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## Absence of viral interference and different susceptibility to interferon between hepatitis B virus and hepatitis C virus in human hepatocyte chimeric mice<sup>☆</sup>

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**Background/Aims:** Both hepatitis B virus (HBV) and hepatitis C virus (HCV) replicate in the liver and show resistance against innate immunity and interferon (IFN) treatment. Whether there is interference between these two viruses is still controversial. We investigated the interference between these two viruses and the mode of resistance against IFN.

**Methods:** We performed infection experiments with either or both of the two hepatitis viruses in human hepatocyte chimeric mice. Huh7 cell lines with stable production of HBV were also established and transfected with HCV JFH1 clone. Mice and cell lines were treated with IFN. The viral levels in mice sera and culture supernatants and messenger RNA levels of IFN-stimulated genes were measured.

**Results:** No apparent interference between the two viruses was seen *in vivo*. Only a small (0.3 log) reduction in serum HBV and a rapid reduction in HCV were observed after IFN treatment, regardless of infection with the other virus. In *in vitro* studies, no interference between the two viruses was observed. The effect of IFN on each virus was not affected by the presence of the other virus. IFN-induced reductions of viruses in culture supernatants were similar to those in *in vivo* study.

**Conclusions:** No interference between the two hepatitis viruses exists in the liver in the absence of hepatitis. The mechanisms of IFN resistance of the two viruses target different areas of the IFN system.

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**Keywords:** Superinfection; JFH-1; IFN-stimulated genes

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**Abbreviations:** GAPDH, glyceraldehydes-3-phosphate dehydrogenase; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCV, hepatitis C virus; IFN, interferon; OAS, 2',5'-oligoadenylate synthetase; PCR, polymerase chain reaction; SCID, severe combined immunodeficiency; uPA, urokinase-type plasminogen activator.

## 1. Introduction

Both hepatitis B virus (HBV) and hepatitis C virus (HCV) infections are serious health problems worldwide. More than 350 million people are infected with HBV, and more than 170 million people are infected with HCV [1,2]. Both types of hepatitis viruses result in the development of chronic liver infection and lead to death due to liver failure and hepatocellular carcinoma [3]. To date, interferon (IFN) remains one of the most important drugs available for the treatment of both types of hepatitis viral infections. Although it is assumed that IFN suppresses viral replication through the effect of IFN-induced gene products such as mixovirus resistance protein A (MxA), RNA-dependent protein kinase (PKR), and 2',5'-oligoadenylate synthetase (OAS) [4], the precise mechanism of action of these proteins on both hepatitis viruses are unknown.

Coinfection with both viruses leads to a rapid and severe progression of chronic liver disease [5], with a higher risk of hepatocellular carcinoma [6]. Currently, there is a debate about whether or not there is interference between the two hepatitis viruses, with some favoring such interference [7] and others arguing against such a concept [8]. A number of mechanisms can cause interference between viruses. A major mechanism of interference is induction of IFN by one virus to prevent replication of the second virus; however, viruses develop their own strategies to resist the effect of IFN. In clinical practice, practitioners often perceive that reduction of HBV in serum by IFN therapy is poorer compared with HCV. HCV levels in sera of IFN-treated patients decrease relatively rapidly, and a proportion of patients eventually show complete eradication of the virus. Furthermore, the recent use of pegylated IFN (PEG-IFN) in combination with ribavirin has improved the eradication rate [9]. Eradication of HBV by IFN, however, is usually difficult, even when using IFN combined with ribavirin [10].

The mechanisms developed by viruses to resist host innate immunity, including IFN signaling, are well established in some viruses. Such mechanisms involve interruption of IFN signaling by interacting molecules that transduce the signal from the IFN receptor through the Janus kinase (Jak) signal transducer and activator of transcription (STAT) pathway [4]. Viral proteins of paramyxoviruses, for example, inhibit IFN signaling [11]. Several studies have also examined the mechanisms by which HCV resists the host immune system. These include degradation of Cardif adaptor protein by NS3A/4 protease [12]. Generally, expression of HCV protein is associated with inhibition of STAT1 function independent of STAT tyrosine phosphorylation [13]. Additionally, expression of the HCV core protein in cultured cells is associated with increased expression levels of the suppressor of cytokine signaling 3 (SOCS-3) [14]. The NS5A and E2 proteins are both inhibitors of PKR

[15]. These strong actions of HCV against innate immunity are consistent with the high chronicity rate of the virus. IFN, however, effectively reduces HCV replicon in Huh7 cells [16], suggesting that the virus has little potential to disturb the actions of IFN.

In contrast to HCV, the mechanisms of IFN resistance by HBV are poorly understood. To date, only a few studies have reported the molecular mechanisms of HBV resistance against the actions of IFN. The HBV-related resistance to IFN, for example, involves upregulation of protein phosphatase 2A (PP2A) as the primary event, which subsequently leads to inhibition of protein arginine methyltransferase 1 (PRMT1) and reduced STAT1 methylation [17]. In addition to these molecular mechanisms, microarray analyses of serial liver biopsies of experimentally infected chimpanzees showed striking differences in the early immune responses to HBV and HCV. HCV, for example, induced early changes in the expression of many intrahepatic genes, including genes involved in type I IFN response [18], whereas HBV did not induce any detectable changes in the expression of intrahepatic genes in the first weeks of infection [19].

HBV–HCV double infection is a good model to use for assessment of the mechanism of IFN resistance by these two viruses because one can test the effect of IFN on one virus in the presence of the other virus. Recently, Bellecave et al. [20] established a novel *in vitro* model system in Huh7 cells that allowed the analysis of both viruses in a replicating context and reported the absence of direct viral interference. To this end, we used human hepatocyte chimeric mice and cell culture systems in the present study. The results showed that the presence of HBV does not affect the actions of IFN on HCV and vice versa. These results suggest the lack of interference between the two viruses in liver cells and indicate that the reported interference between the two viruses might be via inflammation including death of infected cells by cytotoxic T cells, cytokines including IFN- $\alpha$  and IFN- $\beta$ , and interleukins produced by hepatocytes and infiltrating T cells.

## 2. Materials and methods

### 2.1. Transfection of Huh7 cells with HBV DNA and HCV RNA

Huh7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum at 37 °C and under 5% CO<sub>2</sub>. Cloning of HBV DNA and the plasmid construction were performed as described previously [21]. For production of stably transfected cell lines, Huh7 cells were seeded onto 90-mm-diameter culture dishes. Twenty micrograms of the plasmid pTRE-HB-wt [21] was transfected by the calcium phosphate precipitation method. Twenty-four hours after transfection, the cells were split and cultured in Hygromycin B-DMEM selection medium (300  $\mu$ g/ml; Invitrogen Japan K.K., Osaka, Japan), while 50 colonies were isolated and cultured for identification of virus-producing cell lines. Clones positive

for both hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) were selected and further analyzed for production of HBV particles. Finally, three cell lines that produced more than  $10^5$  copies per milliliter of HBV DNA in supernatant were selected and used for further experiments.

For transfection with HCV RNA, we used pJFH1, which contains the complementary DNA of full-length genotype 2a HCV clone JFH1 downstream of the T7 promoter [22]. *In vitro* synthesis of HCV RNA and electroporation into Huh7 cells were performed as described previously [22,23]. Briefly, cells were treated with trypsin, washed twice with ice-cold RNase-free phosphate-buffered saline, and resuspended in Opti-MEM I (Invitrogen, Carlsbad, CA, USA) at a final concentration of  $7.5 \times 10^6$  cells per milliliter. Then, 10  $\mu$ g of HCV RNA to be electroporated was mixed with 0.4 mL of cell suspension and subjected to an electric pulse (950  $\mu$ F and 260 V) using the Gene Pulser II Electroporation System (Bio-Rad, Hercules, CA, USA). After electroporation, the cell suspension was left for 5 min at room temperature and then incubated under normal culture conditions in a 10-cm-diameter cell culture dish.

## 2.2. Generation of human hepatocyte chimeric mice

Generation of the urokinase-type plasminogen activator (uPA)<sup>+/+</sup> and severe combined immunodeficiency (SCID) <sup>$\beta$ 2<sup>-/-</sup></sup> mice and transplantation of human hepatocytes were performed as described recently by our group [21,23,24]. All mice were transplanted with frozen human hepatocytes obtained from the same donor. Infection, extraction of serum samples, and euthanasia were performed under ether anesthesia. The concentration of serum human serum albumin, which correlates with the repopulation index [24], was measured in mice as described previously [21]. All animal protocols described in this study were performed in accordance with the guidelines of the local committee for animal experiments. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan.

## 2.3. Human serum samples

Human serum samples containing high titers of either HBV DNA ( $5.3 \times 10^6$  copies per milliliter) or genotype 1b HCV ( $2.2 \times 10^6$  copies per milliliter) were obtained from patients with chronic hepatitis with a written informed consent. The individual serum samples were divided into small aliquots and separately stored in liquid nitrogen until use. Chimeric mice were injected intravenously with 50  $\mu$ L of either HBV- or HCV-positive human serum. Some mice were injected with HBV-positive human serum at 6 weeks after injection of HCV-positive human serum.

## 2.4. Analysis of HBV and HCV

HBsAg and HBeAg in culture supernatants were measured by commercially available enzyme-linked immunosorbent assay (ELISA) kits (Abbott Japan, Osaka, Japan). DNA was extracted from these samples by SMITEST (Genome Science Laboratories, Tokyo, Japan) and dissolved in 20  $\mu$ L H<sub>2</sub>O [21,25]. RNA was extracted from serum samples by Sepa Gene RV-R (Sankojunyaku, Tokyo), dissolved in 8.8  $\mu$ L RNase-free H<sub>2</sub>O, and reverse transcribed using random primer (Takara Bio Inc., Shiga, Japan) and M-MLV reverse transcriptase (ReverTra Ace, TOYOBO Co., Osaka, Japan) in a 20- $\mu$ L reaction mixture according to the instructions provided by the manufacturer [23]. HCV core antigen in the culture medium was detected with HCV Ag assay (Ortho-Clinical Diagnostics, Rochester, NY, USA).

## 2.5. RNA extraction and measurement of mRNAs of interferon-induced genes by quantitative reverse transcription-polymerase chain reaction

Total RNA was extracted from cell lines using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). One nanogram of each RNA was reverse transcribed with ReverseTra Ace (TOYOBO Co.) and Random

Primer (Takara Bio, Kyoto, Japan). We quantified the transcripts for MxA, OAS, and PKR. Amplification and detection were performed using ABI PRISM 7300 (Applied Biosystems, Foster City, CA, USA). Results were normalized to the transcript levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

## 2.6. Statistical analysis

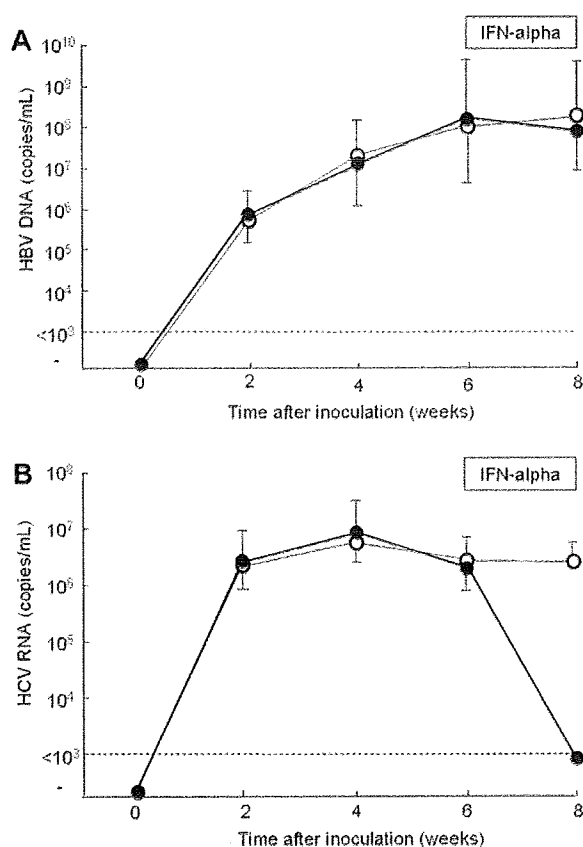
Changes in HBV DNA and HCV RNA in mice sera were compared by Mann-Whitney test and unpaired *t* test. Differences in HBV DNA and HCV core antigen in mice sera and culture supernatants were analyzed by one-way analysis of variance followed by Scheffé's test. A *P* value of <0.05 was considered statistically significant.

