

**Figure 4. Hierarchical clustering analysis of 158 genes associated with IFN treatment.** To analyze the effects of IFN in human hepatocytes, clustering analysis was performed between Group A (without IFN treatment; 4 columns on the left side) and Group B (with IFN treatment; 4 columns on the right side). 152 genes were up-regulated, and 6 genes were down-regulated following IFN treatment. Several well-known interferon-stimulated genes (ISGs), including *CXCL9*, *Mx1*, *ISG20* and *OASL*, were among the up-regulated genes.  
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signaling pathway, in particular, was strongly associated. To verify the effects of HCV infection and/or IFN treatment on gene expression, signal intensities of genes involved in the IFN and JAK-STAT signaling pathways were analyzed. As shown in Figure 6A, among 28 representative genes in the IFN signaling pathway, signal intensities of 22 genes could be analyzed through cDNA microarray analysis. In all genes except *IFNARI*, expression

was up-regulated following HCV infection, whereas IFN responsiveness was suppressed as a result of HCV infection (Figure 6B). 16 out of 22 genes in the JAK-STAT signal pathway could be analyzed via cDNA microarray analysis (Figure 6C), and 12 of the 16 genes were up-regulated following HCV infection, whereas IFN responsiveness was suppressed in 9 genes (Figure 6D).

On the other hand, only 33 genes (21.7%), including several ISGs, such as *GBP1*, *GBP4* and *IFIT3*, remained responsive to IFN in the presence of HCV and were expressed more than 3.0-fold higher in Group D compared to Group C (Table 8). Pathway analysis indicated that these 33 genes were significantly associated with Antimicrobial Response and Inflammatory Response ( $P = 5.22 \times 10^{-10} \sim 1.95 \times 10^{-3}$ ). Changes in mRNA expression for 29 down-regulated genes, including *ISG20*, *WARS*, *Mx1*, *CXCL10*, *IFNGR1* and *IFITM1* were verified by real time PCR (data not shown).

## Discussion

We previously developed a human hepatocyte chimeric mouse model that can be chronically infected with hepatitis B and C viruses [29–31]. This mouse model has enabled us to analyze the effect of viral infection and the response to medication under immunodeficient conditions. Microarray analyses using the human hepatocyte chimeric mouse model with HCV infection have recently been reported, and HCV infection was found to affect expression of genes related to innate antiviral immune response, lipid metabolism and apoptosis via ER stress [34,35]. Whereas these reports were concerned especially with host specific responses to HCV infection, no studies addressing viral modulation of the IFN response have been reported, even though such studies might be important for understanding viral evasion mechanisms in response to IFN therapy and for improving therapy effectiveness for chronic hepatitis C. Therefore, in this study we performed cDNA microarray analysis using a human hepatocyte chimeric mouse model and obtained gene expression profiles to investigate direct influences of HCV infection on IFN responses in human hepatocytes.

First, we evaluated host response to HCV infection in human hepatocytes by comparing profiles between groups A (without HCV infection) and C (with HCV infection). 181 genes were significantly up- or down-regulated following HCV infection. Canonical pathway analysis revealed that genes involved in IFN

