In conclusion, using the Invader Plus *IL28B* SNP genotyping assay, swift and accurate selection of the optimum treatment strategy for individual patients can be improved by combining with other factors.

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DISCLOSURE

The authors have no conflict of interest.

REFERENCES

1. Ray Kim W. (2002) Global epidemiology and burden of hepatitis C. *Microbes and Infect* **4:** 1219–25.
2. Fried M.W., Shiffman M.L., Reddy K.R., Smith C., Marinos G., Goncales F.L. Jr., Haussinger D., Diago M., Carosi G., Dhumeaux D., Craxi A., Lin A., Hoffman J., Yu J. (2002) Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *New Engl J Med* **347:** 975–82.
3. Manns M.P., McHutchison J.G., Gordon S.C., Rustgi V.K., Shiffman M., Reindollar R., Goodman Z.D., Koury K., Ling M., Albrecht J.K. (2001) Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomized trial. *Lancet* **358:** 958–965.
4. Bruno S., Camma C., Di Marco V., Rumi M., Vinci M., Camozzi M., Rebucci C., Di Bona D., Colombo M., Craxi A., Mondelli M.U., Pinzello G. (2004) Peginterferon alfa-2b plus ribavirin for naïve patients with genotype 1 chronic hepatitis C: a randomized controlled trial. *J.Hepatol* **41:** 474–81.
5. Akuta N., Suzuki F., Hirakawa M., Kawamura Y., Yatsuji H., Sezaki H., Suzuki Y., Hosaka T., Kobayashi M., Kobayashi M., Saitoh S., Ayase Y., Ikeda K., Kumada H. (2009) A matched case-controlled study of 48 and 72 weeks of peginterferon plus ribavirin combination therapy in patients infected with HCV genotype 1b in Japan: aimino acid substitutions in HCV core region as predictor of sustained virological response. *J Med Virol* **81:** 452–8.
6. Ghany M.G., Strader D.B., Thomas D.L., Seeff L.B. (2009) Diagnosis, management, and treatment of hepatitis C: an update. *Hepatology* **49:** 1335–74.
7. Hoofnagle J.H., Wahed A.S., BrownJr R.S., Howell C.D., Belle S.H. (2009) Early changes in hepatitis C virus (HCV) levels in response to peginterferon and ribavirin treatment in patients with chronic HCV genotype 1 infection. *J Infect Dis* **199:** 1112–20.
8. Tanaka Y., Nishida N., Sugiyama M., Kurosaki M., Matsuura K., Sakamoto N., Nakagawa M., Korenaga M., Hino K., Hige S., Ito Y., Mita E., Tanaka E., Mochida S., Murawaki Y., Honda M., Sakai A., Hiasa Y., Nishiguchi S., Koike A., Sakaida I., Imamura M., Ito K., Yano K., Masaki N., Sugauchi F.,Izumi N., Tokunaga K., Mizokami M. (2009) Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. *Nat Genet* **10:** 1105–9.
9. Suppiah V., Moldovan M., Ahlenstiel G., Berg T., Weltman M., Abate M.L., Bassendine M., Spengler U., Dore G.J., Powell E., Riordan S., Sheridan D., Smedile A., Fragomeli V., Muller T., Bahlo M., Stewart G.J., Booth D.R., George J. (2009) IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. *Nat Genet* **10:** 1100–4.
10. Ge D., Fellay J., Thompson A.J., Simon J.S., Shianna K.V., Urban T.J., Heinzen E.L., Qiu P., Bertelsen A.H., Muir A.J., Sulkowski M., McHutchison J. G., Goldstein D.B. (2009) Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. *Nature* **461:** 399–401.
11. Rauch A., Kutalik Z., Descombes P., Cai T., Di Iulio J., Mueller T., Bochud M., Battegay M., Bernasconi E., Borovicka J., Colombo S., Cerny A., Dufour J.F., Furrer H., Gunthard H.F., Heim M., Hirschel B., Malinverni R., Moradpour D., Mullhaupt B., Witteck A., Beckmann J.S., Berg T., Bergmann S., Negro F., Telenti A., Bochud P.Y. (2010) Genetic variation in IL28B is associated with chronic hepatitis C and treatment failure: a genome-wide association study. *Gastroenterology* **138:** 1338–45.
12. Rouits E., Boisdron-Celle M., Dumont A., Guerin O., Morel A., Gamelin E. (2004) Relevance of different UGT1A1 polymorphisms in irinotecan-induced toxicity: a molecular and clinical study of 75 patients. *Clin Cancer Res* **10:** 5151–9.
13. Ando Y., Saka H., Ando M., Sawa T., Muro K., Ueoka H., Yokoyama A., Saitoh S., Shimokata K., Hasegawa Y. (2000) Polymorphisms of UDP-glucuronosyltransferase gene and irinotecan toxicity: a pharmacogenetic analysis. *Cancer Res* **60:** 6921–6.
14. Iadonato S.P., Katze M.G. (2009) Genomics: Hepatitis C virus gets personal. *Nature* **461:** 357–8.
15. Ito K., Higami K., Masaki N., Sugiyama M., Mukaide M., Saito H., Aoki Y., Sato Y., Imamura M., Murata K., Nomura H., Hige S., Adachi H., Hino K., Yatsuhashi H., Orito E., Kani S., Tanaka Y., Mizokami M. (2011) The rs8099917 polymorphism, determined by a suitable genotyping methods, is a better predictor for response to pegylated interferon-{alpha}/ribavirin therapy in Japanese patients than other SNPs associated with IL28B. *J Clin Microbiol* **49:** 1853–60.

Increased Levels of Arginase in Patients With Acute Hepatitis B Suppress Antiviral T Cells

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CLINICAL LIVER

BACKGROUND & AIMS: During viral infection, the activities of virus-specific $CD8^+$ T cells are carefully regulated to prevent severe damage of the infected organs. We investigated the mechanisms that control the functions of activated T cells. **METHODS:** We measured the size of the population of activated and proliferating $CD8^+$ T cells and the functional pattern of $CD8^+$ T cells specific for the entire hepatitis B virus proteome and for selected heterologous virus (Epstein–Barr virus, human cytomegalovirus, and influenza virus) using blood samples from 18 patients with acute hepatitis B. We analyzed the effects of different modulatory mechanisms, such as inhibitory molecules, suppressive cytokines (interleukin-10), and arginase, on the activities of $CD8^+$ T cells. **RESULTS:** In patients with acute hepatitis B, the expansion of activated and proliferating (HLA-DR/$CD38^+$, Ki-67^+/Bcl-2^{low}) $CD8^+$ T cells did not quantitatively match their specific functions ex vivo; virus-specific $CD8^+$ T cells had functional impairments that were temporally restricted to the acute phase of viral hepatitis. These impairments in function were not limited to HBV-specific $CD8^+$ T cells but were also observed in $CD8^+$ T cells with specificities for other viruses. We investigated possible causes of antigen-independent $CD8^+$ T cell inhibition and found that the increased levels of arginase observed in patients with acute hepatitis could suppress the function of activated, but not resting, $CD8^+$ T cells. **CONCLUSIONS: The increased level of arginase in patients with acute hepatitis B suppresses the functions of activated $CD8^+$ T cells. This mechanism might limit the amount of liver damage caused by activated $CD8^+$ T cells in patients with acute HBV infection.**

Keywords: Immune Regulation; Liver Disease; Virology; Treatment.

Infection of parenchymal organs with noncytopathic viruses poses problems to the immune system because infected cell elimination might cause organ failure. The immune system can master such a balance because evolution toward fulminant hepatic failure is quite rare (approximately 1%) in patients with acute hepatitis B (AHB)[1] despite infection of most liver cells.[2,3] Patients with AHB are able to eliminate the virus from the hepatocytes by developing an acute liver inflammation that recovers and does not leave any chronic pathological liver conditions.[1]

This ability to control the infection and the degree of liver damage is related to the balance between the quantity of infected hepatocytes and the efficiency of the T-cell response, and several factors are in place to avoid liver destruction by $CD8^+$ T cells.[4] Important self-save mechanisms are represented by the capacity of hepatocytes to eliminate the infecting virus noncytopathically through the action of interferons (IFNs) and tumor necrosis factor (TNF)-α[5,6] and by the hepatocyte resistance to perforin-mediated killing.[7,8] Other mechanisms that regulate $CD8^+$ T cell function have been recently suggested. PD-1 expression on hepatitis B virus (HBV)-specific $CD8^+$ T cells has been reported to be essential to avoid excessive T cell–mediated liver damage, in relation to its ability to inhibit T-cell function,[9] whereas production of interleukin (IL)-10 during the early stages of AHB has been proposed to attenuate natural killer and HBV-specific T-cell responses.[10] Furthermore, pioneering works of Wands and Isselbacher[11] and Chisari et al[12] described more than 30 years ago the presence of soluble factors (then identified as arginase)[13] during viral hepatitis able to suppress T-cell proliferation.

Such a scenario of multiple controls of the T-cell response during acute hepatitis is, however, complicated by our recent observation that acute HBV infection triggers a very vigorous expansion of an activated/proliferating $CD8^+$ T cell population (~25% of total $CD8^+$ T cells).[14] To understand how the function of such a large population of activated $CD8^+$ T cells is regulated during acute viral hepatitis, we studied the frequency and function of HBV- and heterologous virus-specific $CD8^+$ T cells in AHB. We observed that an efficient $CD8^+$ T cell functional suppression, independent from HBV-specific recognition, is operative during acute hepatitis. A characterization of the immunologic mechanisms responsible for such an effect showed that arginase, released either by

Abbreviations used in this paper: AHB, acute hepatitis B; CMV, cytomegalovirus; EBV, Epstein–Barr virus; GFP, green fluorescent protein; HCMV, human cytomegalovirus; ICS, intracellular cytokine staining; IFN, interferon; IL, interleukin; P, patient; PBMC, peripheral blood mononuclear cell; TCR, T-cell receptor; TNF, tumor necrosis factor.

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damaged hepatocytes[13] or by neutrophils[15] or other immune cells,[16] is a major determinant of CD8+ T cell functional suppression.