## 3. Results

### 3.1. Infection of chimeric mouse with HBV and HCV and susceptibility to interferon

To investigate the interference between HBV and HCV and to examine the effect of IFN on both of these two viruses *in vivo*, we used six human chimeric mice. Each of six mice was inoculated intravenously with 50  $\mu$ L of serum samples obtained from either HBV- or HCV-positive patients. The median HBV DNA level in HBV-positive serum-inoculated mice was  $1.4 \times 10^8$  copies per milliliter (range:  $5.3 \times 10^6$ – $3.6 \times 10^9$  copies per milliliter) at 6 weeks after inoculation (Fig. 1A), similar to our recent observation [21]. Similarly, the median HCV RNA level in HCV-positive human serum-inoculated mice was  $1.0 \times 10^7$  copies per milliliter (range:  $1.2 \times 10^6$ – $0.8 \times 10^7$  copies per milliliter) at 4 weeks after inoculation (Fig. 1B), as reported recently by our group [23]. Six weeks after inoculation, three of six HBV- or HCV-infected mice were treated daily with 7000 IU/g per day of intramuscular IFN- $\alpha$  for 2 weeks. Treatment resulted in a decrease of only 0.3 log in mice serum HBV DNA level compared to that in mice without treatment (Fig. 1A). In contrast, the same therapy resulted in a rapid decrease in HCV RNA to undetectable levels, as confirmed by quantitative polymerase chain reaction (PCR; Fig. 1B).

To investigate the direct interference of the two viruses, we performed double-infection experiments. Ten chimeric mice were first inoculated intravenously with 50  $\mu$ L of HCV-positive human serum samples. Six weeks after HCV infection when the mice developed HCV viremia, 50  $\mu$ L of HBV-positive human serum samples were inoculated intravenously in 5 of 10 HCV-infected mice. All five mice became positive for both HBV and HCV at 2 weeks after HBV infection. No significant decrease in HCV RNA levels was observed in these superinfected mice before or after the development of HBV viremia (Fig. 2A). After HBV infection, there was no apparent decrease in HCV titer (Fig. 2B). Moreover, HBV DNA level in HBV-HCV-coinfected mice was comparable with that of only HBV-infected mice (Fig. 2B). These results sug-



**Fig. 1.** Changes in serum virus titers in mice inoculated with hepatitis B virus (HBV) – positive or hepatitis C virus (HCV) – positive human serum samples. (A) HBV DNA levels in six mice inoculated with HBV-positive serum samples. (B) HCV RNA levels in six mice inoculated with HCV-positive serum samples. Six weeks after inoculation, three of six mice were treated daily with (closed circles) or without (open circles) 7000 IU/g per day of interferon- $\alpha$  intramuscularly for 2 weeks. Mice serum samples were extracted every 2 weeks after inoculation. Data are mean plus or minus standard deviation ( $n = 3$ ). The horizontal dashed line represents the detection limit ( $10^3$  copies per milliliter).

gest no interference between the two viruses in mice, which lack immunocytes known to cause hepatitis.

To further investigate if infection with either of the two hepatitis viruses alters the effect of IFN against the other virus, three HBV–HCV-coinfected mice were treated with IFN- $\alpha$  (Fig. 3A). Such treatment resulted in a rapid decrease in HCV RNA in all mice to undetectable levels as confirmed by quantitative PCR (Fig. 3B). In contrast, no significant decrease in HBV DNA titers was observed in these mice (Fig. 3B). These results are similar to the reduction of HCV RNA and HBV DNA in mice that were infected with either of these hepatitis viruses. These results indicate that HCV is more susceptible to IFN- $\alpha$  than HBV and that each virus does not alter the effect of IFN on the other virus. Because the effect of IFN on HCV was not disturbed by HBV, we assumed that HBV has no effect on the signal from IFN receptor to IFN-stimulated genes. It is possible,

however, that HBV and HCV replicated in different cells in these mice. Because it was impossible to detect HCV protein and RNA in HCV-infected mouse liver by histologic examination, we performed *in vitro* experiments.

### 3.2. Production of both HBV- and HCV-producing cells and the effect of interferon

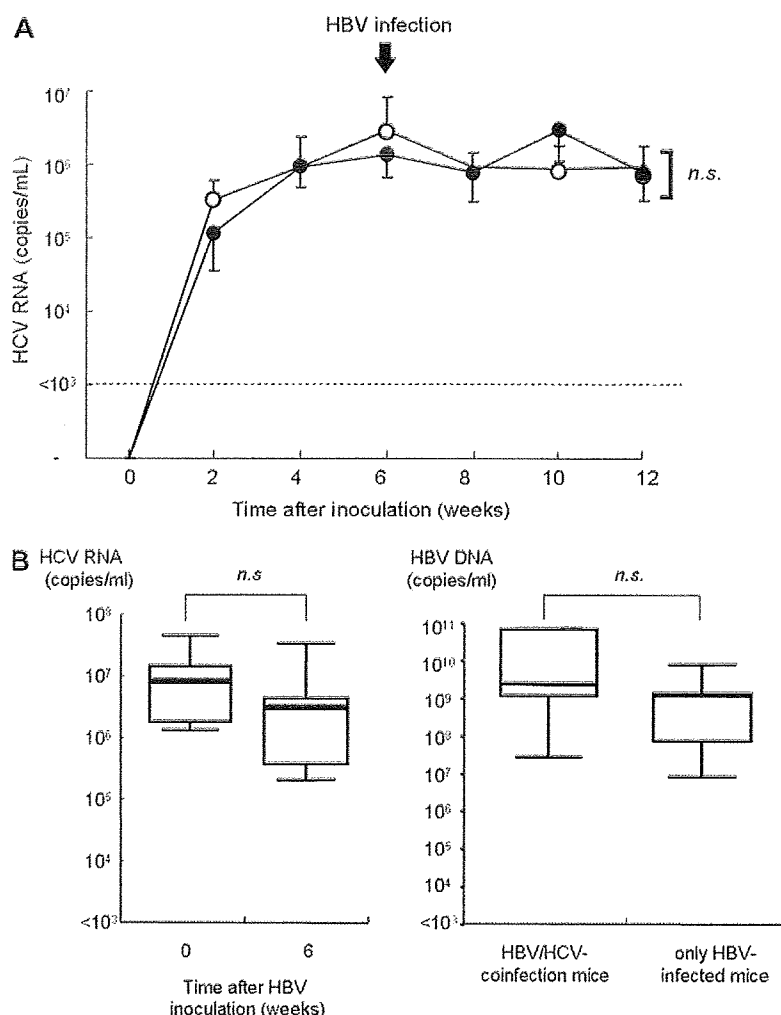
To investigate the effect of IFN on HBV and HCV *in vitro*, we created cell lines that produce both HBV and HCV. First, we established stable HBV-producing Huh7 cell lines. Three cell lines (Clone-39, -42, and -53) that produced HBsAg, HBeAg, and HBV DNA into the supernatant were selected (Table 1). These cell lines continuously produced HBV for more than 3 months (data not shown). Next, JFH1 RNA was transfected into these HBV-producing cell lines to produce both HBV DNA and HCV proteins into the supernatant. HBV DNA levels in the supernatants of these cell lines decreased in Clone-39, increased in Clone-42, and did not change in Clone-53 after JFH1 transfection (Fig. 4A). In contrast, HCV core antigen levels in the supernatants were higher in two of the three cell lines (Clone-39 and -42) than in Huh7 cells, and the level was not different in the remaining cell line (Clone-53) (Fig. 4B). These results indicate that the production of each of the two viruses does not disturb the replication of the other virus.

### 3.3. Effects of interferon on HBV and HCV *in vitro*

The effects of IFN on virus production in both HBV- and HCV-producing cell lines was examined by adding different amounts of IFN- $\alpha$  (0, 50, and 500 IU/mL) into the culture. The mRNA levels of intracellular IFN-stimulated genes such as MxA, OAS, and PKR increased in a dose-dependent manner in all three cell lines as well as in parental Huh7 cells (Fig. 5A). Following the addition of IFN, no apparent reduction of HBV was noted in the supernatant of HBV–HCV-cotransfected cell lines (Fig. 5B). In contrast, the levels of HCV core antigen in the supernatant decreased in all three cell lines treated with IFN, and the decrease was dose-dependent (Fig. 5C).

## 4. Discussion

Although IFN treatment for chronic HCV infection has improved with the advent of PEG-IFN, the rate of viral eradication remains unsatisfactory [9]. The mechanism responsible for failure of IFN to eradicate the virus completely must be clarified. To study the mechanism of viral resistance against IFN, analysis of viral interference may give us some hints because one of the major mechanisms of interference is through the action of IFN.



**Fig. 2.** Comparison of hepatitis C virus (HCV) and hepatitis B virus (HBV) titers in experimentally infected mice. (A) Ten mice were inoculated with HCV-positive serum samples. Six weeks after HCV infection, 5 of the 10 mice were inoculated with HBV-positive human serum samples (closed circles). The remaining five mice (open circles) did not receive HBV inoculation. Data are mean plus or minus standard deviation ( $n = 3$ ). (B) Serum HCV RNA titers in five mice infected with HCV before and at 6 weeks after HBV superinfection (left panel). Serum HBV DNA titers in five mice coinfecting with HBV and HCV were compared with those of five mice with HBV infection only (Fig. 1) at 12 weeks after HCV inoculation (right panel). In these box-and-whisker plots, lines within the boxes represent the median values; the upper and lower lines of the boxes represent the 25th and 75th percentiles, respectively.

Accumulation of mononuclear cells is usually seen in the livers of infected individuals, in association with the state of inflammation. It is thus difficult to examine the interference of hepatitis viruses in infection and replication in liver cells without taking into consideration the effect of these immune cells as well as the chemokines and cytokines produced by these cells. Instead, the present study was designed to examine the interference between HBV and HCV in an experimental setup lacking such inflammatory interferences. The SCID-based human hepatocyte chimeric mouse model is ideal for investigating such interaction. We expected either reduction of HCV after inoculation of HBV in HCV-infected mice or failure to develop HBV viremia or low-level

HBV viremia in these mice due to viral interference; however, no reduction in HCV titers occurred in these mice, and HBV infection developed in a manner similar to that in naïve mice (Fig. 2). We thus confirmed that there is no interference between the two viruses in the absence of immune reaction via the infiltrating lymphocytes in the liver.