**Table 4. The top 35 genes up-regulated with IFN treatment.**

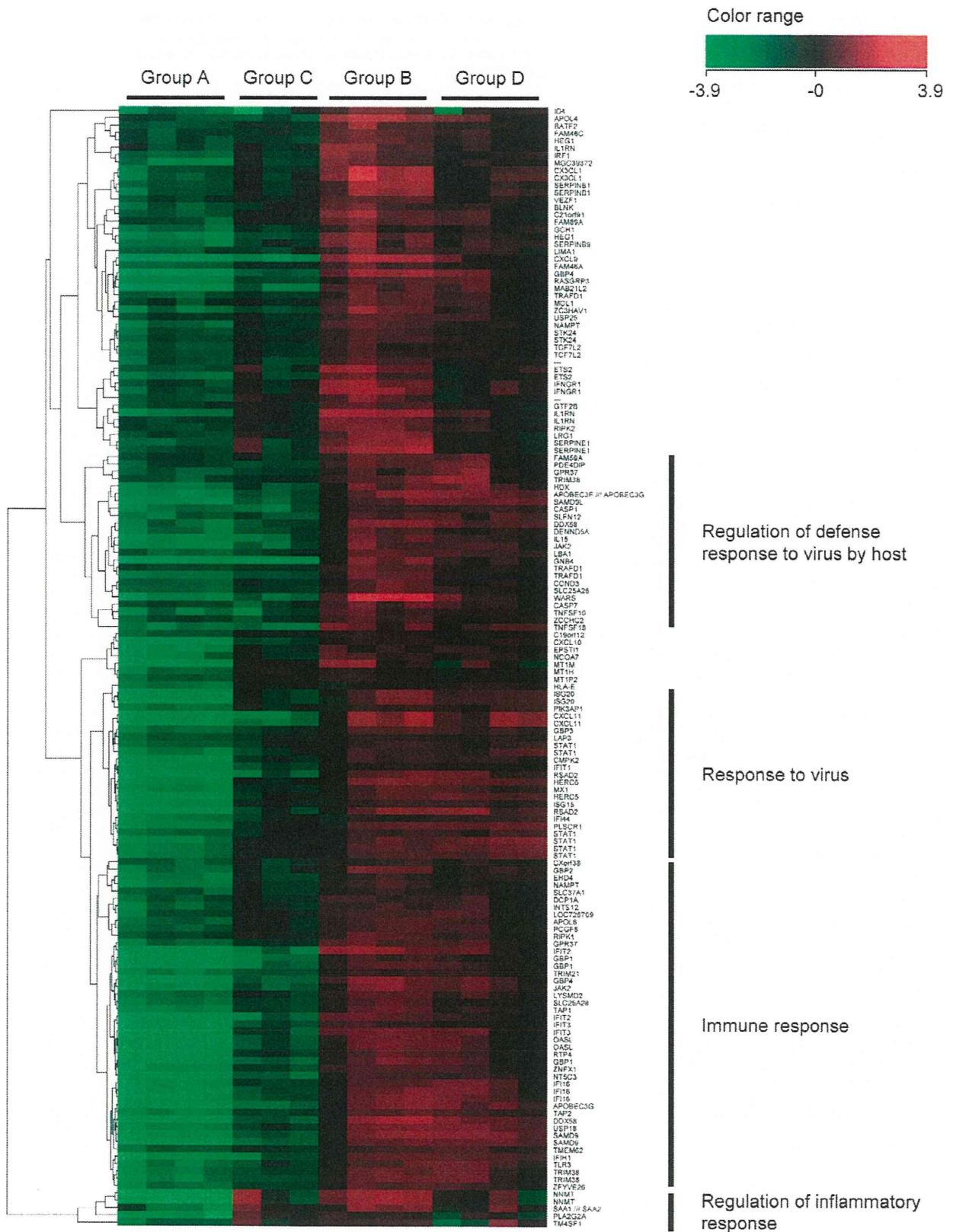
Probe set	Unigene code	Gene symbol	Fold change	P values
211122_s_at	Hs.632592	CXCL11	482.47	1.30E-06
203915_at	Hs.77367	CXCL9	216.26	1.35E-07
242625_at	Hs.17518	RSAD2	101.24	1.26E-05
202237_at	Hs.503911	NNMT	86.80	6.52E-06
217502_at	Hs.437609	IFIT2	75.05	1.73E-06
204533_at	Hs.632586	CXCL10	67.43	2.72E-07
217546_at	Hs.647370	MT1M	46.69	1.12E-04
235175_at	Hs.409925	GBP4	44.94	1.03E-06
204205_at	Hs.660143	APOBEC3G	43.39	5.55E-06
204747_at	Hs.714337	IFIT3	32.73	1.17E-06
218943_s_at	Hs.190622	DDX58	32.19	1.44E-04
33304_at	Hs.459265	ISG20	31.97	3.94E-05
202269_x_at	Hs.62661	GBP1	31.73	5.30E-06
210797_s_at	Hs.118633	OASL	31.59	9.21E-06
200629_at	Hs.497599	WARS	29.65	2.28E-04
206332_s_at	Hs.380250	IFI16	26.37	3.80E-05
210302_s_at	Hs.584852	MAB21L2	24.31	5.31E-07
228531_at	Hs.65641	SAMD9	18.65	1.45E-05
223298_s_at	Hs.487933	NT5C3	17.48	6.65E-06
219863_at	Hs.26663	HERC5	17.02	2.29E-05
225710_at	Hs.173030	GNB4	16.98	3.30E-05
219684_at	Hs.43388	RTP4	16.38	2.55E-05
212657_s_at	Hs.81134	IL1RN	15.18	1.33E-06
219352_at	Hs.529317	HERC6	14.86	1.31E-04
226702_at	Hs.7155	CMPK2	12.50	1.86E-05
205842_s_at	Hs.656213	JAK2	12.49	6.16E-05
230036_at	Hs.489118	SAMD9L	11.98	7.84E-05
214995_s_at	Hs.660143	APOBEC3F/// APOBEC3G	11.62	1.58E-04
823_at	Hs.531668	CX3CL1	11.15	1.07E-04
203153_at	Hs.20315	IFIT1	10.84	5.93E-06
225076_s_at	Hs.371794	ZNFX1	10.40	1.38E-06
213069_at	Hs.477420	HEG1	10.37	3.52E-05
205483_s_at	Hs.458485	ISG15	10.34	1.29E-05
235276_at	Hs.546467	EPST11	10.21	2.10E-04
219209_at	Hs.163173	IFIH1	10.05	4.06E-05

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**Table 5. The top 6 genes down-regulated with IFN treatment.**

Probe set	Unigene code	Gene symbol	Fold change	P value
206211_at		SELE	5.83	6.11E-05
224875_at		C5orf24	5.46	1.11E-04
227256_at	Hs.183817	USP31	3.94	7.27E-05
220070_at	Hs.145717	JMJD5	3.87	5.04E-05
1552482_at	Hs.471162	RAPH1	3.31	1.73E-04
226587_at	Hs.592473	SNRPN	3.17	6.13E-05

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**Figure 5. Hierarchical clustering analysis of 152 genes associated with IFN administration with or without HCV infection.** To analyze the effect of HCV infection on IFN response, gene expression ratios between Groups A and B (gene expression changes by IFN without HCV infection) and those between Groups C and D (gene expression changes by IFN with HCV infection) were compared in the 152 IFN-induced genes. 69.7% of the selected genes showed reduced IFN responsiveness following HCV infection. doi:10.1371/journal.pone.0023856.g005

signaling were the most strongly up-regulated following HCV infection (Figure 3). These findings are mostly consistent with previous studies [36,37]. On the other hand, while no genes involved in lipid metabolism showed any significant induction by HCV infection in this study, Walters et al. reported that HCV-infected chimeric mice exhibited host-specific induction in the expression of lipid metabolism genes [35]. However, we used hepatocytes from a single donor, whereas Walters et al. used hepatocytes from multiple donors, so our results are not necessarily inconsistent with their findings that HCV infection causes induction of lipid metabolism genes in a host-specific manner.