Materials and Methods

Patients and Samples

Samples were taken from patients or healthy volunteers attending clinics in Parma, Italy. The local review board and ethical committee approved the study. Eighteen patients with AHB and 2 patients with drug-induced hepatitis were studied. In 11 of them, Epstein-Barr virus (EBV)-, cytomegalovirus (CMV)-, influenza-, or/and HBV pentamer-positive CD8+ T cells were detected (Table 1). Five healthy volunteers were also enrolled. The age of the subjects ranged from 20 to 54 years. Patients were selected on the basis of appearance of jaundice, high alanine aminotransferase (ALT) levels (>1000 U/L except patient [P] 10; see Table 1), and serologic profile of HBV infection (hepatitis B surface antigen positive, antibody to hepatitis B core antigen immunoglobulin [Ig] M, antibody to hepatitis B core antigen positive, and HBV DNA positive).

Virologic Measurements

Hepatitis B surface antigen, hepatitis B e antigen, antibody to hepatitis B surface antigen, antibody to hepatitis B core antigen IgG and IgM, antibody to hepatitis B e antigen, antibody to hepatitis D virus, antibody to hepatitis C virus, antibody to HIV-1, and antibody to HIV-2 were determined by commercial enzyme immunoassay kits (Abbott Laboratories, Abbott Park, IL; Ortho Clinical Diagnostic, Johnson & Johnson (New York, NY); DiaSorin, Vercelli, Italy). HBV DNA was quantified by PCR Cobas Amplicor Test (Roche Diagnostic, Basel, Switzerland).

Table 1. AHB at Clinical Onset

Patient	ALT (U/L)	Hepatitis B surface antigen	Hepatitis B e antigen	Pentamer positive
P1	3441	Positive	Positive	NF
P2	3029	Positive	Positive	NF
P3	3280	Positive	Positive	NF
P4	1520	Positive	Positive	NF
P5	3188	Positive	Positive	NF
P6	1362	Positive	Positive	NF
P7	5460	Positive	Positive	EBVBZLF1$_{190-197}$ EBVEBNA3A$_{193-201}$
P8	2349	Positive	Positive	EBVLMP2$_{340-349}$ CMVpp65$_{265-275/417-426}$
P9	1443	Positive	Positive	NF
P10	648	Positive	Positive	HBVcore$_{18-27}$ CMVpp65$_{495-504}$
P11	1197	Positive	Positive	HBVcore$_{18-27}$ CMVpp65$_{495-504}$ FLUMP$_{58-66}$
P12	1164	Positive	Positive	HBVcore$_{18-27}$ FLUMP$_{58-66}$
P13	1107	Positive	Positive	HBVcore$_{18-27}$ CMVpp65$_{495-504}$
P14	5650	Positive	Positive	EBVLMP2$_{419-427}$
P15	1207	Positive	Positive	CMVpp65$_{495-504}$ FLUMP$_{58-66}$
P16	1673	Positive	Positive	EBVEBNA1$_{407-417}$ CMVpp65$_{123-131}$ FLUMP$_{58-66}$
P17	2346	Positive	Positive	CMVpp65$_{495-504}$ CMVpp65$_{123-131}$ FLUMP$_{58-66}$ HBVcore$_{18-27}$
P18	2602	Positive	Positive	HBVenv$_{183-191}$ CMVpp65$_{123-131}$

NF, not found.

Reagents

HLA-peptide pentamers were purchased from Proimmune (Oxford, England). All fluorescence-activated cell-sorted antibodies were purchased from BD Biosciences (San Jose, CA). For enzyme-linked immunosorbent spot assays, the capture mouse anti-human IFN-γ antibody, biotinylated anti-human IFN-γ antibody, and streptavidin alkaline phosphatase were purchased from Mabtech (Stockholm, Sweden). Fifteen-mer peptides overlapping by 10 residues (total 313 peptides) were pooled into 16 mixtures as described in Tan et al.[17] The peptides covered the overall sequence of HBV genD (GenBank accession number AF121241) and HBV genB (GenBank accession number AF121243) and were purchased from Chiron Mimotopes (Victoria, Australia). IL-15 and IL-10 were purchased from R&D Systems (Minneapolis, MN), and arginase was from Enzo Life Sciences (Farmingdale, NY). The arginase inhibitor nor-NOHA was purchased from Cayman Chemical (Ann Arbor, MI) and anti-IL-10 antibody from eBioscience (Vienna, Austria).

Functional Assays

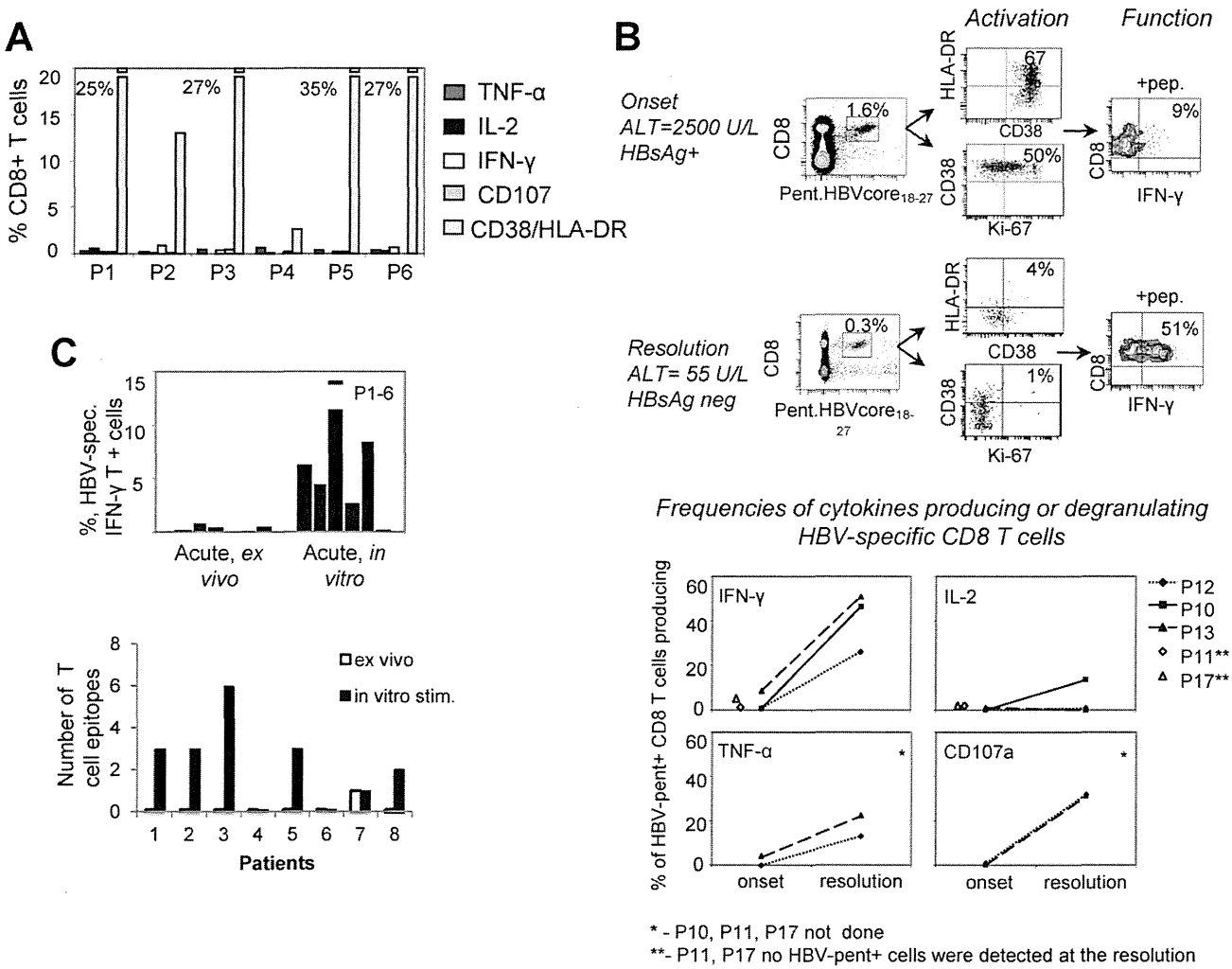
Intracellular cytokine staining and enzyme-linked immunosorbent spot assays were performed as described in Sandalova et al[14] and Tan et al[17] using specific peptides or pools of peptides covering the whole HBV proteome.

Recovery and Inhibition of T-Cell Responses

Peripheral blood mononuclear cells (PBMCs) from patients with AHB or healthy individuals were incubated in vitro in AIM-V medium (Gibco, Invitrogen, Carlsbad, CA) supplemented with 2% AB serum (Invitrogen) or 50% AB/patient serum where indicated for 48 hours. For the serum, IL-10, and arginase inhibition assays, the cells were incubated with or without 50% patients'/healthy serum, 10 pg/mL of IL-10, or 200 μg/mL of arginase. Intracellular cytokine staining (ICS) was performed for pentamer-positive populations, and the percentage of inhibition was calculated based on the percentage of IFN-γ–positive (or CD107a) virus-specific cells.

Inhibition of T-Cell Responses via Killing Assay

HEPG2-Core green fluorescent protein (GFP), a hepatocyte cell line expressing HBV core and transduced with lentiviral vector/GFP, were plated at 100,000 cells/well overnight on a 96-well flat clear-bottom black plate (Greiner Bio-One, Wemmel, Belgium) and used as target. Core 18-27V T-cell receptor (TCR) T cells produced using a healthy donor's PBMCs (described in Gehring et al[18]) were thawed the next day and treated with 0.02 U/mL, 0.2 U/mL, and 2 U/mL recombinant Arginase 1 (Enzo Life Sciences) for 6 hours in AIM-V serum-free medium (Invitrogen) and then added to targets at an effector-to-target ratio of 0.7:1. HEPG2-Core GFP without effectors was used as negative control. The effector cells were removed the next day, and the plate was washed 3 times with 1× phosphate-buffered saline before fixing with 1× phosphate-buffered saline plus 1% formaldehyde. The plate was left to dry and GFP fluorescence mea-



* - P10, P11, P17 not done
**- P11, P17 no HBV-pent+ cells were detected at the resolution

Figure 1. Dysfunction of HBV-specific CD8+ T cells in AHB infection. (*A*) Discrepancy between activated/proliferating and functional CD8+ T cells. *Bars* represent the frequency of TNF-α/IL-2/IFN-γ–producing, degranulating (CD107a+), and CD38/HLA-DR+ CD8+ T cells in 6 patients with AHB ex vivo. (*B*) Direct ex vivo visualization of activation (CD38/HLA-DR+ and CD38/Ki-67+) and IFN-γ production of HBV pentamer–positive CD8+ T cells in representative patients at the onset and after resolution of acute HBV infection. PBMCs were stained with HBV core-18-27 pentamer and CD38, HLA-DR, and Ki67 or stimulated with c18-27 peptide (100 nmol/L) for 4 hours before ICS. Graphs show the frequencies of HBV pentamer–positive CD8+ T cells producing IFN-γ, TNF-α, or IL-2 or degranulating (CD107a+) after peptide stimulation in the indicated patients with AHB at the onset and resolution. (*C*) PBMCs of AHB were stimulated with pools of peptides covering the whole HBV proteome ex vivo or expanded in vitro for 10 days. The *upper graph* compares production of IFN-γ ex vivo and after in vitro expansion. The *lower graph* shows the number of responses that were found ex vivo and after in vitro expansion.

sured with the Tecan Infinite 200 PRO plate reader (Tecan Group Ltd, Männedorf, Switzerland).