Wieland et al. reported that HBV did not induce any genes during entry or expansion in HBV-infected chimpanzee livers and suggested that HBV was a stealthy virus early in the infection [19]. Because no reduction in HCV was noted during and after the development of high-level HBV viremia, we assume that HBV escapes innate immunity via an excellent mechanism without

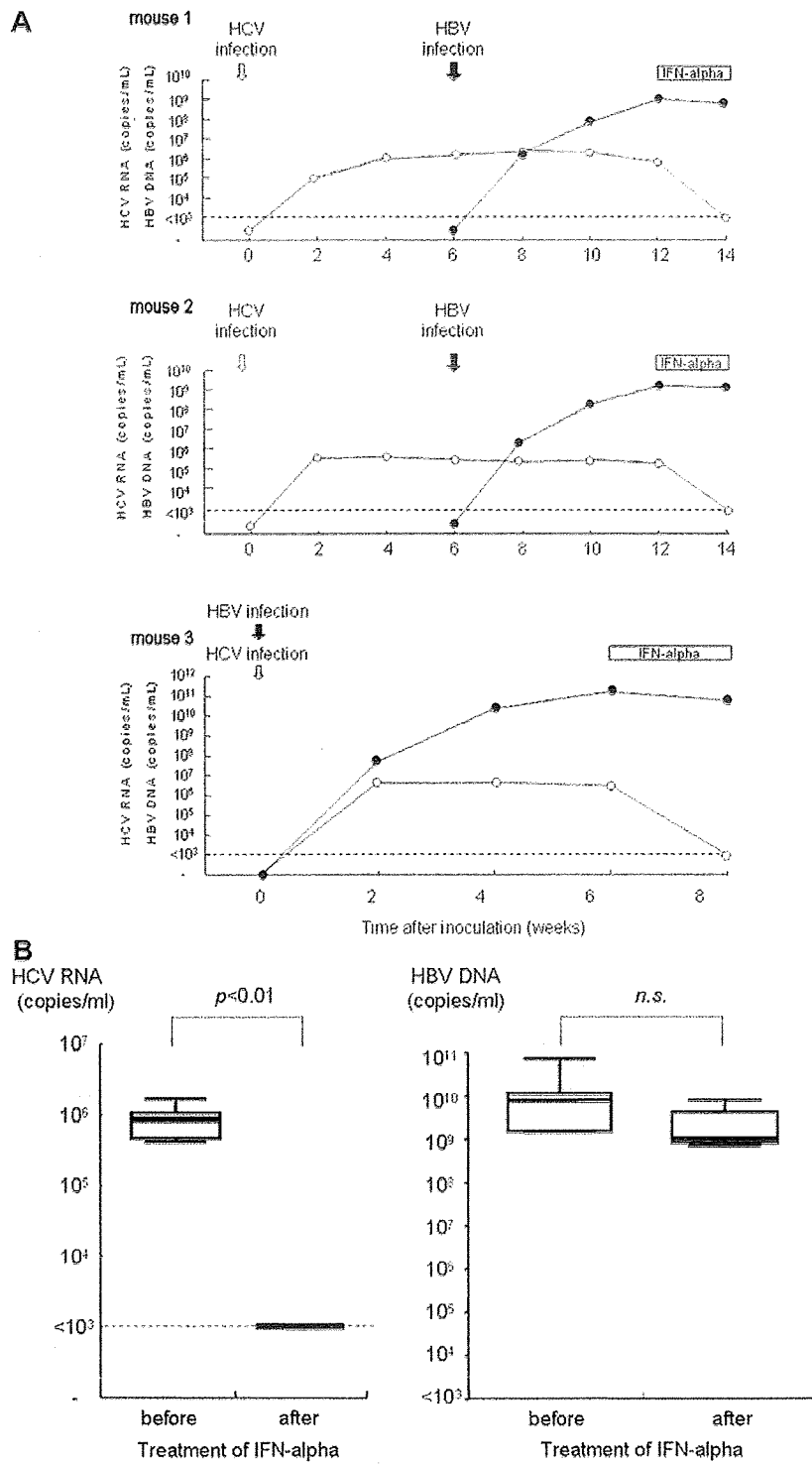


Fig. 3. Changes in serum hepatitis C virus (HCV) RNA and hepatitis B virus (HBV) DNA levels and effects of IFN on HBV–HCV-coinfected mice. Three mice (mouse 1, 2, and 3) were inoculated with both HBV- and HCV-positive human serum samples and treated daily with 7000 IU/g per day of interferon-alpha (IFN- $\alpha$ ) intramuscularly for 2 weeks. Mice sera samples were obtained every 2 weeks after injection, and HCV RNA (open circles) and HBV DNA (close circles) were analyzed by quantitative polymerase chain reaction. (A) The horizontal dashed line represents the detectable limit ( $10^3$  copies per milliliter). (B) Serum HCV RNA and HBV DNA titers in mice before and after 2-week IFN- $\alpha$  treatment. In these box-and-whisker plots, lines within the boxes represent median values; the upper and lower lines of the boxes represent the 25th and 75th percentiles, respectively.

**Table 1**  
Hepatitis B virus (HBV) markers in supernatants of stable HBV-transfected cell lines.

Clone	HBsAg (IU/L)	HBeAg (IU/L)	HBV DNA (log copies per milliliter)
39	0.46	4.57	5.2
42	8.16	1.34	5.3
53	0.08	9.29	5.4

Abbreviations: HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen.

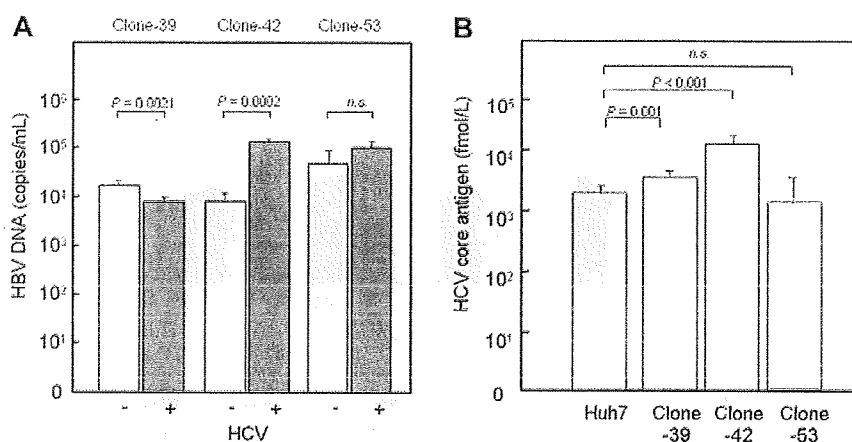
evoking the IFN production system in liver cells. Further study using double-infected mice treated with anti-HBV nucleotide analogs and anti-HCV protease inhibitors should be conducted to confirm the present findings.

With regard to the use of IFN as a treatment, we initially assumed that HBV infection would prevent the effect of IFN on HCV and possibly vice versa in double-infection mice. Unexpectedly, the reduction of HCV by IFN therapy was quite similar in mice infected with HCV only and in those coinfecting with HBV and HCV (Figs. 1 and 3). This finding indicated that HBV does not disturb the effect of IFN through signal transduction from the IFN receptor through the Jak-STAT pathway. It was, however, considered possible that HBV and HCV infect different liver cells in mice and replicated without being affected by each other. It has been reported that the same liver cell could be infected with both HBV and HCV [20,26], but it was difficult in the present study to confirm that these two viruses replicate in the same liver cell of mice because it is difficult to visualize HCV antigen and RNA in pathologic sections of the mouse liver. To address this issue, we transfected HCV to stable HBV-producing cell lines

(Fig. 4). We thought that both HCV and HBV were produced from successfully HCV RNA transfected cells because transfected cells were stable HBV-producing cells. Presence of the both hepatitis viruses in the same hepatocytes has also been shown by a recent report by Bellecave et al. [20]. We showed in our cell line experiments that only HBV-transfected cell lines produced HBV and that cells cotransfected with HBV and HCV did not show a clear effect of HCV replication on HBV production (Fig. 4A). Similarly, stable production of HBV did not alter the replication of HCV (Fig. 4B). These data are consistent with a recent report [20] that showed that HCV could infect cells producing HBV and suggest a lack of interference between the two viruses in liver cells.

Using HCV-transfected HBV-producing cell lines, we demonstrated that presence of HBV did not disturb the actions of IFN on HCV (Fig. 5C). HCV utilizes certain machinery to disrupt the innate immune system; however, once exposed a large concentration of IFN, the virus shows high sensitivity, as shown in the replicon system [16,27]. Thus, HCV seems to have a relatively weak ability to disturb the antiviral actions of IFN compared with HBV. In contrast, HBV showed strong resistance against IFN in cells with diminished HCV replication [28]. The fact that HBV does not disturb IFN signaling but resists the actions of IFN suggests that HBV counteracts the actions of IFN at IFN-induced antiviral product levels.

Although the culture environment is different from the replicon system, the JFH1 strain seems relatively resistant to IFN [29]. This suggests that the core and envelope proteins, which are absent in the replicon system, might play a role in IFN resistance; however, we could not show any effect for HCV infection on the actions of IFN on HBV replication. This finding sug-



**Fig. 4.** Virus titers in supernatants of hepatitis B virus (HBV)-transfected or hepatitis C virus (HCV)-transfected cell lines. Huh7 cells were initially stably transfected with 1.4 genome-length HBV DNA. Three cell lines (Clone-39, -42, and -53) producing HBV DNA into the supernatant were selected. (A) HBV DNA levels in supernatants of HBV-producing cell lines 72 hours after transfection with JFH1 RNA (HCV positive) or control plasmid (HCV negative). (B) HCV core antigen levels in the supernatant of parental Huh7 cells and HBV-producing cell lines 72 h after transfection with JFH1 RNA. Data are mean plus or minus standard deviation ( $n = 3$ ).