Although several cDNA microarray analyses have also been performed using human liver tissues obtained after hepatic resection, the largest difference between human and chimeric mouse livers is the presence or absence of human lymphocytes. According to the previous report using human liver tissues, genes involved in the innate immune response, as well as cell cycle, growth and communication, were up-regulated by HCV infection [38]. In the present study using SCID-derived mice, genes involved in immune response (e.g. *OAS2*, *Mx1*, *IFI27* and *IFI44L*), cell cycle and growth (e.g. *HERC5*) and cell communication (e.g. *HLA-B*) were similarly up-regulated by HCV infection. However, *Apolipoprotein L*, *Cold autoinflammatory syndrome 1*, *CD97 antigen*, and *HLA-DQ*, which are mainly expressed in lymphocytes, were not

observed to be up-regulated by HCV infection in the chimeric mice. These results demonstrate that the chimeric mouse model accurately reflects intracellular responses to HCV infection without the lymphocytic immune response.

To verify the microarray results, expression data were compared with previously published microarray data on the GEO website (<http://www.ncbi.nlm.nih.gov/geo/>). Previously published microarray data showed up-regulation of *IGFBP7*, *IFI27*, *HLA-B*, and *CD74* in HCV-infected liver tissues compared to non-infected liver tissues (fold changes were 2.1, 2.2, 2.1 and 2.3, respectively) [39]. Likewise, we found that *IFI27*, *HLA-B*, and *CD74* were up-regulated following HCV infection (fold changes were 3.6, 3.3, and 6.6, respectively). These three genes are associated with MHC class I activity, suggesting that intra-cellular immunity in human hepatocytes was activated following HCV infection both in human subjects and in chimeric mouse livers. Metallothionein 1G (*MT1G*) expression was also found to be up-regulated by HCV infection in both the current and published studies [39,40]. Although metallothionein isoforms are associated with collagen deposition [41], members of the metallothionein family may be up-regulated and induce liver fibrosis in response to HCV infection.

In this study, genes associated with Organismal Injury and Abnormalities were found to be up-regulated in response HCV infection (Table 3), and some genes in this category, such as *CXCL9*, *CXCL10* and *IFIT3*, maintained high IFN responsiveness under HCV infection (Table 8). These results suggest that protective responses to fibrosis or hepatic injury were activated at the start of HCV infection and remained activated until complete eradication of HCV from hepatocytes was achieved.

Secondly, we compared gene expression profiles between groups A (without IFN treatment) and B (with IFN treatment) to evaluate IFN response without HCV infection. IFN- $\alpha$  stimulates the intracellular IFN-signaling cascade after binding to the IFN- $\alpha$  receptor and mediates the transcriptional activation of IFN-stimulated genes [42–47]. More than 3.0-fold up-regulation was observed 6hrs after IFN treatment in 152 genes. Known ISGs such as those in the *CXCL* family (*CXCL9*, *CXCL10* and *CXCL11*), the *IFIT* family (*IFIT2* and *IFIT3*) and the *APOBEC* family (*APOBEC3G*) were included among the top 20 genes up-regulated following IFN treatment (Table 4). The *APOBEC* family is well known to have anti-viral effects by inducing genomic hypermutation in human immunodeficiency virus and hepatitis B virus [48–57]. *APOBEC3G* expression has been reported to be elevated in patients infected with HCV [58], although it is not clear whether *APOBEC3G* can block HCV replication. On the other hand, *CXCL9* and *IFIT3* were reported to relate to liver fibrosis in chronic hepatitis C patients. Serum *CXCL9* concentrations correlated with the levels of fibrosis in chronic hepatitis C patients, and *CXCL9* has been shown to exert anti-fibrotic effects *in vitro* and *in vivo* [59]. *IFIT3* expression is also reportedly up-regulated in the transition from mild to moderate fibrosis [60]. The results of this study suggest that IFN treatment might lead not only to HCV eradication but also help to prevent and repair liver fibrosis by inducing these key molecules.