Results

HBV-Specific CD8+ T Cell Function During Acute Hepatitis

We have recently shown that patients with AHB present a high frequency of circulating activated/proliferating CD8+ T cells, composed of a large quantity of HBV-specific CD8+ T cells and inflated by IL-15 activated herpes virus–specific CD8+ T cells.[14] To directly quantify the whole HBV-specific CD8+ T cell repertoire, PBMCs of 6 patients with AHB (P1-6) were stimulated directly ex vivo with pools of overlapping peptides covering the whole HBV proteome. Different functional assays including ICS for IFN-γ, TNF-α, and IL-2 and degranulation by CD107a (Figure 1*A*) and IFN-γ enzyme-linked immunosorbent spot assays (Supplementary Figure 1*A*) were used, and the frequency of functional and activated CD8+ T cells was compared. A substantial discrepancy between activated CD8+ T cells and HBV-specific functional T-cell numbers was evident. At the onset of hepatitis, approximately one fourth of CD8+ T cells (see P1, P3, P5, and P6; Figure 1*A*) are activated, but less than 1% of T cells respond to HBV antigens by producing Th1 cytokines or degranulating, suggesting that the majority of activated HBV-specific CD8+ T cells do not respond to peptide stimulation. This finding was confirmed by selecting 4 patients with HLA-A201+ AHB, in whom HBV-specific CD8+ T cells could be directly visualized by HBV-specific pentamers (P10-13 and P17; Table 1). HBV

pentamer–positive CD8+ T cells were then tested directly ex vivo for their expression of activation (CD38 and HLA-DR) and proliferation (Ki-67) markers and for the functional ability to respond to the specific HBV peptide stimulation. Figure 1*B* shows that at the onset of acute hepatitis, at least 90% of HBV-HLA pentamer–positive CD8+ T cells are unresponsive to peptide-specific stimulation, despite expressing activation and proliferation markers, but regain their ex vivo functionality when acute hepatitis progresses toward resolution.

In some subjects, in whom HBV-specific CD8+ T cells could be directly visualized longitudinally, restoration was robust, whereas in others it was only partial (Supplementary Figure 1*B*).

Reverting HBV-Specific T-Cell Functional Suppression

Functional data derived from the ex vivo analysis of the HBV-specific T-cell response in AHB contrasts with the prior description of a strong and multi-specific T-cell response in the same patients.[19] This apparent discrepancy could be related to the different experimental systems used. The concept of strong and multi-specific T-cell responses during acute HBV infection derives from experiments performed after in vitro expansions of T cells,[19] whereas here the functional "exhaustion" was detected by analyzing HBV-specific CD8+ T cells ex vivo. Indeed, when we compared HBV-specific T-cell responses ex vivo and after in vitro expansion, the magnitude and multi-specificity of HBV-specific T cells (Figure 1*C*) were restored after in vitro culture. To better analyze the causes of such restoration, a series of experiments was performed. A negligible restoration of function was detected when ex vivo HBV-specific T-cell function of AHB was tested in the presence of antibodies able to block inhibitory receptors expressed on exhausted T cells (PD-1, CTLA-4)[20–22] or of antiapoptotic drugs[23] or with addition of T-cell stimulatory cytokines (IL-15, IL-2)[24] (Figure 2*A*). In contrast, a 2-day incubation in media containing IL-2, IL-7, and IL-15 led to a robust restoration of the ability of CD8+ T cells to produce IFN-γ (from 5% to 65% of HBV-specific CD8+ T cells IFN-γ+) (Figure 2*B* and *C*). However, the restoration of the HBV-specific response is abrogated if the medium contains 50% of the patient's serum (Figure 2*C*; IL-2/7/15+ serum). Thus, activated HBV-specific CD8+ T cells of AHB need cytokines to survive, but soluble factors present in patients' sera contributed to HBV-specific T-cell functional impairment.

CD8+ T Cell Functional Inhibition During Acute Hepatitis Is Not Restricted to HBV-Specific T Cells

To further analyze whether soluble factors can alter CD8+ T cell functionality, effector memory CD8+ T

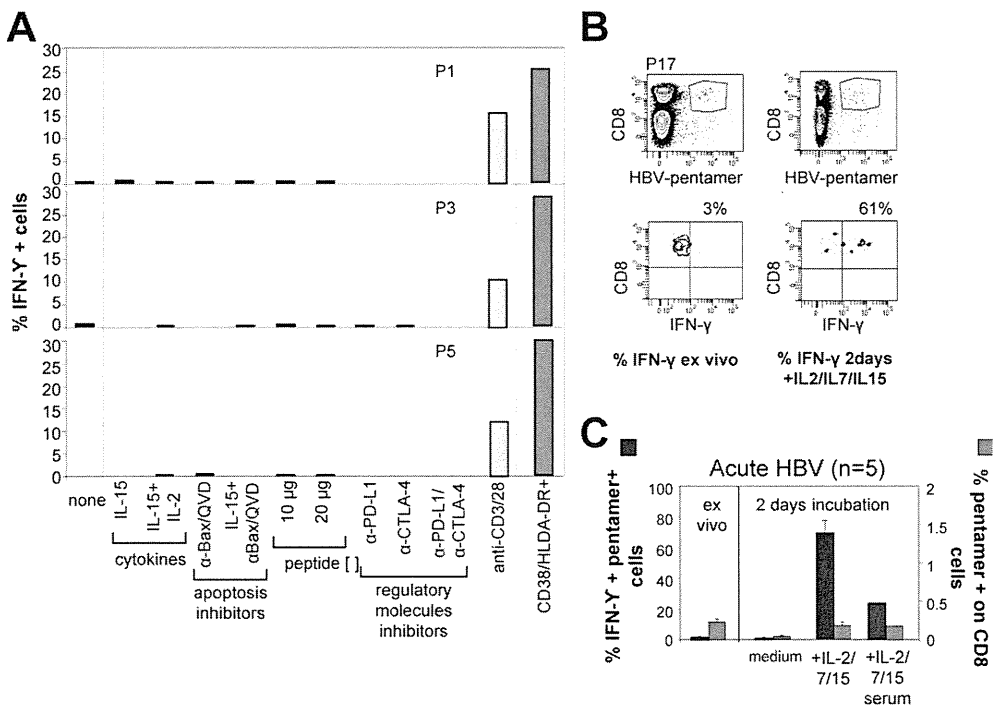


Figure 2. Analysis of functional recovery of HBV-specific CD8+ T cells. (*A*) Patients' PBMCs were stained with CD38 and HLA-DR or stimulated with known specific peptides in different conditions: (1) in medium alone; (2) with IL-15 alone or IL-15 and IL-2; (3) increasing the concentration of specific peptides; (4) with apoptosis inhibitors, anti-Bax peptide, and QVD alone or with IL-15; and (5) blocking PD-L1, CTLA-4, or both. The cells were incubated for 12 hours and then stimulated with specific peptides overnight in the presence of brefeldin A, and ICS was performed. The experiment was performed in 3 individual patients. (*B* and *C*) PBMCs of patients with AHB were stimulated with peptide ex vivo or after 2 days of in vitro culture in medium alone or with IL-2, IL-7, and IL-15 or in 50% patients' serum with IL-2, IL-7, and IL-15. Fluorescence-activated cell sorter plots show the IFN-γ production by HBV pentamer–positive CD8+ T cells ex vivo and after 2 days of culture. Graphs show IFN-γ production by HBV pentamer–positive cells (*black bars*) and the frequency of pentamer-positive cells (*gray bars*).