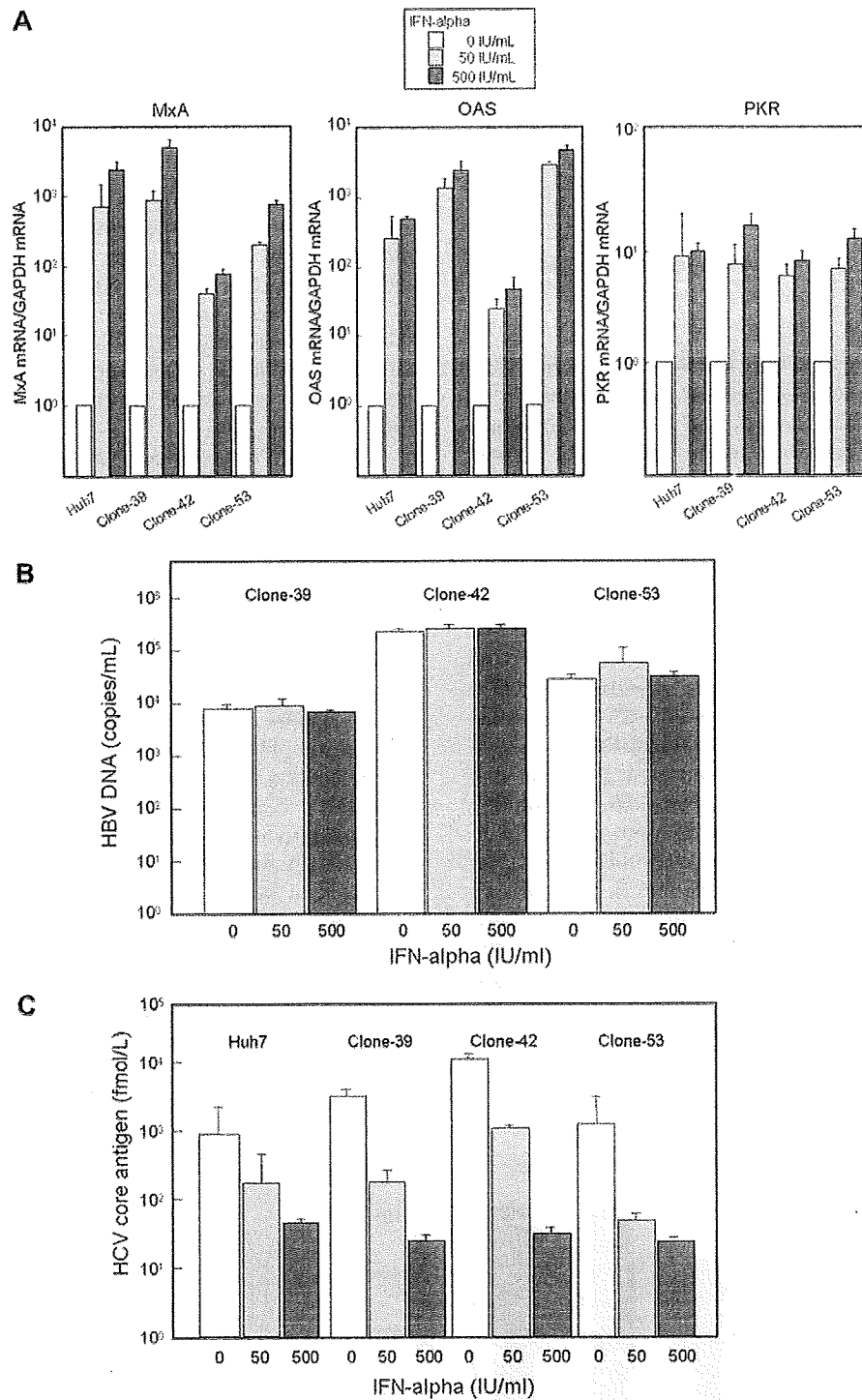


Fig. 5. Effects of interferon (IFN) treatment on hepatitis B virus (HBV) and hepatitis C virus (HCV) *in vitro*. Parental Huh7 cells and three HBV-transfected Huh7 cell lines (Clone-39, -42, and -53) were transfected with JFH1 RNA. Immediately after JFH1 transfection, the cell lines were treated with IFN- $\alpha$  (0, 50, and 500 IU/mL) for 72 h. (A) Intracellular gene expression levels of mixovirus resistance protein A (MxA), 2',5'-oligoadenylate synthetase (OAS), and RNA-dependent protein kinase (PKR) were measured. RNA levels were expressed relative to glyceraldehydes-3-phosphate dehydrogenase (GAPDH) messenger RNA. (B) HBV DNA and (C) HCV core antigen in supernatants were measured. Data are mean plus or minus standard deviation ( $n = 3$ ).

gests that the core and envelope proteins have only a weak effect on IFN resistance.

In clinical practice, HBV shows high resistance against IFN therapy. This is also the case in the cell culture system, as we showed in this study and has been reported in previous studies [20,28]. The mechanism by which hepatitis viruses resist IFN needs to be clarified in order to develop new and effective therapies for eradication of these viruses.

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# Adoptive immunotherapy with liver allograft-derived lymphocytes induces anti-HCV activity after liver transplantation in humans and humanized mice

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After liver transplantation in HCV-infected patients, the virus load inevitably exceeds pre-transplantation levels. This phenomenon reflects suppression of the host-effector immune responses that control HCV replication by the immunosuppressive drugs used to prevent rejection of the transplanted liver. Here, we describe an adoptive immunotherapy approach, using lymphocytes extracted from liver allograft perfusate (termed herein liver allograft-derived lymphocytes), which includes an abundance of NK/NKT cells that mounted an anti-HCV response in HCV-infected liver transplantation recipients, despite the immunosuppressive environment. This therapy involved intravenously injecting patients 3 days after liver transplantation with liver allograft-derived lymphocytes treated with IL-2 and the CD3-specific mAb OKT3. During the first month after liver transplantation, the HCV RNA titers in the sera of recipients who received immunotherapy were markedly lower than those in the sera of recipients who did not receive immunotherapy. We further explored these observations in human hepatocyte-chimeric mice, in which mouse hepatocytes were replaced by human hepatocytes. These mice unfailingly developed HCV infections after inoculation with HCV-infected human serum. However, injection of human liver-derived lymphocytes treated with IL-2/OKT3 completely prevented HCV infection. Furthermore, an *in vitro* study using genomic HCV replicon-containing hepatic cells revealed that IFN- $\gamma$ -secreting cells played a pivotal role in such anti-HCV responses. Thus, our study presents what we believe to be a novel paradigm for the inhibition of HCV replication in HCV-infected liver transplantation recipients.

## Introduction

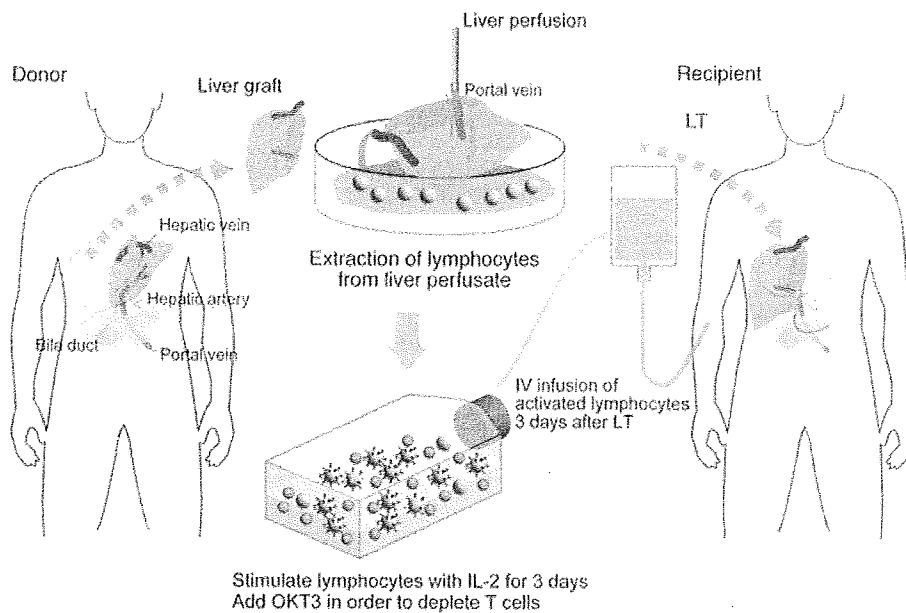
Liver failure and hepatocellular carcinoma (HCC) due to chronic hepatitis C infection are the most common indications for liver transplantation (LT), and the incidences of both have been projected to increase further in the future. Recurrent HCV infection of the allograft is universal, occurs immediately after LT, and is associated with accelerated progression to cirrhosis, graft loss, and death (1, 2). This reflects the suppression of those host-effector immune responses that usually control HCV replication, suggesting that the immunosuppressive environment may play a major role in the rapid progression of recurrent HCV infection after LT (3, 4). Further, the immunosuppressive condition described above is considered to increase the incidence of cancer recurrence after LT in HCC patients. We recently proposed the novel strategy of adjuvant immunotherapy for preventing the recurrence of HCC after LT; this immunotherapy involves intravenously injecting LT recipients with activated liver allograft-derived NK cells (5, 6). Since the immunosuppressive regimen currently used after LT reduces the adaptive immune components but effectively maintains the innate components of cellular immunity (7–9), the augmenta-

tion of the NK cell response, which is thought to play a pivotal role in innate immunity, may be a promising immunotherapeutic approach (6). We confirmed that the IL-2/anti-CD3 mAb-treated (IL-2/OKT3-treated) liver allograft-derived NK cells expressed a significantly high level of the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), which is a critical molecule for tumor cell killing. Further, these cells showed high cytotoxicity against HCC cells, with no such effect on normal cells (5). After obtaining approval from the ethical committee of our institute, we successfully administered adoptive immunotherapy with IL-2/OKT3-treated liver lymphocytes to liver cirrhosis patients with HCC in a phase I trial. Although the long-term benefits of this approach with regard to the control of HCC recurrence after LT remain to be elucidated, this trial provided a unique opportunity to study whether the adoptive administration of IL-2/OKT3-treated liver lymphocytes could also mount an anti-HCV response in HCV-infected LT recipients.

Previous studies have highlighted the important roles of innate lymphocytes in developing immunity against hepatotropic viruses, including HCV (10, 11). In this regard, it is known that patients with chronic HCV infection show diminished NK and NKT cell responses (12–14). In the case of an LT, it has recently been reported that the host CD56<sup>+</sup> innate lymphocyte population,

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**Figure 1**

Schematic outline of adoptive immunotherapy with lymphocytes extracted from liver allograft perfusate. The therapy involved giving an intravenous injection of IL-2/OKT3-treated liver lymphocytes to LT recipients. The lymphocytes were extracted from the donor liver graft perfusate. After 3 days of culture with IL-2 (100 JRU/ml), the activated liver NK cell-enriched lymphocytes were administered to the LT recipients through venous circulation. OKT3 (1  $\mu$ g/ml) was added to the culture medium 1 day before this administration in order to prevent GVHD.

consisting of NK and NKT cells, is appreciably associated with the severity of HCV recurrence after LT (15). These insights into the immunopathogenesis of HCV recurrence indicate that the innate immune components mentioned above are potential targets for therapeutic manipulation. In this study, we have demonstrated for the first time to our knowledge that adoptive immunotherapy with IL-2/OKT3-treated liver lymphocytes, including abundant NK and NKT cells, shows anti-HCV activity after LT, even in an immunosuppressive environment.

## Results

**Adoptive transfer of IL-2/OKT3-treated liver lymphocytes.** The human liver contains a significant number of resident lymphocytes. These cells include abundant CD56<sup>+</sup> NK and NKT cells, many of which differ phenotypically and functionally from the circulating cells (14, 16). In our previous study, we performed ex vivo perfusion of the liver through the portal vein, which was necessary in order to flush blood from the liver graft before implantation. Liver-resident lymphocytes were then extracted from the perfusates (number of lymphocytes extracted from normal liver perfusates,  $0.5 \pm 0.1$  cells per gram of liver weight;  $n = 14$ ) (5). Proportions of CD56<sup>+</sup>CD3<sup>-</sup> NK cells and CD56<sup>+</sup>CD3<sup>+</sup> NKT cells among the lymphocytes extracted from the liver perfusates (NK cells,  $46.4\% \pm 4.2\%$ ; NKT cells,  $17.2\% \pm 2.3\%$ ;  $n = 14$ ) were significantly ( $P < 0.05$ ) higher than those among the lymphocytes derived from the peripheral blood of the same donors (NK cells,  $21.9\% \pm 3.7\%$ ; NKT cells,  $3.8\% \pm 0.9\%$ ;  $n = 14$ ). Extensive preclinical studies have shown that liver allograft-derived resident NK cells mediate remarkably higher cytotoxic activity against HCC cells than do peripheral blood NK cells (5). On this basis, we undertook a clinical trial of adjuvant immunotherapy with IL-2/OKT3-treated liver lymphocytes for preventing the recurrence of HCC after LT in 14 recipients with HCC (Figure 1 and Tables 1 and 2). The therapy involved administering a single intravenous injection of IL-2/OKT3-treated liver lymphocytes to recipients 3 days after LT ( $2-5 \times 10^8$  cells injected per subject). In order to prevent graft-versus-host disease (GVHD),

i.e., to inactivate CD3<sup>+</sup> alloreactive T cells, we added an anti-CD3 mAb, OKT3, to the culture medium a day before the inoculation. During the follow-up period (mean, 23.4 months; range, 10.7-32.9 months), neither any remarkable adverse effects nor rejection episodes occurred. All 14 subjects who received the immunotherapy were alive without recurrence of HCC after LT (including 5 patients with HCC exceeding the Milan criteria; ref. 17). At our institute, the survival rate and recurrence rate of historical control patients with HCC exceeding the Milan criteria were 78% (30 of 37) and 10.8% (4 of 37), respectively. The lymphocytes in the peripheral blood of LT recipients who received immunotherapy in the early postoperative period showed significantly enhanced cytotoxicity against an HCC cell line (HepG2) as compared with those in the peripheral blood of LT recipients who did not receive the therapy in the same period (Figure 2A). Although the gross proportions of NK/NKT cells in the peripheral blood of patients treated with immunotherapy did not differ from those in the peripheral blood of untreated patients, the proportions of TRAIL<sup>+</sup> NK cells significantly increased after immunotherapy in the peripheral blood of the former patients. This increase in the TRAIL<sup>+</sup> NK cells in the peripheral blood lymphocytes was not observed in untreated patients (Figure 2B). Furthermore, there was a significant correlation between the frequency of TRAIL<sup>+</sup> NK cells in the peripheral blood lymphocytes and the NK cytolytic activity of the peripheral blood lymphocytes at 7 days after LT (Spearman rank-order correlation coefficient = 0.54,  $P = 0.01$ ; Figure 2C), indicating the anti-HCC effect of adoptively injected TRAIL<sup>+</sup> NK cells. It would be pertinent to conduct additional clinical trials of this immunotherapy for preventing HCC recurrence after LT.