We focused on the 152 genes up-regulated (> 3.0 fold) as a result of IFN administration and evaluated the effect of HCV infection on IFN response among these genes. As shown in Table 8, although several ISGs still showed high response to IFN

**Table 6.** The top 20 genes in which IFN-induced up-regulation is inhibited following HCV infection.

Probe Set ID	Gene symbol	Fold change		P value
		HCV infection (-)	HCV infection (+)	
235175_at	GBP4	44.94	5.50	2.93E-07
231577_s_at	GBP1	24.60	4.89	6.15E-07
218943_s_at	DDX58	32.19	5.56	1.26E-05
226702_at	CMPK2	12.50	2.13	2.35E-05
225973_at	TAP2	7.07	2.84	3.31E-05
229450_at	IFIT3	20.66	2.74	6.37E-05
217739_s_at	NAMPT	5.91	1.95	6.51E-05
213797_at	RSAD2	69.70	4.50	7.01E-05
210797_s_at	OASL	31.59	3.53	1.02E-04
218508_at	DCP1A	3.56	1.65	1.36E-04
228531_at	SAMD9	18.65	5.87	1.45E-04
204804_at	TRIM21	5.37	2.69	1.47E-04
219209_at	IFIH1	10.05	4.69	1.67E-04
219684_at	RTP4	16.38	2.55	1.98E-04
239186_at	MGC39372	7.61	2.57	2.27E-04
219211_at	USP18	8.72	3.74	2.40E-04
225076_s_at	ZNF1	10.40	2.91	2.91E-04
204698_at	ISG20	31.29	3.33	3.00E-04
223192_at	SLC25A28	5.05	2.20	3.25E-04
228439_at	BATF2	4.59	1.83	3.28E-04

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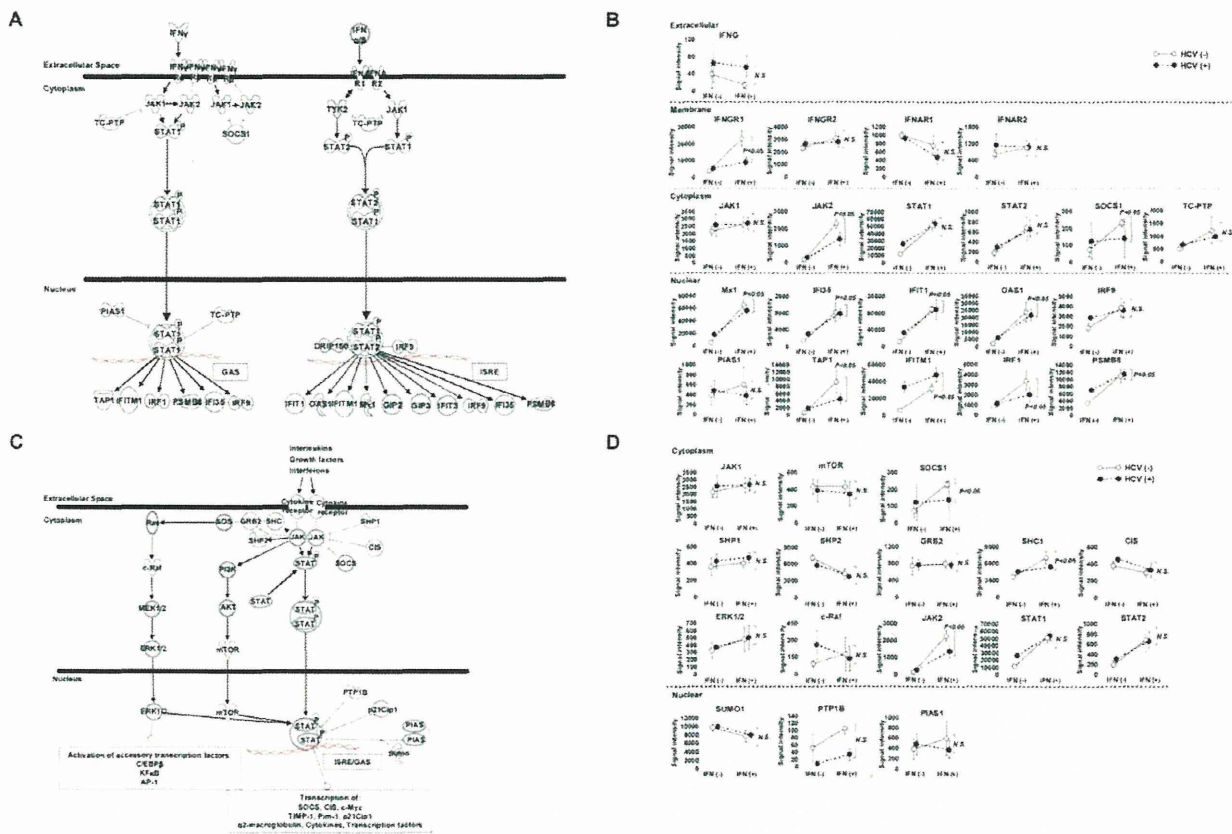
**Table 7.** The top 5 canonical pathways associated with the 106 genes in which IFN response is suppressed by HCV infection.

Category	P value	Ratio	Associated genes
Interferon Signaling	4.41E-11	7/30 genes	IFIT1, IFIT3, IFNGR1, IRF1, MX1, STAT1, TAP1
Type I Diabetes Mellitus Signaling	1.08E-04	5/119 genes	HLA-E, IFNGR1, IRF1, RIPK1, STAT1
Antigen Presentation Pathway	5.29E-04	3/39 genes	HLA-E, TAP1, TAP2
Primary Immunodeficiency Signaling	1.64E-03	3/63 genes	BLNK, TAP1, TAP2
Activation of IRF by Cytosolic Pattern Recognition Receptors	2.95E-03	3/73 genes	ISG15, RIPK1, STAT1

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treatment in the presence of HCV infection, 7 genes in the IFN Signaling pathway became unresponsive (Table 6). Reduction in IFN responsiveness was also observed for *STAT1* (4.27 fold in the

absence of HCV to 2.29 fold in the presence of HCV,  $P=4.04 \times 10^{-4}$ ), as well as 5 of 7 genes downstream of *STAT1* (*IFIT1*, *IFIT3*, *IRF1*, *MX1*, and *TAP1*). As shown in Figure 3, IFN