CLINICAL LIVER

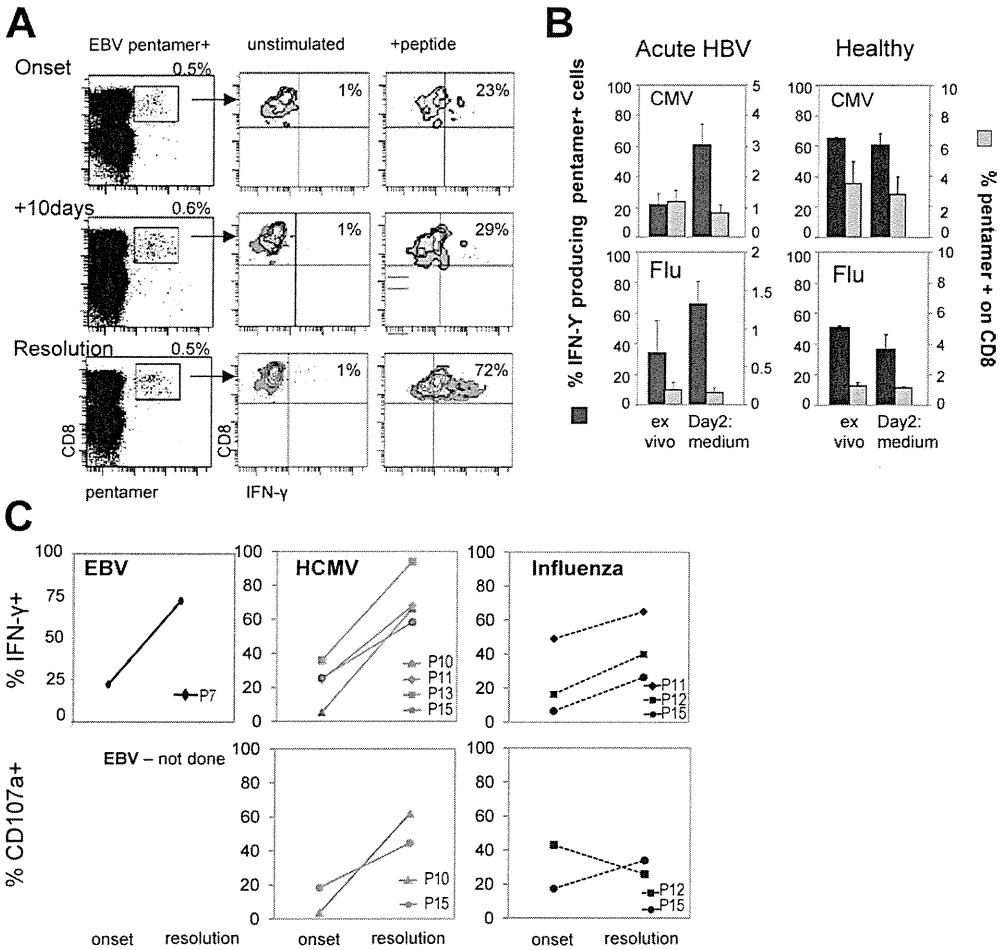


Figure 3. Functional inhibition of heterologous CD8+ T cells during AHB. (*A*) Production of IFN-γ by EBV pentamer–positive CD8+ T cells during AHB. PBMCs were stained with EBV BZLF1190–197 and EBNA193–201 pentamers and stimulated with the relevant peptides. After 4 hours of incubation with brefeldin A, ICS was performed as described in Materials and Methods. The cells were gated on CD3+, CD8+, and EBV pentamer positive. Data of a representative patient at the onset, 10 days after onset, and at resolution of acute HBV infection are shown. (*B*) Altered IFN-γ production and CD107a+ expression on EBV, HCMV, and influenza pentamer–positive CD8+ T cells in 5 patients with AHB over time. (*C*) Frequencies of CMV (n = 4) or influenza (n = 3) pentamer-positive CD8+ T cells and IFN-γ–positive pentamer-positive cells in patients with AHB compared with healthy controls (n = 3) ex vivo and after 2 days in culture with IL-2, IL-7, and IL-15.

cells specific for viruses unrelated to HBV (human CMV [HCMV], EBV, and influenza-named heterologous viruses) were studied. Heterologous virus-specific CD8+ T cells in AHB were visualized with specific HLA pentamers, and synthetic peptides corresponding to the specific viral epitopes were used to stimulate the cells directly ex vivo. The frequency of heterologous virus-specific CD8+ T cells was constant over time without signs of attrition (Figure 3*A* and data not shown), but their functionality (IFN-γ production and degranulation) was partially inhibited during the acute phase of hepatitis (Figure 3*A* and *B*).

Similar to HBV-specific CD8+ T cells, functional recovery of heterologous virus-specific T-cell function was observed by incubating PBMCs of AHB for 2 days in culture (Figure 3*C*). In this case, the addition of cytokines (IL-2, IL-7, and IL-15) was optional, because the survival of heterologous virus-specific CD8+ T cells present in acute hepatitis was not dependent on the presence of IL-2, IL-7, and IL-15. In vitro incubation did not affect the functionality of heterologous virus-specific CD8+ T cells present in healthy individuals (Figure 3C and data not shown).

Thus, these data show that antigen-independent mechanisms of T-cell suppression are active during acute hepatitis and that soluble factors are likely implicated. Both HBV- and heterologous-specific CD8+ T cells regain function when incubated with normal media with cytokines, but not with acute hepatitis sera (Figures 2*E* and 3*C*).

Soluble Factors Present in the Serum of Patients With Acute Hepatitis Inhibit CD8+ T Cell Function

The role of soluble factors in inhibiting T-cell proliferation in patients with acute and chronic hepatitis was suggested 30 years ago.[11,12] Arginase released by the injured liver parenchyma was shown to affect T-cell responsiveness[13] and more recently has been implicated in causing low proliferation of intrahepatic T cells in patients with chronic hepatitis B.[25] Furthermore, arginase can be produced by other cell types such as neutrophils,[15] which participate in the development of acute hepatitis.[26] One other soluble factor, IL-10, is an immune regulatory cytokine that has been associated with the delayed expansion of HBV-specific T-cell response during acute hepatitis.[10]

To understand whether IL-10 and arginase can be responsible for the inhibition of CD8+ T cell function, we first measured their concentrations in patients with AHB. IL-10 and arginase values were elevated at the onset of acute hepatitis in comparison with disease resolution (Figure 4*A* and *B*). The linear regression analysis showed significant correlation of serum ALT and arginase values;

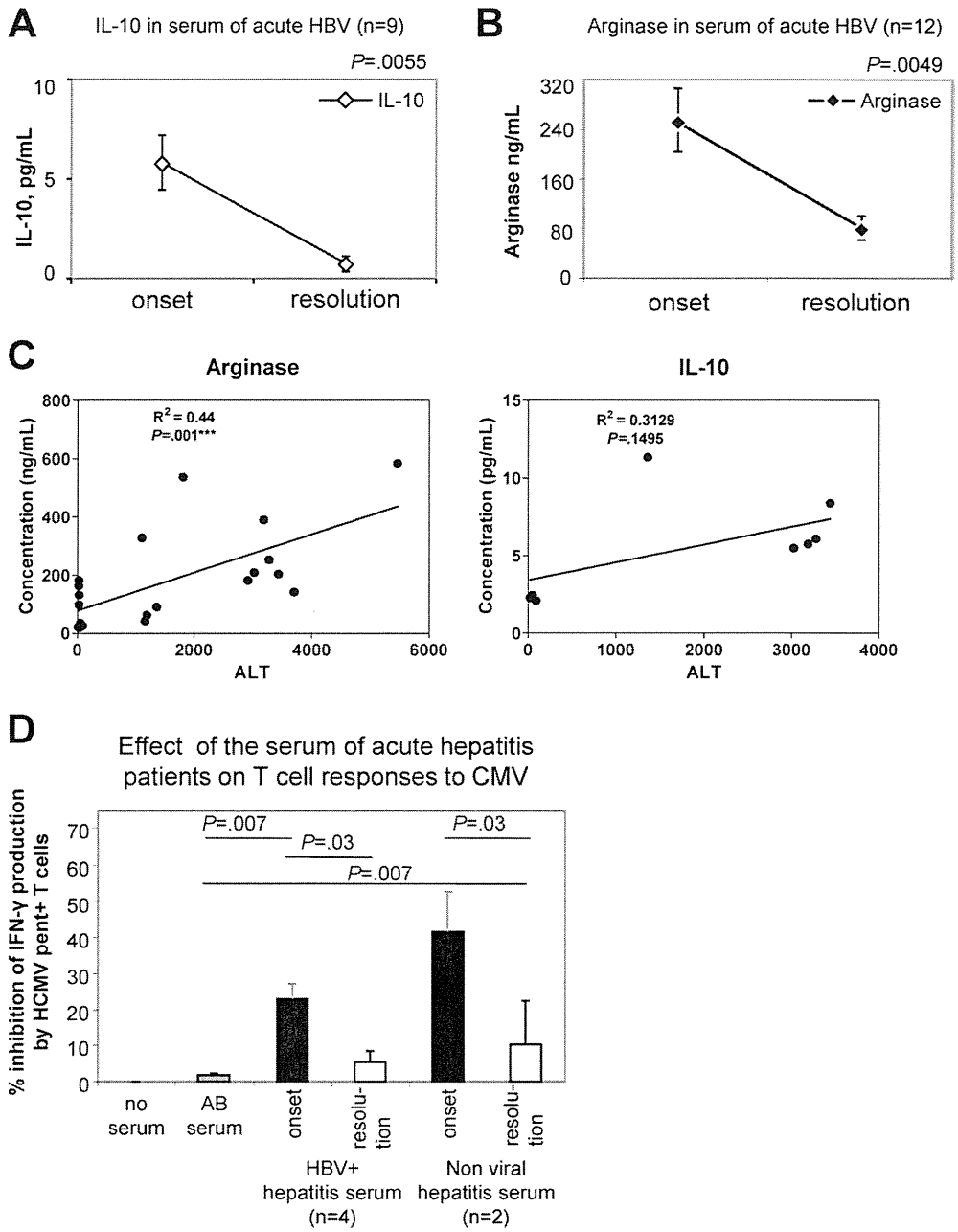


Figure 4. Sera of patients with acute hepatitis inhibit CD8+ T cell function. (*A*) IL-10 and (*B*) arginase were measured in patients' serum (n = 9; n = 12) at the onset of acute hepatitis and at the resolution. (*C*) Correlation of serum levels of arginase I and IL-10 with ALT. Linear regression analysis of serum arginase I levels (*left panel*) and IL-10 levels (*right panel*) against ALT from patients with acute HBV (n = 12) was performed. A *P* value of <.05 indicates a gradient with significant deviation from 0. (*D*) Effect of the serum of AHB on T-cell responses to HCMV. PBMCs of healthy individuals with a defined CMV response were cultured for 48 hours in presence of IL-15 (10 ng/mL) and 50% of the serum of HBV-positive patients or patients with drug-induced hepatitis at onset or resolution (n = 4); alternatively, healthy AB serum or no serum was used. (Acute onset sera ALT levels ranged from 3500 to 2600 U/L, arginase from 220 to 160 pg/mL, and IL-10 from 8 to 6 pg/mL.) After incubation, ICS was performed for IFN-γ after 5 hours of stimulation with specific peptide. Percent inhibition by serum was calculated based on the percent of IFN-γ–positive CMV-specific CD8+ T cells. The number of patients' sera tested is indicated.

however, the correlation of ALT and IL-10 levels was not significant (Figure 4*C*).

We then tested whether the serum of AHB could inhibit virus-specific CD8+ T cell functions. PBMCs containing HCMV-specific CD8+ T cells were incubated with IL-15 for 48 hours (which activates HCMV-specific T cells[14]) in the presence of serum (50% of cell medium) of healthy individuals or patients with AHB (n = 4) collected during the acute phase (ALT level >2000 U/L) and after recovery (ALT level <60 U/L). After incubation, IFN-γ production was tested by ICS after 5 hours of stimulation with specific peptides. Incubation with patients' sera collected during the acute phase inhibited IFN-γ production of HCMV-specific activated T cells (Figure 4*D*). Interestingly, sera of 2 patients with drug-related subfulminant hepatitis (ALT level >1500 U/L) were equally suppressive (Figure 4*D*).