**Anti-HCV activity after adoptive immunotherapy.** Of the 14 LT recipients who received the immunotherapy, 7 had chronic HCV infection. During the period of this trial, 5 other HCV-infected LT recipients who did not agree to receive immunotherapy served as controls; the background of the controls, including HCV genotype, age, and immunosuppressive therapy, was similar to that of the immunotherapy recipients (Table 3). It has been reported



**Table 1**  
Recipient and tumor characteristics

Patient no.	Age (yr)	Sex	MELD	Hepatitis virus infection	A	HLA B	C	Milan criteria	AFP (ng/ml)	PIVKA-II (AU/ml)	Tumor no.	Maximum tumor size (mm)	Path. vascular invasion	Path. stage	Postop. months	Outcome
1	67	M	19	B	24,-	13,40	03,-	OUT	-	2,584	5	35	-	III	32.9	Alive
2	53	M	16	B	2603,3303	4002,4403	0304,1403	IN	25.3	43	4	11	-	II	31.0	Alive
3	54	M	7	B	0206,3101	3501,5101	0303,1402	OUT	5.7	213	1	26	-	III	29.4	Alive
4	64	F	16	C	2601,2603	3501,4801	0303,-	IN	5.9	142	-	-	-	-	28.5	Alive
5	59	F	14	B	0206,2601	4002,5502	0102,0304	OUT	<5	65	1	13	b1	II	27.8	Alive
6	47	F	8	C	2402,2601	3501,5201	0303,1202	IN	18	46	3	12	-	II	26.2	Alive
7	57	M	29	B	2402,3101	5101,5201	1202,1402	IN	40.3	514	1	25	-	II	25.4	Alive
8	65	F	18	C	1101,2402	5401,5901	0102,-	IN	-	-	3	6	-	II	24.4	Alive
9	60	F	8	-	1101,3001	1302,4006	0602,0801	OUT	32.8	3,026	2	40	w1	IVA	22.7	Alive
10	56	M	8	C	2402,3303	5201,5801	0302,1202	OUT	-	304	11	22	-	III	19.1	Alive
11	56	M	9	C	0207,-	4601,-	0102,-	IN	47	20	3	25	-	III	17.5	Alive
12	58	M	22	C	1101,3101	1501,3501	0102,0415	IN	-	62	1	17	-	I	16.5	Alive
13	59	M	6	C	1101,2402	1507,1501	0303,0401	IN	202.9	19	3	16	-	II	15.8	Alive
14	51	M	16	B	1101,2601	4002,5401	0102,0304	IN	-	29	1	-	-	-	10.7	Alive

The Milan criteria specifies that liver cancer patients with a single tumor of 5 or fewer centimeters in diameter or 3 or fewer tumors, each no more than 3 cm in diameter, and with no macrovascular invasion, can expect an excellent outcome after LT, with only a 10% risk of cancer recurrence (31). AFP, alpha fetoprotein; F, female; M, male; MELD, model for end-stage liver disease; PIVKA-II, protein induced by vitamin K absence; Path., pathological; Postop., postoperative.

that HCV RNA concentrations sharply decrease a day after LT and increase rapidly thereafter (3). In some of the patients, who did not receive the immunotherapy, HCV RNA titers remained lower than that of the pretransplant titer 1 week after LT, suggesting the individual variation of increasing tempo. However, in almost all patients, HCV RNA titers exceeded the pretransplantation levels by 2 weeks after LT. Notably, HCV infection disappeared in 2 LT recipients after the immunotherapy, but this was not observed in the case of any HCV-infected LT recipients who did not receive the therapy. In one of these patients (who had the lowest HCV RNA levels before LT), HCV RNA has not been detected to date (20 months after LT), even with a qualitative assay. In the other patient, HCV RNA became detectable at 2 months after LT. On the other hand, the 2 patients with the highest HCV viral loads did not respond at all to the immunotherapy. Thus, the effects of immunotherapy were dependent on the HCV virus load before LT, probably because of the proportion of effectors and targets. All patients with HCV viremia are currently being treated with pegylated IFN- $\alpha$ 2b and ribavirin. Nevertheless, during the first month after LT, the HCV RNA titers in the sera of LT recipients who received the immunotherapy were statistically lower than those in the sera of LT recipients who did not receive the therapy ( $P < 0.05$ ) (Figure 3). Among the LT recipients who received the immunotherapy, at 2 weeks after LT, HCV RNA remained undetectable in 4 patients (responders), whereas it was detectable in the other 3 patients (nonresponders). The serum ALT levels did not differ between the responders and nonresponders (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI38374DS1), suggesting that the immunotherapy did not inhibit HCV RNA by injuring HCV-infected hepatocytes.

*In vitro evidence to prove the anti-HCV activity of IL-2/OKT3-treated liver lymphocytes by using HCV replicon-containing hepatic cells.* The liver allograft-derived lymphocytes were cultured in complete medium with and without IL-2 for 3 days. This was followed by adding OKT3 to the culture medium 1 day before coculturing the lymphocytes with HCV replicon-containing hepatic cells in a transwell system, at an indicated time. While the freshly isolated liver allograft-derived lymphocytes inhibited HCV replication in the HCV replicon-containing hepatic cells to some extent, the cultivation of these lymphocytes with IL-2/OKT3 markedly promoted anti-HCV activity. Absence of exposure to either IL-2 or OKT3 resulted in reduced anti-HCV activity of the lymphocytes (OKT3 had a more profound influence than IL-2) (Figure 4A). When the lymphocytes were treated with IL-2 alone, the CD56<sup>+</sup> fraction, including NK and NKT cells, that had been isolated by magnetic cell sorting inhibited HCV replication more strongly than the CD56<sup>-</sup> fraction; further, the CD3<sup>-</sup>CD56<sup>+</sup> NK cell and CD3<sup>+</sup>CD56<sup>+</sup> NKT cell subfractions showed equivalent anti-HCV activity (Figure 4, B and C). On the other hand, when the lymphocytes were treated with both IL-2 and OKT3, the CD56<sup>+</sup> and CD56<sup>-</sup> fractions showed similar levels of anti-HCV activity (Figure 4B). After the treatment with IL-2 and OKT3, IFN- $\gamma$  was the predominant cytokine in the culture supernatant of the lymphocytes (Figure 5A), and intracellular IFN- $\gamma$  expression was induced in the CD3<sup>-</sup>CD56<sup>+</sup> NK, CD3<sup>+</sup>CD56<sup>+</sup> NKT, and CD3<sup>+</sup>CD56<sup>-</sup> T cells (Figure 5B). There was no difference between the proportions of TRAIL<sup>+</sup> and TRAIL<sup>-</sup>CD3<sup>-</sup>CD56<sup>+</sup> NK cells producing IFN- $\gamma$  (Supplemental Figure 2). Adding mAb against IFN- $\gamma$  to the coculture of lymphocytes with HCV replicon cells markedly weakened the anti-HCV effects. The incomplete restoration of the anti-HCV effect by anti-IFN- $\gamma$  treat-



**Table 2**  
Donor and graft characteristics

Donor no.	Donor age (yr)	Donor sex	HLA			Relationship	Graft	Graft weight (g)	No. of cells administered ( $\times 10^6$ )
			A	B	C				
1	41	M	24,-	07,40	03,07	Offspring	Right	608	172
2	24	M	2402,2603	4002,2603	0304,5201	Offspring	Right	658	38
3	51	F	0201,2402	0702,3901	0702,-	Spouse	Right	670	129
4	34	M	2601,2603	4001,4801	0303,0401	Offspring	Left	414	143
5	31	M	0206,2402	4002,5401	0102,0304	Offspring	Posterior	702	135
6	53	F	2402,-	5201,5401	0102,1202	Sibling	Right	538	411
7	24	M	2601,3101	4006,5201	0801,1202	Offspring	Right	642	350
8	34	M	1101,-	4001,5401	0102,1502	Offspring	Right	846	229
9	37	M	0201,1101	1501,4006	0702,0801	Offspring	Left	402	811
10	28	M	1101,3303	5502,5801	0102,0302	Offspring	Right	686	517
11	28	M	0207,2402	4601,5201	0102,5201	Offspring	Right	558	414
12	27	M	0201,1101	1501,3501	0303,0415	Offspring	Right	628	509
13	54	F	1101,2402	1501,1507	0303,0401	Sibling	Right	650	460
14	21	F	2601,2603	1501,5401	0102,0303	Offspring	Right	436	382

ment suggests the possibility that other inflammatory cytokines may also be responsible for the anti-HCV effect, although we have not defined them at present (Figure 5C). Thus, the vigorous anti-HCV activity of IL-2/OKT3-treated liver lymphocytes was dependent, at least in part, on their IFN- $\gamma$ -secreting activity.

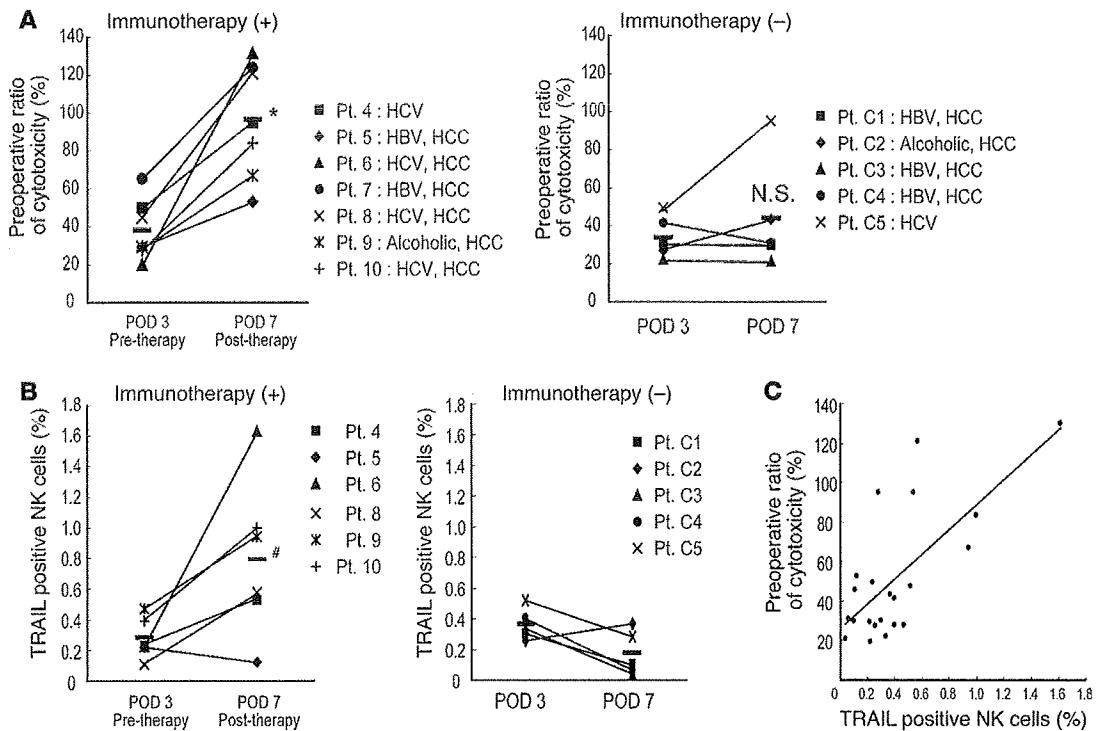
*IFN- $\gamma$ -secreting activity in LT recipients after adoptive immunotherapy.* At 14 days after LT, the number of IFN- $\gamma$ -secreting cells in the peripheral blood of LT recipients who received adoptive immunotherapy was significantly higher than that in the peripheral blood of LT recipients who did not receive immunotherapy during the trial period (Figure 6). This result was consistent with the results of the in vitro studies showing the crucial role of IFN- $\gamma$  produced in IL-2/OKT3-treated liver lymphocytes.