**Figure 6. Changes in expression of genes in the IFN and JAK-STAT signaling pathways under HCV infection and/or IFN administration.** A) An overview of the IFN signaling pathway consisting of 26 representative genes is shown. Genes illustrated as gray shapes were not included in this study. B) Relative expression levels of genes with/without HCV infection and/or IFN administration were plotted (closed dots: with HCV infection; open dots: without HCV infection) using microarray data. The slopes of the dashed and solid lines represent IFN responsiveness with and without HCV infection, respectively. In 21 of the 22 examined genes in the IFN signaling pathway, signal intensities increased and IFN responsiveness was repressed following HCV infection. Student's t-test was used for statistical analysis. C) An overview of the JAK-STAT signaling pathway consisting of 22 representative gene products is shown. Genes illustrated as gray shapes were not included in this study. D) Relative expression levels of genes with/without HCV infection and/or IFN administration were plotted using microarray data (closed dots: with HCV infection; open dots: without HCV infection). Signal intensities increased following HCV infection in 16 of 22 genes in the JAK-STAT signaling pathway, and IFN response was suppressed in 9 genes. Statistical analysis was performed using Student's t-test. doi:10.1371/journal.pone.0023856.g006

**Table 8.** The 33 genes that remained more than 3-fold up-regulated following IFN treatment in HCV-infected mice.

ID	Symbol	Location	Type(s)
214995_s_at	APOBEC3F	unknown	enzyme
204205_at	APOBEC3G	Nucleus	enzyme
206011_at	CASP1	Cytoplasm	peptidase
204533_at	CXCL10	Extracellular Space	cytokine
210163_at	CXCL11	Extracellular Space	cytokine
203915_at	CXCL9	Extracellular Space	cytokine
218943_s_at	DDX58	Cytoplasm	enzyme
231577_s_at	GBP1 (includes EG:2633)	Cytoplasm	enzyme
235175_at	GBP4 (includes EG:115361)	Cytoplasm	enzyme
225710_at	GNB4	Plasma Membrane	enzyme
1553646_at	HDX	unknown	other
213069_at	HEG1	unknown	other
206332_s_at	IFI16	Nucleus	transcription regulator
219209_at	IFIH1	Nucleus	enzyme
217502_at	IFIT2	unknown	other
204747_at	IFIT3	Cytoplasm	other
205992_s_at	IL15	Extracellular Space	cytokine
204698_at	ISG20	Nucleus	enzyme
205841_at	JAK2	Cytoplasm	kinase
210302_s_at	MAB21L2	unknown	other
223298_s_at	NT5C3	Cytoplasm	phosphatase
205660_at	OASL	unknown	enzyme
205801_s_at	RASGRP3	Cytoplasm	other
242625_at	RSAD2	unknown	enzyme
228531_at	SAMD9	unknown	other
230036_at	SAMD9L	unknown	other
219885_at	SLFN12	Nucleus	enzyme
206271_at	TLR3	Plasma Membrane	transmembrane receptor
214329_x_at	TNFSF10	Extracellular Space	cytokine
221371_at	TNFSF18	Extracellular Space	cytokine
203610_s_at	TRIM38	unknown	other
219211_at	USP18	Cytoplasm	peptidase
200629_at	WARS	Cytoplasm	enzyme

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signaling was activated in the presence of HCV, and the expression of *STAT1* was more than 3.0 fold up-regulated by HCV infection (data not shown). *STAT1* expression was highest in mice with both HCV infection and IFN treatment, but downstream genes such as *MX1*, *IFIT1* and *IFIT3* showed reduced IFN response. Sarasin-Filipowicz et al. reported that IFN-induced *STAT1* phosphorylation was stronger in rapid responders than in non-rapid responders [61]. Reduced induction of genes downstream of *STAT1* by IFN under HCV infection might reflect reduced phosphorylation of *STAT1*, although we did not quantify *STAT1* phosphorylation in this study.

Recently, an *IL-28B* genetic polymorphism strongly associated with response to IFN- $\alpha$  plus ribavirin combination therapy [12], as well as with hepatic ISG expression [62], was identified. Further studies using chimeric mice transplanted with hepatocytes carrying different genotypes of candidate genes such as *IL-28B* will be important in order to elucidate possible mechanisms underlying host-specific responses.

In conclusion, we performed cDNA microarray analysis using HCV-infected human hepatocyte chimeric mice, which allowed us to analyze the direct effects of IFN treatment and HCV infection without the confounding effects of the lymphocytic immunological response. These results might provide molecular insights into possible mechanisms used by HCV to evade IFN-induced immune responses, as well as suggest novel therapeutic targets and a potential new indication for interferon therapy. Further analysis of the genes identified in our study would be worthwhile in order to improve efficacy of the therapy for chronic hepatitis C.

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## Author Contributions

Conceived and designed the experiments: MT YF NH MI ST KC. Performed the experiments: MT YF NH FM 'T'. Analyzed the data: MT

YF YZ MO TK HA DM. Contributed reagents/materials/analysis tools: MT NH MI FM TT KC. Wrote the paper: MT YF ST HO CNH KC.