Arginase Suppresses Activated CD8+ T Cell Function

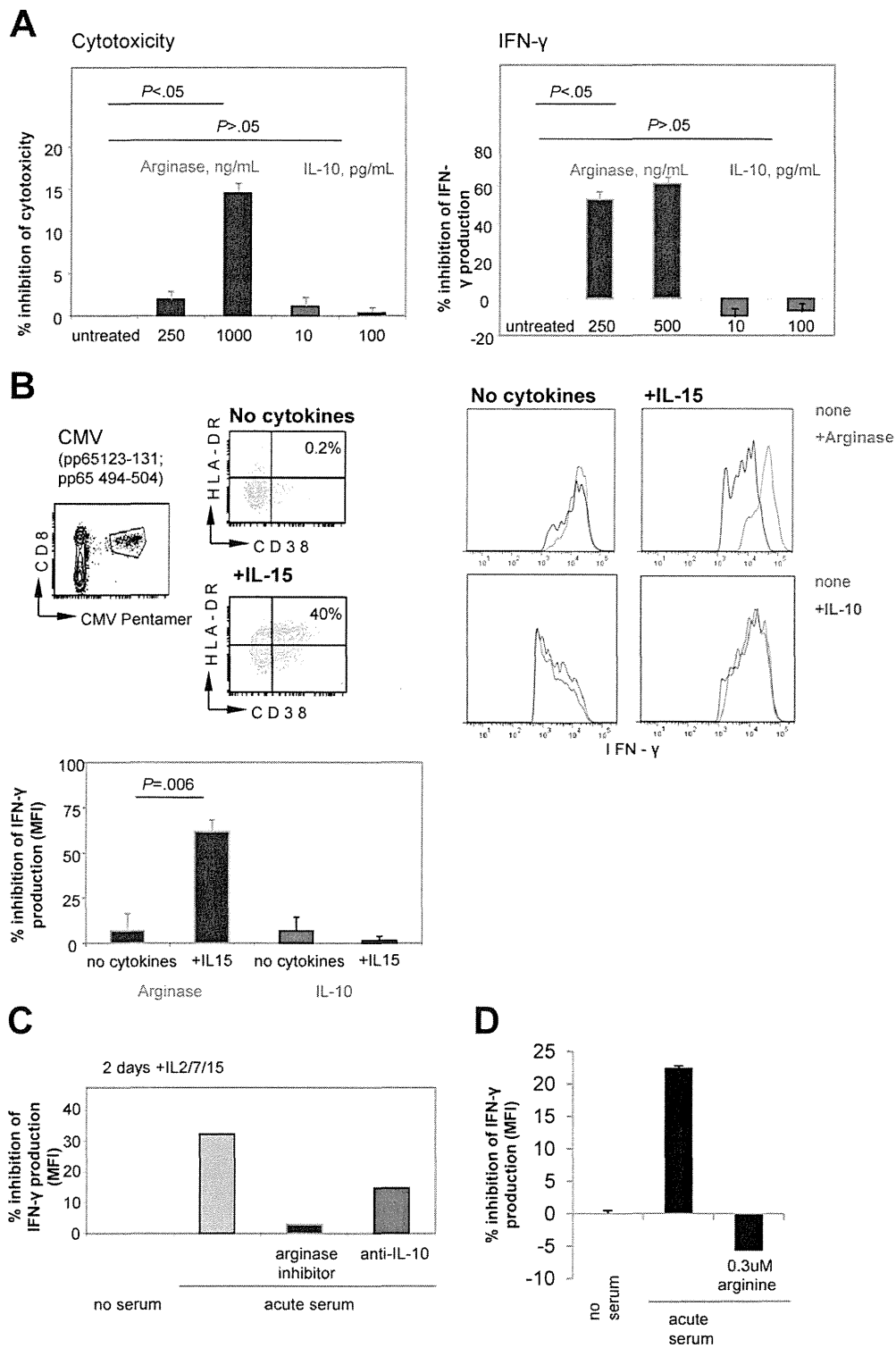
To decipher whether IL-10 or arginase can mediate the observed inhibitory effect observed, HBV-specific CD8+ T cells or PBMCs of healthy donors containing HCMV-specific CD8+ T cells were incubated with IL-10 and arginase and their ability to recognize specific targets was tested.

Arginase inhibited HBV- and HCMV-specific CD8+ T cell function (cytotoxicity and cytokine production), confirming the ability of this enzyme to alter the function of

effector T cells[16] (Figure 5*A* and *B*). Note, however, that concentrations of arginase higher than those detected in the sera of acute patients (which are ~250 ng/mL) were necessary to inhibit cytotoxicity (Figure 5*A* and Supplementary Figure 2).

In contrast, IL-10 did not show any inhibitory effects on the function of effector T cells, in line with its preferential effect on antigen-presenting cells and T-cell induction.[27] Interestingly, only cytokine-activated HCMV-specific CD8$^+$ T cells were suppressed by arginase (Figure 5*A* and *B*), whereas the function of resting HCMV-specific CD8$^+$ T cells was altered neither by arginase nor by IL-10. Similar results were confirmed on EBV and influenza-specific CD8$^+$ T cells (Supplementary Figure 3).

To confirm that arginase present in the serum of patients with acute hepatitis is responsible for the inhibitory



effect on activated T cells, arginase inhibitors (nor-NOHA) and anti-IL-10 antibodies were added to the patient's serum. Figure 5C shows that the arginase inhibitor almost completely blocked the inhibitory effect of acute hepatitis serum, whereas the effect of anti-IL-10 was only partial. Furthermore, the inhibitory effect of acute hepatitis sera was reversed by the addition of L-arginine in cell culture (Figure 5D).

Discussion

In this work, we analyzed how the HBV-specific CD8$^+$ T cell response is modulated in AHB. We showed that arginase could inhibit the T-cell function during the acute phase of liver inflammation, preferentially acting on activated CD8$^+$ T cells. This mechanism is likely to play a crucial regulatory role in limiting excessive liver pathology of different etiologies caused by host adaptive immunity.

By measuring the quantity of activated/proliferating T cells and their function, we observed that HBV infection triggers a robust expansion of activated and proliferating T cells,[14] most of which are, however, impaired in their ability to produce cytokines and degranulate. The observation, by itself, is not novel. A "stunned functional phenotype" was previously described in acute HCV infection[28] and was also partially described in a previous analysis of acute HBV infection.[29,30] In addition, the inability to produce cytokines was observed in murine HBV-specific CD8$^+$ T cells adoptively transferred and activated in HBV-transgenic mice.[31] However, this altered functional state of virus-specific CD8$^+$ T cells was interpreted as an antigen-specific tolerogenic phenomenon, caused by excessive antigenic stimulation,[29] lack of cytokines,[24] and overexpression of inhibitory molecules such as PD-1 typically upregulated in exhausted T cells.[21]

Here, we argued that a further critical mechanism able to suppress CD8$^+$ T cell function during acute hepatitis is non-antigen specific and mediated by arginase, a class of enzymes that metabolize L-arginine and have been shown to suppress T-cell function in different pathological conditions.[16]

The importance of such a mechanism in vivo during hepatitis was clearly indicated by the important decrease in the ability of CD8$^+$ T cells specific for heterologous viruses to produce antiviral cytokines at the peak of liver damage compared with the recovery phase. The relative major impact of the non-antigen-specific mechanisms of CD8$^+$ T cell regulation in comparison to antigen-specific mechanisms was also further supported by the fact that hyperactivation of negative costimulatory or apoptotic pathways was not primarily involved because PD-1 and CTLA-4 blockade as well as the use of antiapoptotic compounds had no effect on ex vivo HBV-specific CD8$^+$ T cell responses. Moreover, ex vivo T-cell responsiveness could not be restored by the simple addition of T-cell stimulatory cytokines (IL-2, IL-7, IL-15) and by increasing doses of antigen.

In contrast, we could observe that either HBV-specific or heterologous virus-specific CD8$^+$ T cells could recover their functionality by cell culture in complete medium. More importantly, the ability of acute viral hepatitis serum to inhibit CD8$^+$ T cell function revealed that the suppressive effect detected at the peak of acute hepatitis was mediated by soluble factors. Because this effect was not only observed by incubating CD8$^+$ T cells with the serum of patients with AHB but also with sera of patients with virus-unrelated hepatitis, such an effect could not be mediated by HBV antigens, which have been reported to inhibit innate immune responses,[32] but by soluble factors associated with liver damage. We believed that IL-10 and arginase could be the 2 potential candidates. IL-10 is increasing during acute and chronic hepatic flares[10,33] and has been implicated in delaying the expansion of virus-specific CD8$^+$ T cells in AHB.[10] However, because it acts directly on antigen-presenting cells, IL-10 preferentially exerts its effect on the induction and not on the effector phase of T-cell responses.[27] Arginase instead suppresses the function of terminally differentiated effector CD8$^+$ T cells.[16] In line with this prediction, data presented here showed that levels of both arginase and IL-10 were elevated during the peak of acute hepatitis and correlated temporally with the inhibition of HBV-specific and heterologous virus-specific CD8$^+$ T cell functions. However, only arginase, and not IL-10, at the concentrations detected in the circulation of patients can inhibit the virus-specific CD8$^+$ T cell function, and only arginase inhibitor or addition of L-arginine, but not anti-IL-10, prevents the

Figure 5. Arginase, but not IL-10, inhibits CD8$^+$ T cell function. (*A*) HBV core18-27 specific CD8$^+$ T cell clone was incubated with arginase or IL-10 at indicated concentration for 2 days, and killing assay and intracellular cytokine staining for IFN-γ was performed with GFP-expressing HepG2 cells as targets. The plots show percent inhibition of cytotoxicity or IFN-γ production (measured as mean fluorescent intensity) compared with that of untreated T cells. (*B*) Healthy PBMCs were incubated either with IL-10 (10 pg/mL) or arginase (250 pg/mL) with or without IL-15 for 48 hours and specific IFN-γ production was evaluated. The cells were gated on an HCMV pentamer–positive population (*B, left plot*). The level of activation of HCMV-specific T cells is shown based on HLA-DR/CD38$^+$, the histogram plots show the levels of IFN-γ production by HCMV-specific T cells, and the bar charts indicate the percent inhibition of IFN-γ production. Experiments were performed in 2 individuals 3 times. (*C*) Arginase inhibitor reverted the suppressive effect of the sera from patients with AHB. HBV pentamer–positive CD8$^+$ T cells present in patients with AHB were stimulated with HBV peptide after 2 days of culture with IL-2, IL-7, and IL-15 with or without patients' serum (ALT 3100 U/L, arginase 210 pg/mL, IL-10 7 pg/mL), arginase inhibitor (nor-NOHA), or anti–IL-10 antibody. The percent inhibition of IFN-γ production by HBV-specific CD8$^+$ T cells compared with culture without serum is shown. (*D*) Addition of L-arginine reverted the suppressive effect of the sera from patients with AHB. A total of 0.3 μmol/L arginine was added to PBMCs cultured in the presence of sera from patients with AHB (ALT 3100 U/L, arginase 210 pg/mL). Here, the arginine level was adjusted according to the arginine concentration in culture medium (AIM-V), used to restore T-cell responses. HCMV-specific IFN-γ response was evaluated after 5 hours of activation. Percent inhibition is shown. Experiments were performed in 2 individuals 3 times.