*In vivo evidence to prove the anti-HCV activity of adoptive immunotherapy by using HCV-infected human hepatocyte-chimeric mice.* HCV-infected mice have previously been developed by inoculating HCV-infected human serum into chimeric urokinase-type plasminogen activator-SCID (uPA-SCID) mice with engrafted human hepatocytes (18). This HCV-infected mouse model has been reported to be useful for evaluating anti-HCV drugs such as IFN- $\alpha$  and anti-NS3 protease (19). We also generated a human hepatocyte-chimeric mouse model, in which mouse hepatocytes were almost completely replaced by human hepatocytes (20). These mice consistently developed long-term HCV infections, showing high viral titers after inoculation with HCV genotype 1b-infected human serum (50  $\mu$ l/mouse) (Supplemental Figure 3). Intraperitoneal injection of IL-2/OKT3-treated liver lymphocytes ( $20 \times 10^6$  cells/mouse), at 2 weeks after inoculation with the infected serum, consistently prevented the development of HCV infection in

the human hepatocyte-chimeric mice (Figure 7A). Such anti-HCV effects were countered by anti-IFN- $\gamma$  neutralizing antibodies in some chimeric mice, suggesting the potential role played by IFN- $\gamma$  in the anti-HCV effects of the immunotherapy. The administration of recombinant human IFN- $\gamma$  markedly and consistently prevented the development of HCV infection in the human hepatocyte-chimeric mice. Once the HCV RNA became undetectable in the sera of chimeric mice receiving either IL-2/OKT3-treated liver lymphocytes or recombinant IFN- $\gamma$ , it could not be detected again. The constant levels of human serum albumin in the chimeric mice indicated that neither the immunotherapy nor recombinant IFN- $\gamma$  administration had significant adverse effects on human hepatocytes in those mice (Figure 7B). Once HCV infection had developed in the human hepatocyte-chimeric mice, who showed high titers of HCV RNA in their sera (over  $10^3$  copies/ml) 4 weeks after the inoculation of HCV-infected serum, the preventive effects of the adoptive immunotherapy or recombinant IFN- $\gamma$  on HCV infection were no longer observed (Figure 7C).

**Table 3**  
Characteristics of HCV-infected LT recipients that received and did not receive immunotherapy

No.	Age	Sex	HCV genotype	MELD	Pre-HCV RNA (KIU/ml)	Postoperative months	Immunosuppressant
<b>With immunotherapy</b>							
4	64	F	1b	16	210	29	Basiliximab+FK506+MMF
6	47	F	1b	8	5,000	26	Basiliximab+CsA+MMF
8	65	F	1b	18	2,400	24	Basiliximab+CsA+MMF
10	56	M	1b	8	970	19	Basiliximab+FK506+MMF
11	56	M	1b	9	1,700	17	Basiliximab+FK506+MMF
12	58	M	1b	22	19	17	Basiliximab+FK506+MMF
13	59	M	1b	6	2,200	16	Basiliximab+FK506+MMF
<b>Without immunotherapy</b>							
A	51	M	1b	27	420	42	Basiliximab+FK506+MMF
B	44	M	1b	10	1,600	32	Basiliximab+FK506+MMF
C	54	M	1b	8	180	22	Basiliximab+CsA+MMF
D	56	M	2a	10	470	20	Basiliximab+FK506+MMF
E	57	M	1b	12	3,200	6	Basiliximab+FK506+MMF



**Figure 2**

Adoptive immunotherapy with IL-2/OKT3-treated liver lymphocytes promoted the cytotoxic activity and TRAIL expression of NK cells in LT recipients. (A) The NK cytotoxic activities of the indicated effectors against their target cells were analyzed by the <sup>51</sup>Cr-release assay. The dot plot represents the NK cytotoxic activities of freshly isolated peripheral blood lymphocytes obtained from recipients who received immunotherapy (+) (*n* = 7) and did not receive immunotherapy (-) (*n* = 5) against HepG2 target cells (effector/target [E/T] ratio, 40:1) 3 and 7 days after LT. NK cytotoxic activities are represented as a proportion (percentage) of the preoperative cytotoxicity in each patient. Horizontal lines indicate the mean. Statistical analyses were performed using the 2-tailed, paired Student's *t* test. \**P* < 0.05 for day 7 versus day 3. (B) The frequency of TRAIL<sup>+</sup> NK cells increased remarkably in the peripheral blood of LT recipients who received the immunotherapy. Horizontal lines indicate the mean. Statistical analyses were performed using the Mann-Whitney *U* test. #*P* = 0.013 for immunotherapy group versus untreated group in postoperative day 7. (C) Correlation between TRAIL<sup>+</sup> NK cell ratio and NK cytotoxicity after LT (Spearman rank-order correlation coefficient = 0.54, *P* = 0.01). Statistical analyses were performed using the Spearman rank-order correlation coefficient. The diagonal line indicates a linear regression line. Each dot indicates the cytotoxicity and TRAIL<sup>+</sup> NK cell percentage of each patient. C1, control 1; POD, postoperative day; Pt., patient.

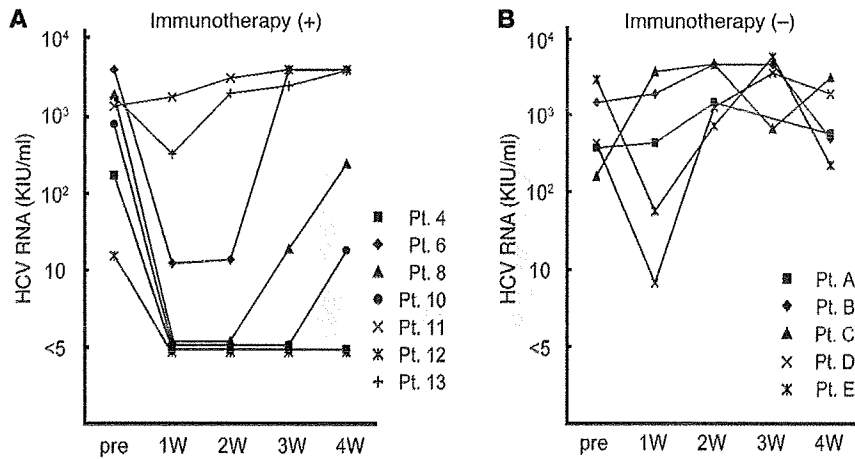
**Discussion**

The consequences of recurrent hepatitis C on the survival of graft and LT recipients can only be avoided by the development of safe and effective antiviral strategies that can not only prevent initial graft infection but also eradicate established hepatitis C recurrence (3, 4). With regard to initial graft infection, the circulating virions infect the liver graft immediately after LT. HCV RNA concentrations usually increase a few days after LT, reflecting active HCV replication in the liver graft. In general, in such an early phase of a viral infection, the first line of host defense may be effective in removing the virus; however, recent reports have indicated that HCV effectively escapes the innate immune system comprising NK and NKT cells, resulting in persistent infection (21, 22). It has been reported that cross-linking of CD81 on NK cells by the major envelope protein of HCV, HCV-E2, blocks NK cell activation, IFN- $\gamma$  production, cytotoxic granule release, and proliferation (21). Engagement of CD81 on NK cells blocks tyrosine phosphorylation through a mechanism that is distinct from the negative signaling pathways associated with NK cell inhibitory receptors for major histocompatibility complex class I molecules (22). These

facts prove that HCV-E2-mediated inhibition of NK cells is an efficient HCV evasion strategy, which involves targeting the early antiviral activities of NK cells and allowing the virus to establish itself as a chronic infection.

We have explored whether CD81 cross-linking-induced inhibitory effects occur even in IL-2-stimulated NK cells. CD81 cross-linking by a mAb specific for CD81 inhibited antitumor cytotoxicity and anti-HCV activity mediated by resting NK cells, but this manipulation did not alter both these activities of IL-2-stimulated NK cells (Supplemental Figure 4). This indicated that exposure to IL-2 before CD81 cross-linking abrogates subsequent inhibitory signals in the NK cells. This would be one mechanism whereby the adoptive immunotherapy with IL-2/OKT3-treated liver lymphocytes inhibited HCV replication at the early phase of infection after LT.

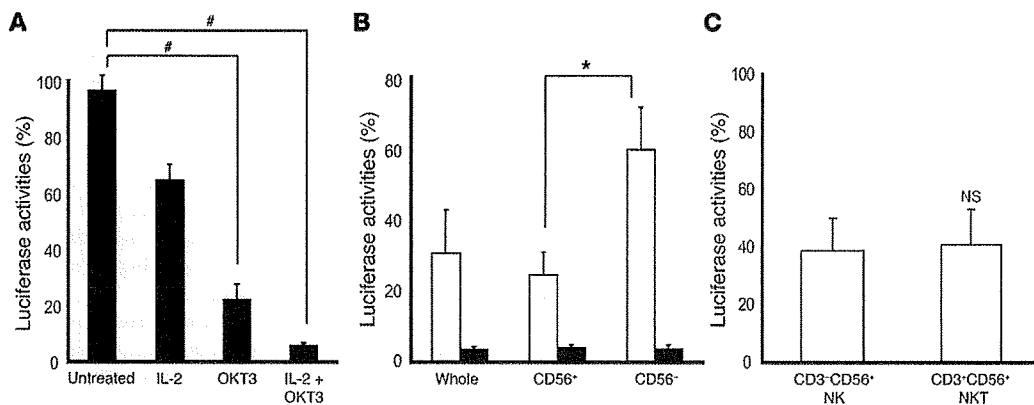
Although the role of NK cells in controlling HCV infection and replication has not been completely elucidated, a recent report has indicated that NK cells do not exert a direct cytolytic effect on the HCV replicon-containing hepatic cells but release IFN- $\gamma$ , suppressing HCV RNA expression (11). The role of IFN- $\gamma$  in the expression of NK cell-mediated anti-HCV activity has been proved by the observa-



**Figure 3** Serial measurement of the HCV RNA titers of LT recipients after LT. The HCV RNA titers in the sera of LT recipients who received immunotherapy were markedly lower than those in the sera of LT recipients who did not receive the therapy during the first month after LT. Each line with a different symbol represents serial HCV RNA titers from an LT recipient who received (+) (A;  $n = 7$ ) and 1 who did not receive (-) (B;  $n = 5$ ) the immunotherapy after LT. KIU, kilo international unit; pre, pre LT; W, week.