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## Effects of Hepatitis B Virus Infection on the Interferon Response in Immunodeficient Human Hepatocyte Chimeric Mice

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**Complementary DNA microarray analysis of human livers cannot exclude the influence of the immunological response. In this study, complementary DNA microarray analysis was performed under immunodeficient conditions with human hepatocyte chimeric mice, and gene expression profiles were analyzed by hepatitis B virus (HBV) infection and/or interferon treatment. The expression levels of 183 of 525 genes upregulated by interferon treatment were significantly suppressed in response to HBV infection. Suppressed genes were statistically significantly associated with the interferon signaling pathway and pattern recognition receptors in the bacteria/virus recognition pathway ( $P = 1.0 \times 10^{-8}$  and  $P = 1.2 \times 10^{-8}$ , respectively). HBV infection attenuated virus recognition and interferon response in hepatocytes, which facilitated HBV escape from innate immunity.**

Chronic hepatitis B virus (HBV) infection is associated with the development of virus-related liver diseases, including chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. Interferon  $\alpha$  (IFN- $\alpha$ ) has been used for the treatment of chronic hepatitis B, and many large clinical trials and meta-analyses have

demonstrated the effectiveness of interferon [1–3]. However, the effect of IFN- $\alpha$  therapy is unsatisfactory, and the molecular basis for tolerance to IFN- $\alpha$  is not clearly defined.

DNA microarray technology has enabled genome-wide analysis of gene transcript levels with the use of clinical tissues and animal models, which has yielded insights into the molecular features of several liver diseases [4–6]. However, it has been difficult to determine whether the changes in gene expression were caused by viral interference or by the human immune response, because all of these studies that used clinical and experimental samples were analyzed under the influence of adaptive immune responses. Recently, Mercer and colleagues developed a human hepatocyte chimeric mouse model [7]. These mice were derived from severe combined immunodeficiency (SCID) mice, which are severely immunocompromised, and the mouse liver cells were extensively replaced with human hepatocytes [7, 8]. With the use of this chimeric mouse model, in which HBV can continuously infect human hepatocytes, the effect of drugs and the response of viral infection can be analyzed in human hepatocytes under immunodeficient conditions [9]. In this study, we performed microarray analysis with human hepatocyte chimeric mouse livers to assess the direct impacts of HBV infection and IFN treatments on gene expression profiles. We successfully demonstrated that HBV infection attenuated the expression of IFN-stimulating genes under immunodeficient conditions, which suggests that HBV proteins might afford escape mechanisms from cellular innate immunity.

### METHODS

A serum sample was obtained from a HBV carrier after obtaining written informed consent for the donation and evaluation of the blood sample. The inoculum was positive for Hepatitis B surface and Hepatitis B e antigens, with slightly elevated levels of serum alanine aminotransferase and high-level viremia (HBV DNA load, 7.1 log copies/mL). The studied patient was infected with HBV genotype C. The experimental protocol conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the Hiroshima University Hospital ethical committee (approval ID: D08-9).

The uPA<sup>+/+</sup>/SCID<sup>+/+</sup> mice were prepared and the human hepatocytes were transplanted as described elsewhere [8]. The experiments were performed in accordance with the guidelines of the local committee for animal experiments at Hiroshima University.

Sixteen chimeric mice, in which >90% of the liver tissue was replaced with human hepatocytes, were divided into

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4 experimental groups. Group A contained 4 mice that were neither infected with HBV nor treated with IFN. Group B consisted of 3 mice that were treated with IFN- $\alpha$  for 6 h (7,000 IU per gram of body weight) just before being humanely killed but were not infected with HBV. Mice in groups C and D were inoculated via the mouse tail vein with human serum containing  $6 \times 10^6$  copies of HBV. After inoculation, we collected mouse serum samples every 2 weeks and analyzed HBV DNA titers by real-time polymerase chain reaction (PCR) and human albumin levels by means of a human albumin enzyme-linked immunosorbent assay quantitation kit (Bethyl Laboratories), as described elsewhere [9]. Virus and human albumin titer levels are shown in Supplementary data 1. All 9 mice developed measurable viremia 4 weeks after inoculation. Eight weeks after inoculation, 4 of the 9 infected mice (group C) were humanely killed without IFN treatment and the remaining 5 mice (group D) were humanely killed after 6 h of IFN- $\alpha$  treatment (7,000 IU per gram of body weight). The mice were infected, had serum samples extracted, and were killed humanely under ether anesthesia, as described elsewhere [8].

All 16 chimeric mice were killed humanely, and human hepatocytes were finely dissected from the mouse livers and stored in liquid nitrogen after submerging in RNA later solution (Applied Biosystems). Total RNA was extracted with TRIzol reagent (Invitrogen) and labeled with cyanine 3 by use of a low RNA input linear amplification kit (Agilent Technologies) after amplification. Cyanine-3-labeled complementary RNA was hybridized to a 44-K whole human genome oligo microarray (Agilent). Detailed protocols are described in Supplementary data 2.

Gene expression profiles were analyzed using GeneSpring GX software (version 10.0.2; Tomy Digital Biology). The detailed protocol is described in Supplementary data table 3. Complete linkage hierarchical clustering analysis was applied using Euclidean distance, and differentially expressed genes were annotated using information from the Gene Ontology (GO) Consortium. Global molecular networks and comparisons of canonical pathways were generated using Ingenuity Pathway Analysis (IPA) software (version 8.6; Ingenuity Systems).