T-cell inhibitory effect of the sera of patients with acute hepatitis.

The inhibition of T-cell function caused by arginase through depletion of L-arginine was suggested to act through TCR ζ chain down-regulation.[16] We analyzed the expression of the TCR ζ chain on total and activated T cells present at the peak of acute hepatitis, but we were unable to detect any reproducible evidence of TCR ζ chain down-regulation (data not shown). This could be explained by the fact that inhibition of T-cell function by arginase can also occur in the absence of TCR ζ chain down-regulation, for example, in T-cell infiltrating human prostate cancers.[3,4] Alternatively, our measurement of TCR ζ chain expression was likely performed also on cells with minimal functional inhibition. Indeed, because of sample availability, TCR ζ chain expression was measured not only on the highly unresponsive HBV-specific $CD8^+$ T cells, but on total activated $CD8^+$ T cells that also comprise activated heterologous-specific $CD8^+$ T cells that, even though inhibited, still conserve a degree of antiviral functionality.

Thus, despite acting in a non–antigen-specific way, arginase maintains a level of selectivity because it acts on activated $CD8^+$ T cells with highly active metabolism and therefore preferentially inhibits HBV-specific $CD8^+$ T cells that are stimulated by a high quantity of viral antigen and are in a higher activation state than heterologous virus-specific $CD8^+$ T cells. This relative selectivity is the likely explanation of the absence of clinical signs of generalized immunosuppression in patients with acute hepatitis. Note that arginase reduces the overall quantity of cytokine produced by the activated T cells but does not seem to interfere with the initial $CD8^+$ T cell triggering. This differential effect can explain why HBV-specific $CD8^+$ T cells are activated at the peak of acute hepatitis despite the presence of arginase. This interpretation is also consistent with our previous observation that activated heterologous virus-specific $CD8^+$ T cells during acute viral hepatitis have a low activation threshold.[14]

Overall, our data contribute to describe the existence of distinct mechanisms of T-cell functional regulation, which have the potential to modulate different phases of the antiviral T-cell immunity in the liver.

A representation of such distinct mechanisms is displayed in Figure 6. We propose that early after HBV infection, T-cell expansion can be modulated by IL-10 and by the balance between different costimulatory signals, such as those mediated by PD-1.[9] However, despite the effect of these negative regulatory mechanisms, HBV-specific T cells expand vigorously and are likely the principal effectors of liver cell lysis. Release of arginase ensuing from hepatocyte damage[13] or from neutrophils[15] can in turn suppress efficiently effector T-cell function by virtue of a preferential effect on activated T cells (Figure 6).

Thus, arginase can act as a simple direct suppressive mechanism helping to preserve liver viability in the course of the exuberant T-cell activation triggered by acute HBV infection. Interestingly, such an effect is not only restricted to acute hepatitis, because we have recently observed inhibition of heterologous (HCMV and influenza) virus-specific $CD8^+$ T cell IFN-γ production in hepatic flares of chronic HBV infection (unpublished observation, August 2010). In conclusion, arginase-mediated inhibition is a robust antigen-independent mechanism of T-cell functional suppression that is active during hepatitis and may play an important role in preserving the viability of infected parenchymal organs when they are targeted by large quantities of activated virus-specific $CD8^+$ T cells.

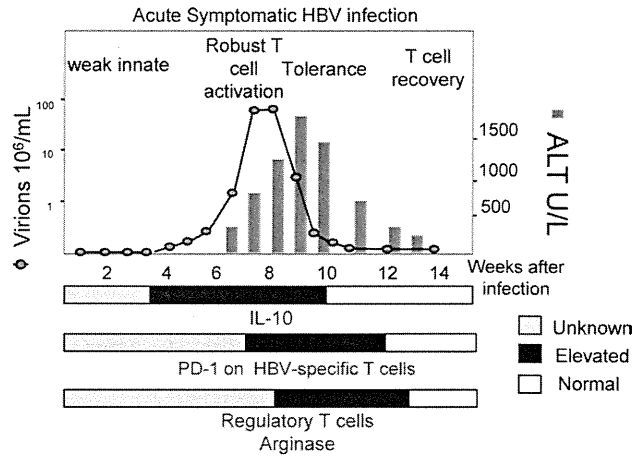


Figure 6. Regulation of immune T-cell response during AHB infection. Schematic representation of different mechanisms of T-cell functional modulation potentially active during AHB.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at http://dx.doi.org/10.1053/j.gastro.2012.03.041.

References

1. Ganem D, Prince AM. Hepatitis B virus infection—natural history and clinical consequences. N Engl J Med 2004;350:1118–1129.
2. Jilbert A, Wu T, England J, et al. Rapid resolution of duck hepatitis B virus infections occurs after massive hepatocellular involvment. J Virol 1992;66:1377–1388.
3. Kajino K, Jilbert AR, Saputelli J, et al. Woodchuck hepatitis virus infections: very rapid recovery after prolonged viremia and infection of virtually every hepatocyte. J Virol 1994;68:5792–5803.
4. Guidotti L, Chisari F. To kill or to cure: options in host defense against viral infection. Curr Opin Immunol 1996;8:478–483.
5. Guidotti LG, Ishikawa T, Hobbs MV, et al. Intracellular inactivation of the hepatitis B virus by cytotoxic T lymphocytes. Immunity 1996;4:25–36.
6. Biermer M, Puro R, Schneider RJ. Tumor necrosis factor alpha inhibition of hepatitis B virus replication involves disruption of capsid Integrity through activation of NF-kappaB. J Virol 2003;77:4033–4042.
7. Kafrouni MI, Brown GR, Thiele DL. Virally infected hepatocytes are resistant to perforin-dependent CTL effector mechanisms. J Immunol 2001;167:1566–1574.
8. Barrie MB, Stout HW, Abougergi MS, et al. Antiviral cytokines induce hepatic expression of the granzyme B inhibitors, proteinase inhibitor 9 and serine proteinase inhibitor 6. J Immunol 2004;172:6453–6459.

9. Zhang Z, Jin B, Zhang JY, et al. Dynamic decrease in PD-1 expression correlates with HBV-specific memory CD8T-cell development in acute self-limited hepatitis B patients. J Hepatol 2009;50: 1163–1173.
10. Dunn C, Peppa D, Khanna P, et al. Temporal analysis of early immune responses in patients with acute hepatitis B virus infection. Gastroenterology 2009;137:1289–1300.
11. Wands KR, Isselbacher KJ. Lymphocyte cytotoxicity to autologous liver cells in chronic active hepatitis. Proc Natl Acad Sci U S A 1975;72:1301–1303.
12. Chisari FV, Routenberg JA, Fiala M, et al. Extrinsic modulation of Human T-lymphocyte E rosette function associated with prolonged hepatocellular injury after viral hepatitis. J Clin Invest 1977;59: 134–142.
13. Chisari FV, Nakamura M, Milich DR, et al. Production of two distinct and independent hepatic immunoregulatory molecules by the perfused rat liver. Hepatology 1985;5:735–743.
14. Sandalova E, Laccabue D, Boni C, et al. Contribution of herpesvirus specific CD8T cells to anti-viral T cell response in humans. PLoS Pathog 2010;6.
15. Munder M, Schneider H, Luckner C, et al. Suppression of T-cell functions by human granulocyte arginase. Blood 2006;108: 1627–1634.
16. Bronte V, Zanovello P. Regulation of immune responses by L-arginine metabolism. Nat Rev Immunol 2005;5:641–654.
17. Tan AT, Loggi E, Boni C, et al. Host ethnicity and virus genotype shape the hepatitis B virus-specific T-cell repertoire. J Virol 2008; 82:10986–10997.
18. Gehring AJ, Xue SA, Ho ZZ, et al. Engineering virus-specific T cell immunity to target chronic hepatitis B virus infection and HBV-related hepatocellular carcinoma cell lines. J Hepatol 2011;55: 103–110.
19. Chisari F. Cytotoxic T cells and viral hepatitis. J Clin Invest 1997; 99:1472–1477.
20. Maier H, Isogawa M, Freeman GJ, et al. PD-1:PD-L1 interactions contribute to the functional suppression of virus-specific CD8+ T lymphocytes in the liver. J Immunol 2007;178:2714–2720.
21. Fisicaro P, Valdatta C, Massari M, et al. Antiviral intrahepatic T-cell responses can be restored by blocking programmed death-1 pathway in chronic hepatitis B. Gastroenterology 2010;138:682–693.
22. Schurich A, Khanna P, Lopes AR, et al. Role of the coinhibitory receptor cytotoxic T lymphocyte antigen-4 on apoptosis-Prone CD8T cells in persistent hepatitis B virus infection. Hepatology 2011;53:1494–1503.
23. Lopes AR, Kellam P, Das A, et al. Bim-mediated deletion of antigen-specific CD8T cells in patients unable to control HBV infection. J Clin Invest 2008;118:1835–1845.
24. Radziewicz H, Ibegbu CC, Fernandez ML, et al. Liver-infiltrating lymphocytes in chronic human hepatitis C virus infection display an exhausted phenotype with high levels of PD-1 and low levels of CD127 expression. J Virol 2007;81:2545–2553.
25. Das A, Hoare M, Davies N, et al. Functional skewing of the global CD8T cell population in chronic hepatitis B virus infection. J Exp Med 2008;205:2111–2124.
26. Sitia G, Isogawa M, Kakimi K, et al. Depletion of neutrophils blocks the recruitment of antigen-nonspecific cells into the liver without affecting the antiviral activity of hepatitis B virus-specific cytotoxic T lymphocytes. Proc Natl Acad Sci U S A 2002;99: 13717–13722.
27. Groux H, Bigler M, de Vries JE, et al. Inhibitory and stimulatory effects of IL-10 on human CD8+ T cells. J Immunol 1998;160: 3188–3193.
28. Lechner F, Wong D, Dunbar P, et al. Analysis of succesful immune responses in person infected with hepatitis C virus. J Exp Med 2000;191:1499–1512.
29. Maini MK, Boni C, Ogg GS, et al. Direct ex vivo analysis of hepatitis B virus-specific CD8+ T cells associated with the control of infection. Gastroenterology 1999;117:1386–1396.
30. Boettler T, Panther E, Bengsch B, et al. Expression of the interleukin-7 receptor alpha chain (CD127) on virus-specific CD8+ T cells identifies functionally and phenotypically defined memory T cells during acute resolving hepatitis B virus infection. J Virol 2006;80:3532–3540.
31. Isogawa M, Furuichi Y, Chisari FV. Oscillating CD8(+) T cell effector functions after antigen recognition in the liver. Immunity 2005; 23:53–63.
32. Wu J, Meng Z, Jiang M, et al. Hepatitis B virus suppresses toll-like receptor-mediated innate immune responses in murine parenchymal and nonparenchymal liver cells. Hepatology 2009;49:1132–1140.
33. Tan AT, Koh S, Goh W, et al. A longitudinal analysis of innate and adaptive immune profile during hepatic flares in chronic hepatitis B. J Hepatol 2010;52:330–339.
34. Bronte V, Kasic T, Gri G, et al. Boosting antitumor responses of T lymphocytes infiltrating human prostate cancers. J Exp Med 2005; 201:1257–1268.

