. J Med Virol. 2011;83:412–8.
11. Barber DL, Wherry EJ, Masopust D, Zhu B, Allison JP, Sharpe AH, et al. Restoring function in exhausted CD8 T cells during chronic viral infection. Nature. 2006;439:682–7.
12. Velu V, Kannanganat S, Ibegbu C, Chennareddi L, Villinger F, Freeman GJ, et al. Elevated expression levels of inhibitory receptor programmed death 1 on simian immunodeficiency virus-specific CD8 T cells during chronic infection but not after vaccination. J Virol. 2007;81:5819–28.
13. Zhang JY, Zhang Z, Wang X, Fu JL, Yao J, Jiao Y, et al. PD-1 up-regulation is correlated with HIV-specific memory CD8+ T-cell exhaustion in typical progressors but not in long-term nonprogressors. Blood. 2007;109:4671–8.
14. Urbani S, Amadei B, Tola D, Massari M, Schivazappa S, Missale G, et al. PD-1 expression in acute hepatitis C virus (HCV) infection is associated with HCV-specific CD8 exhaustion. J Virol. 2006;80:11398–403.
15. Kimura K, Ando K, Tomita E, Ohnishi H, Ishikawa T, Kakumu S, et al. Elevated intracellular IFN-gamma levels in circulating $CD8^+$ lymphocytes in patients with fulminant hepatitis. J Hepatol. 1999;31:579–83.
16. Xu D, Fu J, Jin L, Zhang H, Zhou C, Zou Z, et al. Circulating and liver resident $CD4^+$ $CD25^+$ regulatory T cells actively influence the antiviral immune response and disease progression in patients with hepatitis B. J Immunol. 2006;177:739–47.
17. Blackburn SD, Shin H, Haining WN, Zou T, Workman CJ, Polley A, et al. Coregulation of $CD8^+$ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. Nat Immunol. 2009;10:29–37.
18. Wherry EJ, Ha SJ, Kaech SM, Haining WN, Sarkar S, Kalia V, et al. Molecular signature of $CD8^+$ T cell exhaustion during chronic viral infection. Immunity. 2007;27:670–84.
19. Guidotti LG, Rochford R, Chung J, Shapiro M, Purcell R, Chisari FV. Viral clearance without destruction of infected cells during acute HBV infection. Science. 1999;284:825–9.
20. Au WY, Lie AK, Liang R, Liu CL, Shek TW, Lau GK. Aggressive hepatocellular carcinoma complicating pregnancy after autologous bone marrow transplantation for non-Hodgkin's lymphoma. Bone Marrow Transplant. 2002;29:177–9.
21. Kojima H, Abei M, Takei N, Mukai Y, Hasegawa Y, Iijima T, et al. Fatal reactivation of hepatitis B virus following cytotoxic chemotherapy for acute myelogenous leukemia: fibrosing cholestatic hepatitis. Eur J Haematol. 2002;69:101–4.
22. Seth P, Alrajhi AA, Kagevi I, Chaudhary MA, Colcol E, Sahovic E, et al. Hepatitis B virus reactivation with clinical flare in allogeneic stem cell transplants with chronic graft-versus-host disease. Bone Marrow Transplant. 2002;30:189–94.
23. Kusumoto S, Tanaka Y, Ueda R, Mizokami M. Reactivation of hepatitis B virus following rituximab-plus-steroid combination chemotherapy. J Gastroenterol. 2011;46:9–16.
24. Liang R, Lau GK, Kwong YL. Chemotherapy and bone marrow transplantation for cancer patients who are also chronic hepatitis B carriers: a review of the problem. J Clin Oncol. 1999;17:394–8.
25. Araki K, Gangappa S, Dillehay DL, Rouse BT, Larsen CP, Ahmed R. Pathogenic virus-specific T cells cause disease during treatment with the calcineurin inhibitor FK506: implications for transplantation. J Exp Med. 2010;207:2355–67.
26. Wherry EJ. T cell exhaustion. Nat Immunol. 2011;12:492–9.
27. Alatrakchi N, Koziel M. Regulatory T cells and viral liver disease. J Viral Hepat. 2009;16:223–9.
28. Mackall CL, Fry TJ, Gress RE. Harnessing the biology of IL-7 for therapeutic application. Nat Rev Immunol. 2011;11:330–42.
29. Sawa Y, Arima Y, Ogura H, Kitabayashi C, Jiang JJ, Fukushima T, et al. Hepatic interleukin-7 expression regulates T cell responses. Immunity. 2009;30:447–57.