Total RNA was extracted from the implanted human hepatocytes in the mouse livers by use of an RNeasy mini kit (Qiagen) and was reverse transcribed. The selected messenger RNA (mRNA) was quantified by real-time PCR using the 7300 real-time PCR system (Applied Biosystems), and the expression of glyceraldehyde-3-phosphate dehydrogenase served as a control. The amplification protocol and primer sequences are described in Supplementary data 4 and 5.

## RESULTS

To analyze the direct effects of IFN in human hepatocytes, we compared the gene expression profiles between groups A (mice

without IFN treatment) and B (mice with IFN treatment). Of the 1403 genes that remained after screening with the Welch *T* test, 685 genes showed a  $>3.0$ -fold change between groups. Of these 685 genes, 525 genes were up-regulated and the other 160 genes down-regulated by IFN. The top 20 IFN-regulated genes are listed in Supplementary data table 6. GO analysis revealed that 8 (40%) of the top 20 genes that were upregulated with IFN treatment were related to immune response.

To analyze the effect of HBV infection in human hepatocytes, we compared the gene expression profiles between groups A (mice without HBV infection) and C (mice with HBV infection). Among the 1,714 genes that remained after screening, 373 genes showed a  $>3.0$ -fold change between groups. Of these 373 genes, 159 genes were up-regulated and the other 214 genes down-regulated by HBV. The top 20 HBV-regulated genes are listed in Supplementary data table 7. Several oncogenic genes such as growth differentiation factor 15 and glial cell derived neurotrophic factor were included in the top group. Most of the top 20 genes that were downregulated with HBV infection were associated with transcriptional regulation.

To examine whether HBV infection may alter the effect of IFN response in human hepatocytes, we compared gene expression profiles among all groups. As mentioned above, 525 genes were upregulated by  $>3.0$ -fold by IFN in the absence of HBV infection. A comparison of groups C (mice with HBV infection but no IFN treatment) and D (mice with both HBV infection and IFN treatment) revealed that 183 (34.9%) of the 525 genes showed statistically significantly reduced IFN response with HBV infection ( $P < .01$ ) (Supplementary data 8A). The top 20 genes in which IFN response was significantly changed by HBV infection are shown in Table 1. The mRNA expression levels of 11 selected genes among the 183 genes with reduced IFN response were also analyzed by real-time PCR, and the reductions in IFN response by HBV infection were verified (Supplementary data 8B). Additionally, we used IPA software to analyze the influence of HBV infection on the IFN response of these 183 genes by means of a pathway-oriented approach. Pathway analysis revealed that several pathways were affected by HBV infection (Table 2). The IFN response was statistically significantly attenuated by HBV infection in the pathways related to IFN signaling and pattern recognition of bacteria and viruses ( $P = 1.0 \times 10^{-8}$  and  $P = 1.2 \times 10^{-8}$ , respectively).

## DISCUSSION

Elsewhere we have demonstrated a human hepatocyte chimeric mouse model that can be chronically infected with hepatitis B and C viruses [9–11]. This mouse model facilitates analysis of the effect of viral infection and the response to medication under immunodeficient conditions. In this study, we performed complementary DNA microarray analysis using the chimeric mouse model and obtained gene expression profiles to analyze



**Table 1. Genes With Interferon Responsiveness Downregulated by Hepatitis B Virus (HBV) Infection**

Gene symbol	GenBank accession no.	Function	Fold change in expression level		<i>P</i>
			Without HBV infection	With HBV infection	
ENST00000322831	None	Unknown	4.52	-1.45	$4.15 \times 10^{-7}$
AA593970	AA593970	EST	9.70	1.61	$5.58 \times 10^{-7}$
THC2533996	None	Unknown	3.74	-2.50	$6.97 \times 10^{-7}$
LOC388532	None	Unknown	3.11	-2.48	$1.61 \times 10^{-6}$
<i>ZNF267</i>	NM_003414	Transcription regulator	7.66	1.79	$2.30 \times 10^{-6}$
<i>ZNF217</i>	NM_006526	Transcription regulator	3.69	1.03	$3.62 \times 10^{-6}$
<i>CRSP3</i>	NM_015979	Transcription regulator	7.50	-1.02	$4.06 \times 10^{-6}$
MGC39372	BC025340	Hypothetical protein	30.92	7.03	$5.74 \times 10^{-6}$
BF972140	BF972140	EST	16.91	4.71	$5.78 \times 10^{-6}$
LOC731599	XR_015536	Hypothetical protein	3.17	-4.18	$8.58 \times 10^{-6}$
LOC645676	AK126559	Hypothetical protein	3.76	1.35	$9.13 \times 10^{-6}$
THC2650457	None	Unknown	78.07	6.28	$1.29 \times 10^{-5}$
<i>ZNF24</i>	NM_006965	Transcription regulator	3.69	1.36	$1.64 \times 10^{-5}$
<i>CCDC68</i>	NM_025214	Unknown	5.88	-2.83	$1.89 \times 10^{-5}$
<i>SP110</i>	NM_004510	Transcription regulator	5.00	10.77	$2.00 \times 10^{-5}$
FLJ21272	AK024925	Hypothetical protein	14.70	2.49	$3.18 \times 10^{-5}$
<i>PLEKHF1</i>	NM_024310	Unknown	6.65	1.84	$4.70 \times 10^{-5}$
AK026418	AK026418	Unknown	9.50	2.58	$5.02 \times 10^{-5}$
hCG_1790262	XM_001133847	Unknown	3.13	-2.94	$6.25 \times 10^{-5}$
<i>CEBPD</i>	NM_005195	Transcription regulator	8.16	1.56	$7.03 \times 10^{-5}$
<i>FLJ20273</i>	NM_019027	RNA binding	3.37	1.11	$7.11 \times 10^{-5}$