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Reprint requests

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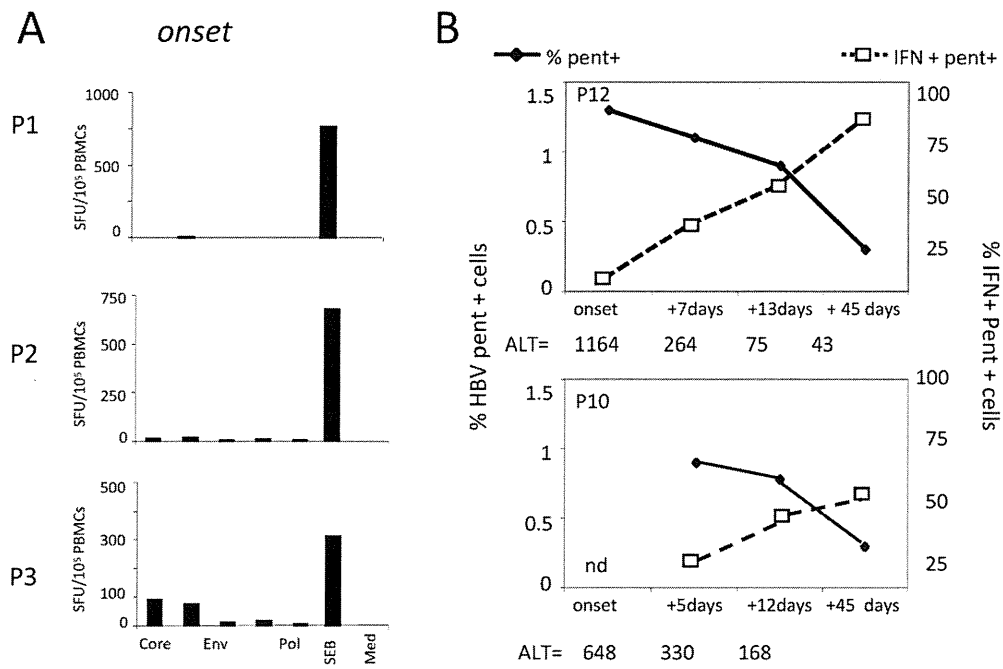
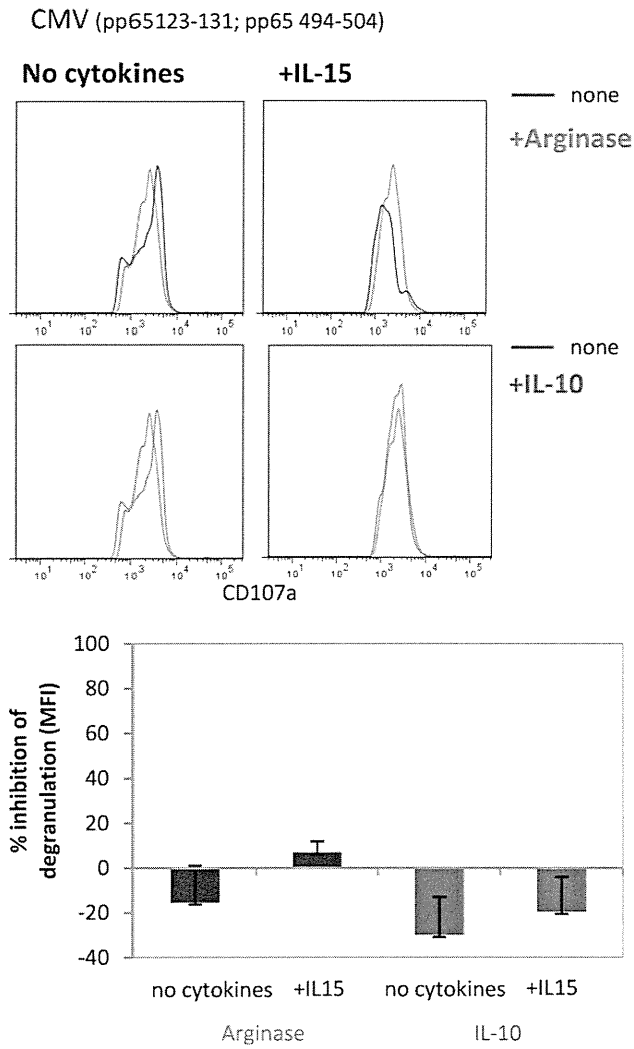
Conflicts of interest

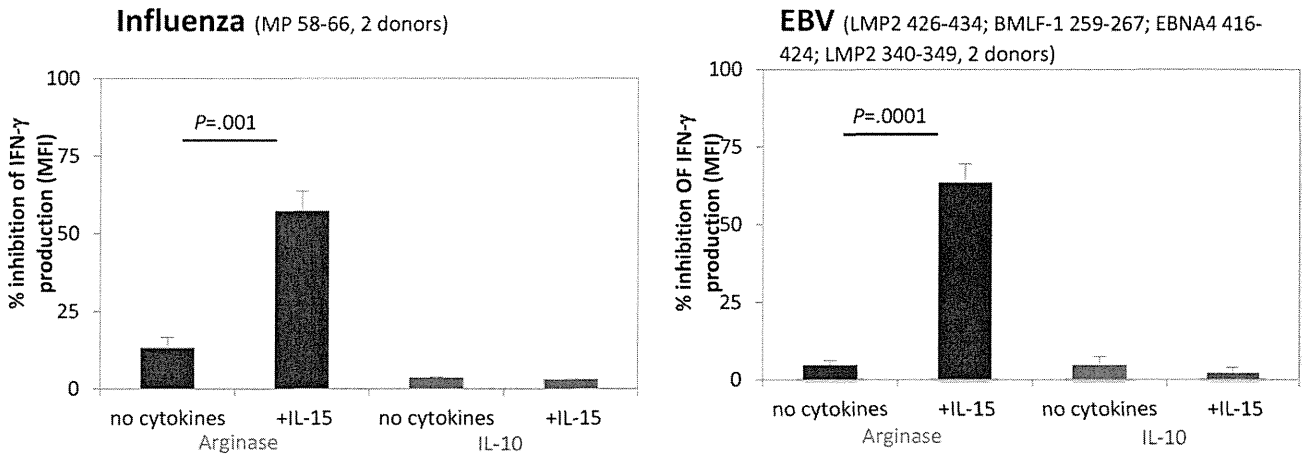
The authors disclose no conflicts.



Supplementary Figure 1. (*A*) Dysfunction of HBV-specific CD8$^+$ T cells in AHB infection. PBMCs of patients with AHB were stimulated with pools of peptides covering the whole HBV proteome, or with staphylococcal enterotoxin B, and the cells were incubated for 12 hours for enzyme-linked immunospot assay. Results of an enzyme-linked immunospot assay for 3 representative acute patients are displayed. (*B*) The frequencies of IFN-γ pentamer–positive CD8$^+$ T cells are plotted together with total HBV core 18-27 pentamer–positive cells in 2 representative patients over time. Corresponding ALT values are shown below each graph.



Supplementary Figure 2. Healthy PBMCs were incubated with either IL-10 (10 pg/mL) or arginase (250 ng/mL) with or without IL-15 for 48 hours, and specific CD107a up-regulation was evaluated. The cells were gated on an EBV or influenza pentamer–positive population. The bar charts indicate the percent inhibition of degranulation (CD107a up-regulation). Experiments were performed in 2 individuals 3 times.



Supplementary Figure 3. Arginase, but not IL-10, inhibits EBV- and influenza-specific responses. Healthy PBMCs were incubated with either IL-10 (10 pg/mL) or arginase (250 ng/mL) with or without IL-15 for 48 hours, and specific IFN-γ production was evaluated. The cells were gated on an EBV or influenza pentamer–positive population. The bar charts indicate the percent inhibition of IFN-γ production. Experiments were performed in 2 individuals 3 times.

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OPEN ACCESS

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]

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.

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.

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

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.

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

Donor hepatocytes†	No of chimeric mice	Inoculum	Test compound	Dose			
				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.