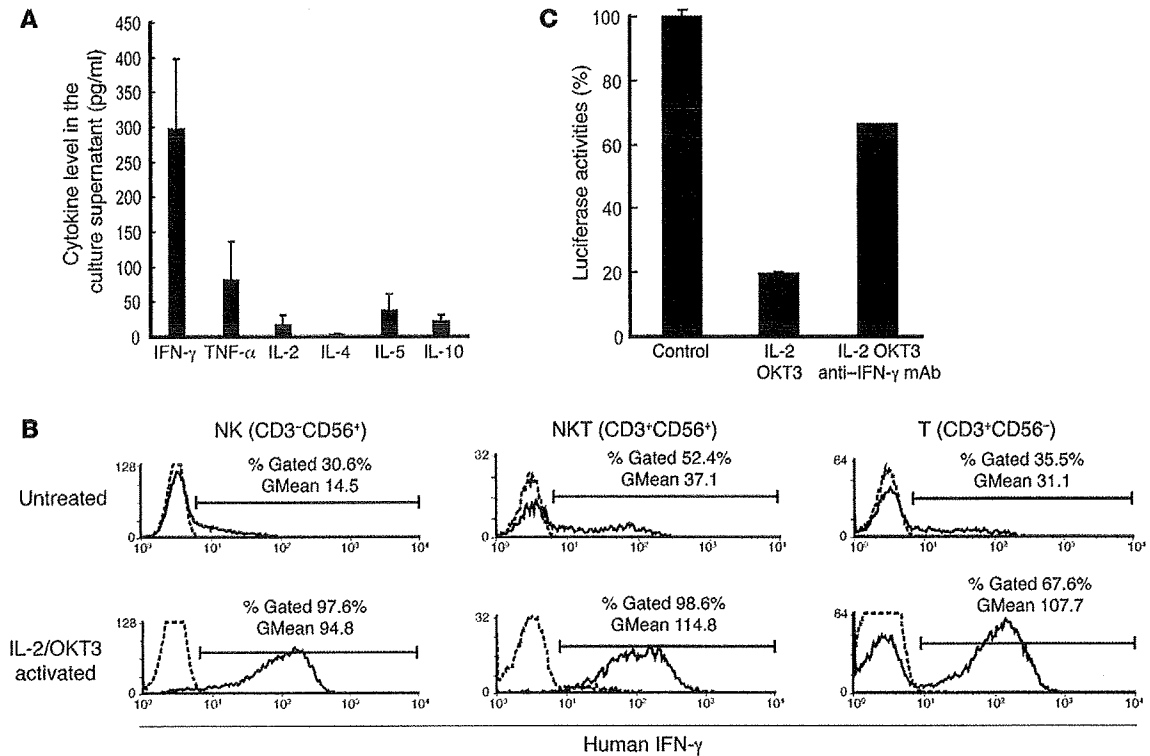
tion that NK cell-conditioned media have an enhanced expression of signal transducer and activator of transcription 1, a nuclear factor that is essential in IFN- $\gamma$ -mediated antiviral pathways. It has also been reported that hepatocytes cultured in NK cell-conditioned media express higher levels of IFN- $\alpha/\beta$ , IFN regulatory factor 3, and IFN regulatory factor 7, confirming that NK cells play a key role in suppressing HCV infection of and replication in human hepatocytes in an IFN-dependent manner (23). Similar to recent reports, in the present study, we demonstrated that the NK cells among the IL-2/OKT3-treated liver lymphocytes released soluble factors, predominantly IFN- $\gamma$ , thus suppressing HCV replication (Figures 5-7). In addition to NK cells, NKT cells are thought to be involved in eliciting innate responses against infection; however, the role

of NKT cells in controlling HCV infection/replication remains unclear. One report has indicated that the number of NKT cells in patients with chronic HCV infection does not differ from that in healthy donors; however, activated NKT cells in HCV-infected patients produce higher levels of IL-13 — but comparable levels of IFN- $\gamma$  — than those in healthy subjects, showing that NKT cells are biased toward T-helper 2-type responses in chronic HCV infection (24). Another recent report has shown that the sustained response of patients with chronic hepatitis C to treatment with IFN- $\alpha$  and ribavirin is closely associated with increased dynamism of NK and NKT cells in the liver, implicating an NKT cell-mediated mechanism in anti-HCV activity (25). Here, we have described that NKT as well as NK cells in the IL-2/



**Figure 4** The cultivation of liver lymphocytes with IL-2/OKT3 markedly promoted anti-HCV activity. (A) Activation by IL-2 and OKT3 significantly promoted the anti-HCV effect of the liver allograft-derived lymphocytes that were cultured in complete medium with and without IL-2 (100 JRU/ml) for 3 days. OKT3 (1  $\mu\text{g/ml}$ ) was then added 1 day before coculturing with HCV replicon cells, at the indicated time. The bar graphs indicate the luciferase activities of the cells in each group. Data are presented as mean  $\pm$  SEM ( $n = 5$ ). Statistical analyses were performed using the Mann-Whitney  $U$  test with Bonferroni correction after the Kruskal-Wallis  $H$  test. # $P < 0.01$  for OKT3 and IL-2/OKT3 treatment versus no treatment. (B)  $\text{CD56}^+$  fraction, including NK and NKT cells, strongly inhibited HCV replication. The culture conditions are described in A. By magnetic cell sorting,  $\text{CD56}^+$  and  $\text{CD56}^-$  fractions were isolated from the activated lymphocytes and analyzed for anti-HCV activity. The bar graphs indicate the luciferase activities of the cells in each group (IL-2-treated group, white bars; IL-2 plus OKT3-treated group, black bars). Whole, whole lymphocytes. Data are presented as mean  $\pm$  SEM ( $n = 5$ ). Statistical analyses were performed using the Mann-Whitney  $U$  test. \* $P < 0.05$  for  $\text{CD56}^+$  fraction versus  $\text{CD56}^-$  fraction. (C) Anti-HCV effect of NK cells was almost identical to that of NKT cells after IL-2 activation. The liver allograft-derived lymphocytes were cultured in complete medium with IL-2 (100 JRU/ml) for 3 days. By magnetic sorting,  $\text{CD3}^+\text{CD56}^-$  (NK) and  $\text{CD3}^+\text{CD56}^+$  (NKT) fractions were isolated from the activated lymphocytes and analyzed for anti-HCV activity. Data are presented as mean  $\pm$  SEM ( $n = 6$ ).





**Figure 5**

Anti-HCV activity of IL-2/OKT3-treated liver lymphocytes was dependent on their IFN- $\gamma$  secretion ability. (A) IFN- $\gamma$  was the major cytokine released from the cultured cells. The bar graphs indicate the concentrations of various cytokines (IFN- $\gamma$ , TNF- $\alpha$ , IL-2, IL-4, IL-5, and IL-10) detected in the coculture supernatant by CBA. Data are presented as mean  $\pm$  SEM ( $n = 3$ ). (B) The effects of IL-2 and OKT3 (100 JRU/ml and 1  $\mu$ g/ml, respectively) on IFN- $\gamma$  production by stimulated CD3<sup>+</sup>CD56<sup>+</sup> NK, CD3<sup>+</sup>CD56<sup>+</sup> NKT, and CD3<sup>+</sup>CD56<sup>-</sup> T cells were evaluated by a combination of cell surface and cytoplasmic mAb staining and subsequent flow cytometric analysis. Histograms represent the log fluorescence intensities obtained upon staining for IFN- $\gamma$  after gating of each fraction. Dotted lines represent negative control staining with isotype-matched mAbs. Horizontal lines indicate the gated portion of lymphocytes. GMean, geometric mean fluorescent intensity. (C) Blocking of IFN- $\gamma$  with mAb (100  $\mu$ g/ml) elucidated the marked role played by IFN- $\gamma$  in producing the anti-HCV effect. The bar graphs indicate the luciferase activities of the cells in each group. Data are presented as mean  $\pm$  SEM of a representative triplicate sample.

OKT3-treated liver lymphocytes could play a vital role in controlling HCV replication in hepatic cells via an IFN- $\gamma$ -associated mechanism (Figures 5 and 6).

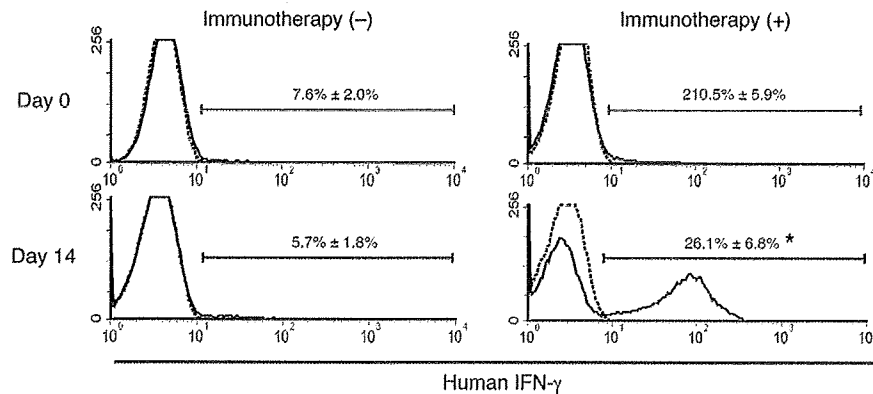
Therefore, in the early phase of HCV reinfection after LT, the effects of IFN- $\gamma$  secretion from adoptively injected liver lymphocytes may include inhibition of HCV virion production, which is probably caused by suppression of viral RNA and protein synthesis without immune lysis of intact hepatic cells. This IFN- $\gamma$  secretion from both CD3<sup>+</sup>CD56<sup>+</sup> NKT cells and CD3<sup>+</sup> T cells was markedly upregulated after treatment with OKT3, which was originally used to prevent GVHD (Figure 5B). This is possibly because of the potent mitogenic activity of OKT3 that induces the activation of CD3<sup>+</sup>CD56<sup>+</sup> NKT cells and CD3<sup>+</sup> T cells. However, the administration of OKT3-coated cells in vivo results in the opsonization and subsequent trapping and/or lympholysis of cells by the reticuloendothelial system (26–28). Thus, GVHD is prevented in LT recipients treated with adoptive immunotherapy.

Our finding that the IL-2/OKT3-treated liver lymphocytes controlled HCV replication via an IFN- $\gamma$ -associated mechanism can lead to the clinical application of recombinant IFN- $\gamma$  for anti-HCV treatment. However, a clinically applicable dose of recombinant IFN- $\gamma$  could not induce significant inhibitory effects on HCV

viremia in the previous study (29). Based on the accumulation of adoptively injected IL-2/OKT3-treated liver lymphocytes in the liver of human hepatocyte-chimeric mice (data not shown), the immunotherapy with the liver lymphocytes would provide sufficient IFN- $\gamma$  to the HCV-infected site.

It has been recently reported that HCV-specific CD8<sup>+</sup> T cells exert strong antiviral effects by both cytopathic and IFN- $\gamma$ -mediated noncytopathic effector functions (30). However, in patients with chronic HCV infection, dysfunction and functional restoration of HCV-specific CD8<sup>+</sup> T cell responses have been reported (31). Since HCV-specific CD8<sup>+</sup> T cell defects may be important in persistent HCV infections, correcting these defects is considered to our knowledge to be a novel approach to treat HCV infection. Further studies are required to investigate whether activation of NK or NKT cells functionally restores HCV-specific CD8<sup>+</sup> T cells.

In conclusion, adoptive immunotherapy using IL-2/OKT3-treated liver lymphocytes containing abundant NK and NKT cells could mount remarkable anti-HCV responses in HCV-infected LT recipients, although its effects were incomplete or transient. Treatment-related improvements, such as defining the best schedule and frequency of cell inoculation and developing more potent effectors, could improve clinical benefits.