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]

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;

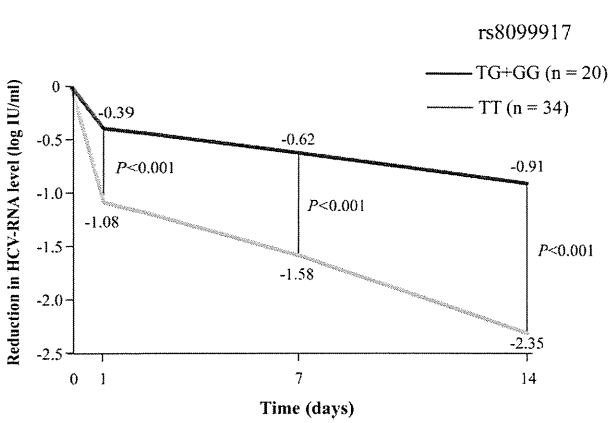

Figure 1 Rapid reduction of median hepatitis C virus (HCV)-RNA levels (log IU/ml) at 1, 7 and 14 days between *IL28B* single nucleotide polymorphisms rs8099917 genotype TT (n=34) and TG/GG (n=20) in HCV genotype 1-infected patients treated with peg-IFN-α plus ribavirin.

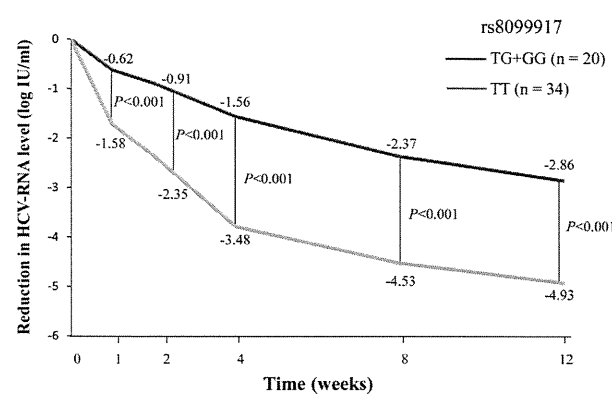

Figure 2 Weekly reduction of median hepatitis C virus (HCV)-RNA levels (log IU/ml) at 1, 2, 4, 8 and 12 weeks between *IL28B* single nucleotide polymorphisms rs8099917 genotype TT (n=34) and TG/GG (n=20) in HCV genotype 1-infected patients treated with pegylated interferon α plus ribavirin.

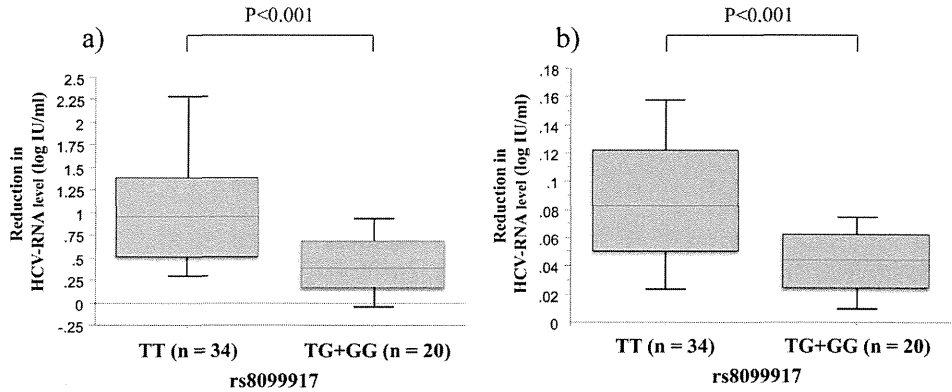

Figure 3 (A) The first-phase viral decline slope per day (Ph1/day) and (B) second-phase viral decline slope per week (Ph2/week) in hepatitis C virus (HCV) genotype 1-infected patients treated with pegylated interferon α plus ribavirin. The lines across the boxes indicate the median values. The hash marks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

−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

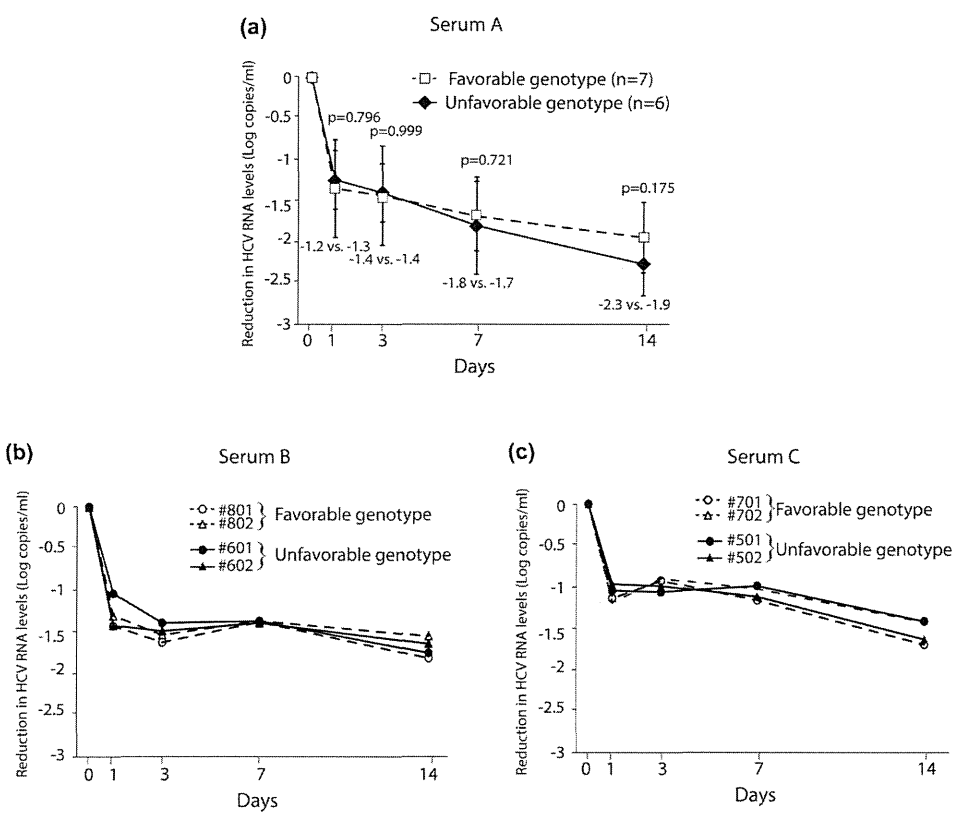

Figure 4 Median reduction of hepatitis C virus (HCV)-RNA levels (log copies/ml) after administering pegylated interferon α to chimeric mice having human hepatocytes containing various single nucleotide polymorphisms around the *IL28B* gene as favourable (rs8099917 TT) and unfavourable (rs8099917 TG) 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.

(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 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

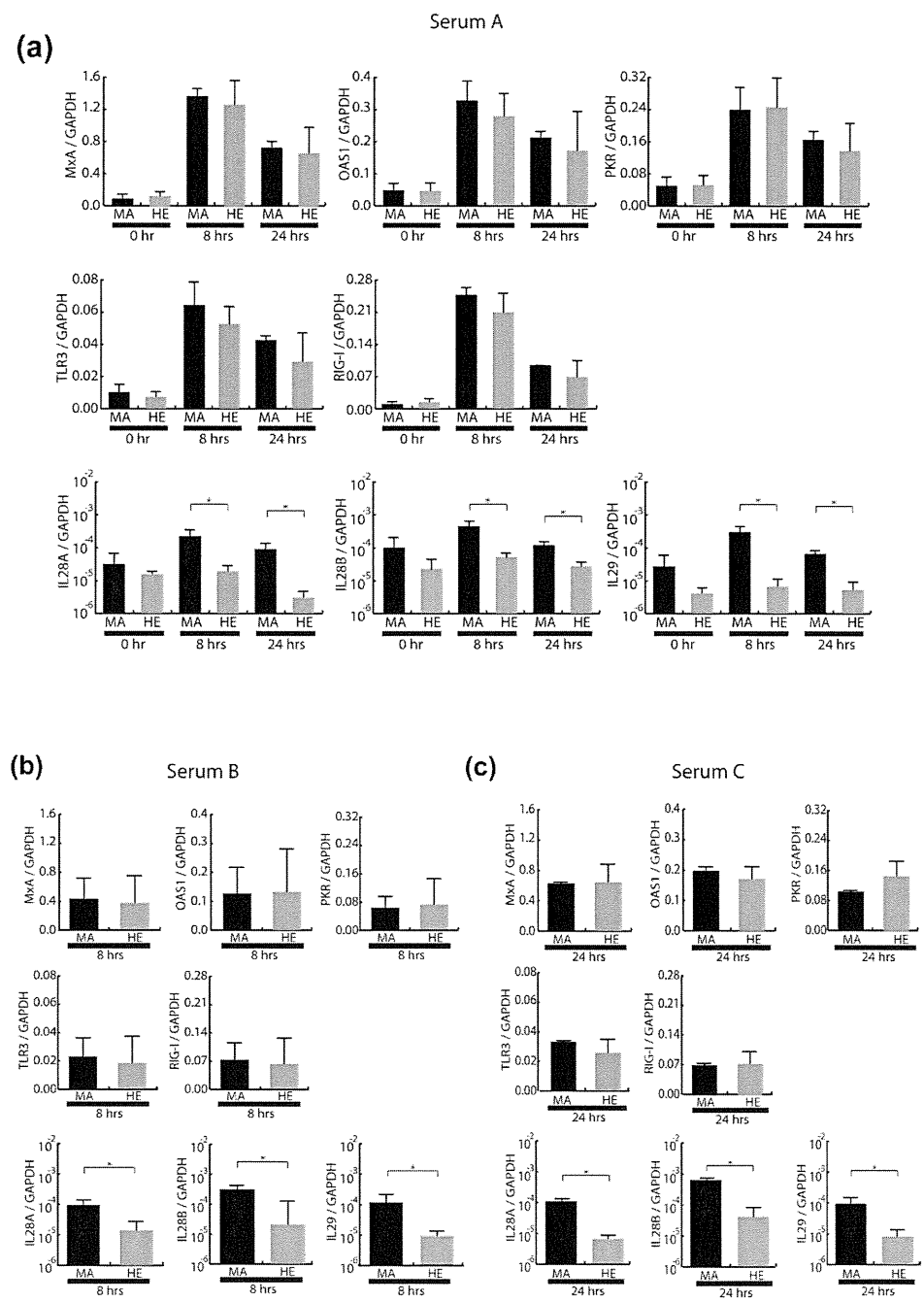

Figure 5 Intrahepatic interferon (IFN)-stimulated gene (ISG) expression levels in the pegylated interferon α (peg-IFN-α)-treated chimeric mice having human hepatocytes containing homozygous favourable allele (rs8099917 TT; MA) and heterozygous unfavourable allele (rs8099917 TG; HE) were measured and 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.

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 *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.

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.