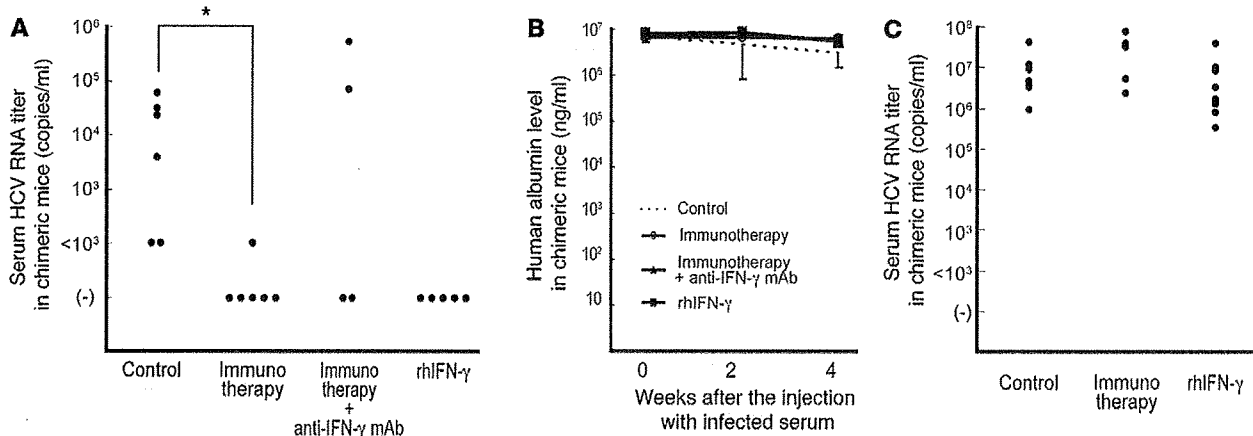
**Figure 6**

Adoptive immunotherapy with IL-2/OKT3-treated liver lymphocytes induced the production of IFN- $\gamma$  in the LT recipients. At 14 days after LT, the number of IFN- $\gamma$ -secreting cells in the peripheral blood of LT recipients treated with the adoptive immunotherapy (+) with IL-2/OKT3-treated liver lymphocytes, including NK and NKT cells, was significantly higher than that in the peripheral blood of untreated LT recipients (-). Histograms represent the proportion (percentage) of IFN- $\gamma$ -positive cells among the mononuclear cells obtained from the peripheral blood of the immunotherapy ( $n = 4$ ) and control group ( $n = 4$ ) LT recipients. Dotted lines represent negative control staining with isotype-matched mAbs. Horizontal lines indicate the gated portion of lymphocytes. Data are presented as mean  $\pm$  SEM. Histogram profiles shown are representative of 4 independent experiments. Statistical analyses were performed using the Mann-Whitney  $U$  test. \* $P < 0.05$  for immunotherapy group versus control group.

## Methods

**Subjects.** All the human liver samples were collected at Hiroshima University Hospital. Tissue specimens were collected after approval from the Institutional Review Board of Hiroshima University and after written informed consent was obtained from the patients. The use of immunotherapy with IL-2/OKT3-treated liver lymphocytes was approved by the Clinical Institutional Ethical Review Board of Hiroshima University. Written informed consent was

obtained from all of the patients. This approach was successfully used in 14 cirrhotic patients with HCC undergoing clinical LT (Tables 1 and 2). Of these 14 patients, 7 had chronic HCV infection. Five other LT recipients with chronic HCV infection did not agree to receive this immunotherapy during the trial period. HCV RNA was qualitatively detected in the sera of these patients by a standardized qualitative RT-PCR assay (Amplicor HCV monitor, version 2.0; Roche Diagnostics) every week during the first month after LT.

**Figure 7**

Adoptive immunotherapy with IL-2/OKT3-treated liver lymphocytes prevented HCV infection in human hepatocyte-chimeric mice. (A) Human hepatocyte-chimeric mice were intravenously injected with human serum samples positive for HCV genotype 1b. Two weeks after injecting the infected serum, the mice were intraperitoneally inoculated with IL-2/OKT3-treated liver lymphocytes ( $20 \times 10^6$  cells/mouse;  $n = 6$ ) for adoptive immunotherapy. When indicated, anti-human IFN- $\gamma$  mAb was injected intraperitoneally 1 day before the immunotherapy ( $n = 4$ ). Intraperitoneal injection of recombinant human IFN- $\gamma$  (rhIFN- $\gamma$ ) was commenced at 2 weeks after injecting the infected serum ( $n = 5$ ). The untreated mice served as controls ( $n = 6$ ). The dot plots represent serum HCV RNA titers in each chimeric mouse 4 weeks after the injecting the infected serum. Statistical analyses were performed using the Mann-Whitney  $U$  test. \* $P < 0.01$  for immunotherapy group versus control group. (B) The lines represent serial changes in human serum albumin levels in the sera of the mice indicated above. Data are presented as mean  $\pm$  SEM. (C) IL-2/OKT3-treated liver lymphocytes ( $20 \times 10^6$  cells/mouse) were intraperitoneally inoculated 4 weeks after the injection with the infected serum ( $n = 5$ ) for adoptive immunotherapy. Intraperitoneal injection of recombinant human IFN- $\gamma$  was commenced 4 weeks after the injecting the infected serum ( $n = 9$ ). The untreated mice served as controls ( $n = 9$ ). The dot plots represent serum HCV RNA titers in each chimeric mouse 6 weeks after injection with the infected serum.



**Isolation of lymphocytes from liver allograft perfusate.** Donor hepatectomy and the transplantation procedure were performed as described previously (32). After hepatectomy, *ex vivo* perfusion of the liver allograft was performed through the portal vein. Liver allograft-derived lymphocytes were isolated by gradient centrifugation with Ficoll-Paque (GE Healthcare Bio-Sciences AB).

**Adoptive transfer of IL-2/OKT3-treated liver lymphocytes.** Liver lymphocytes were cultured with human recombinant IL-2 (100 Japanese reference units/ml [JRU/ml]; Takeda) in complete medium at 37°C in a 5% CO<sub>2</sub> incubator for 3 days. One day before the infusion, 1 µg/ml of OKT3 (Janssen-Kyowa) was added in order to opsonize the CD3<sup>+</sup> fraction. On the day of infusion, the cells were washed twice with 0.9% sodium chloride and resuspended with 5% human serum albumin in 0.9% sodium chloride for injection (Figure 1). The viability of the cells was assessed by the dye-exclusion test, and the cells were checked twice for possible contamination by bacteria, fungi, and endotoxins.

**Cytotoxicity assay.** A <sup>51</sup>Cr-release assay was done as previously described (5), using HepG2 tumor cells (Japanese Cancer Research Resources Bank) as targets. Briefly, <sup>51</sup>Cr-labeled target tumor cells were added for 4 hours at 37°C to effector cells in round-bottomed 96-well microtiter plates (BD Biosciences – Discovery Labware). The percentage of specific <sup>51</sup>Cr release was calculated as follows: % cytotoxicity = [(cpm of experimental release – cpm of spontaneous release)/(cpm of maximum release – cpm of spontaneous release)] × 100. All the assays were performed in triplicate.

**Flow cytometry.** Flow cytometric analyses were performed using a FACSCalibur dual-laser cytometer (BD Biosciences). The following mAbs were used for the surface staining of the lymphocytes: FITC-conjugated anti-CD3 mAb (clone HIT3a; BD Biosciences – Pharmingen); PE-conjugated anti-CD56 mAb (clone B159; BD Biosciences – Pharmingen); and biotinylated anti-TRAIL (biotin-conjugated anti-TRAIL) mAb (clone RIK-2; eBioscience). The biotinylated mAb was visualized using APC-streptavidin (BD Biosciences – Pharmingen). Dead cells identified by light scatter and propidium iodide staining were excluded from the analysis. IFN-γ production in the lymphocytes was measured by a combination of cell surface and cytoplasmic mAb staining and subsequent flow cytometric analysis, as described previously (33).

**Isolation of CD56<sup>+</sup> and CD56<sup>-</sup> fractions and that of NK and NKT cells.** Liver allograft-derived lymphocytes were separated into a CD56<sup>+</sup> fraction – including NK and NKT cells – and a CD56<sup>-</sup> fraction by using auto MACS (Miltenyi Biotec) with anti-human CD56 microbeads (Miltenyi Biotec) according to the manufacturer's instructions. The NK and NKT cells were also isolated by magnetic cell sorting, using the human NK cell isolation kit or human CD3<sup>+</sup>CD56<sup>+</sup>NKT cell isolation kit (Miltenyi Biotec). The purity of the isolated fractions was assessed by flow cytometric analysis, and only the fractions with purities greater than 90% were used for functional studies.

**Coculture with HCV replicon-containing hepatic cells.** An HCV subgenomic replicon plasmid, pRep-Feo, was derived from pRep-Neo (originally, pHC-VIbneo-delS; ref. 34). The pRep-Feo carries a fusion gene comprising firefly luciferase (*Fluc*) and neomycin phosphotransferase, as described elsewhere (35, 36). After culture in the presence of G418 (Invitrogen), pRep-Feo cell lines stably expressing the replicons were established. For coculture experiments, transwell tissue culture plates (pore size, 1 µm; Costar) were used. HCV replicon-containing hepatic cells (10<sup>5</sup> cells) were incubated in the lower compartment with different numbers of lymphocytes in the upper compartment. The hepatic cells in the lower compartments were collected 48 hours after coculture for the luciferase assay. Luciferase activities were

measured with a luminometer (Lumat LB9501; Promega), using the Bright-Glo Luciferase Assay System (Promega).

**Cytometric bead array.** Cytokine (IFN-γ, TNF-α, IL-2, IL-4, IL-5, IL-10) levels in the coculture assay supernatants were measured with the FACSCalibur dual-laser cytometer (BD Biosciences), using a BD Human Th1/Th2 Cytokine Cytometric Bead Array (CBA) Kit according to the manufacturer's instructions.

**Generation of human hepatocyte-chimeric mice.** Generation of the *uPA*<sup>+/+</sup>*SCID*<sup>+/+</sup> mice and transplantation of human hepatocytes were performed as described recently by our group (20, 37). Mouse serum concentrations of human serum albumin correlated with the repopulation index (20), and these were measured as described previously (37).

**In vivo studies using human hepatocyte-chimeric mice.** Human hepatocyte-chimeric mice were intravenously injected with 50 µl of the human serum samples positive for HCV genotype 1b. The serum HCV RNA titer in human hepatocyte-chimeric mice was detected by nested PCR, as previously described (38, 39). All animal protocols described in this study were performed in accordance with the guidelines and with approval of the Ethics Review Committee of Animal Experimentation of the Graduate School of Biomedical Sciences, Hiroshima University. Either 2 or 4 weeks after injecting the infected serum, the mice were intraperitoneally inoculated with IL-2/OKT3-treated liver lymphocytes (20 × 10<sup>6</sup> cells/mouse) for adoptive immunotherapy. When indicated, anti-human IFN-γ mAb (R&D Systems) (1.5 mg/mouse) was injected intraperitoneally 1 day before the immunotherapy. In a separate experiment, intraperitoneal injection of recombinant human IFN-γ (Imunomax-γ; Shionogi & Co. Ltd.) was commenced at either 2 or 4 weeks after injecting the infected serum. IFN-γ was administered as follows: 1 × 10<sup>5</sup> IU on the first day and thereafter 2 × 10<sup>4</sup> IU/day for 13 days.

**Statistics.** Data are presented as mean ± SEM. The statistical differences of the results were analyzed by 2-tailed, paired Student's *t* test, Mann-Whitney *U* test, and Mann-Whitney *U* test with Bonferroni correction after the Kruskal-Wallis *H* test, using the Stat View program. *P* values of 0.05 or less were considered statistically significant.

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