**NOTE.** *P* values were analyzed by the Welch *T* test. *CEBPD*, CCAAT/enhancer binding protein (C/EBP) delta; *CCDC68*, coiled-coil domain containing 68; *CRSP3*, mediator complex subunit 23 (*MED23*); EST, expressed sequence tag; *FLJ20273*, RNA binding motif protein 47 (*RBM47*); *PLEKHF1*, pleckstrin homology domain containing, family F (with FYVE domain) member 1; *SP110*, SP110 nuclear body protein; *ZNF24*, zinc finger protein 24; *ZNF217*, zinc finger protein 217; *ZNF267*, zinc finger protein 267.

the direct influence of HBV infection and IFN- $\alpha$  treatment on human hepatocytes.

To avoid contamination with mouse tissue, human hepatocyte chimeric mice, in which liver tissue is largely (>90%) replaced by human hepatocytes, were used in the present study. However, a small amount of mouse-derived cells, such as interstitial cells, bile duct cells, and vascular cells, still remain in the chimeric mouse livers. Because of high homology between the human and mouse genomes, the signals from microarray analyses may be influenced by cross-hybridization with mouse mRNA. It is difficult to produce uPA<sup>+/+</sup>/SCID<sup>+/+</sup> mice >10 weeks old without hepatocyte transplantation, and a previous study demonstrated that it is feasible to use microarray analysis in a functional genomics analysis of chimeric mice [12]. Therefore, to compensate for the contamination, the mice in group A, which were neither infected with HBV nor treated with IFN, were used as negative controls.

To analyze the effect of IFN treatment, we compared gene expression profiles between groups A (mice without IFN treatment) and B (mice with IFN treatment); 525 genes with >3.0-fold upregulation following IFN treatment were observed. Among them, chemokine (C-X-C motif) ligand 9, chemokine (C-X-C motif) ligand 10, and chemokine (C-X-C motif) ligand 11, which promote T cell adhesion, were remarkably highly

induced with IFN treatment (Supplementary data table 6) [13]. These results suggest that the antiviral effects of IFN might involve not only direct activation of IFN-stimulated proteins such as myxovirus resistance protein A and double strand RNA-dependent protein kinase but also activation of immunity via chemokines.

Second, we compared the profiles between groups A (mice without HBV infection) and C (mice with HBV infection). As shown in Supplementary data table 7, more than half (12) of the top 20 genes upregulated by HBV infection localized to the cell membrane or the extracellular region, but 14 (70%) of the 20 downregulated genes localized to the nucleus. In addition, GO analysis demonstrated that genes related to cell cycle and DNA modification were affected by HBV infection. We speculate that HBV infection promotes cell growth and DNA damage in the hepatocyte nucleus and activates the immune response in the cytoplasm. From the clinical standpoint, some healthy HBV carriers develop hepatocellular carcinoma without chronic hepatitis or cirrhosis. The present results strongly support this observation, showing that most of the affected genes are known to be associated with carcinogenesis.

Clinically, HBV is known to develop tolerance to IFN treatment in patients with chronic hepatitis B, although the mechanism is not clear. We analyzed the IFN response with and

**Table 2. Pathway Analysis of 183 Interferon-Induced Genes With Interferon Responsiveness Downregulated by Hepatitis B Virus Infection**

Canonical pathways	P	Genes
Interferon signaling	$1.00 \times 10^{-8}$	<i>IFIT3, SOCS1, IFIT1, MX1, IFNGR1, JAK2, STAT1, TAP1, IRF1</i>
Role of pattern recognition receptors in recognition of bacteria and viruses	$1.20 \times 10^{-8}$	<i>IL12A, OAS2, OAS3(includes EG:4940), IFIH1, PIK3R3, TLR4, NOD2, TICAM1, DDX58, CASP1, NOD1, TLR3, RIPK2</i>
Type 1 diabetes mellitus signaling	$2.00 \times 10^{-4}$	<i>SOCS1, IL12A, RIPK1, GAD1, SOCS6, SOCS2, IFNGR1, JAK2, STAT1, IRF1</i>
Prolactin signaling	$2.70 \times 10^{-4}$	<i>PIK3R3, SOCS1, SOCS6, SOCS2, NMI, JAK2, STAT1, IRF1</i>
<i>TREM1</i> signaling	$3.50 \times 10^{-4}$	<i>TLR4, NOD2, ICAM1, CASP1, JAK2, TLR3, CASP5</i>
Production of nitric oxide and reactive oxygen species in macrophages	$3.90 \times 10^{-4}$	<i>PIK3R3, TLR4, RND3, PPP2R2A, PPM1J, RHOU, IFNGR1, MAP3K8, IRF8, JAK2, STAT1, IRF1</i>
Pathogenesis of multiple sclerosis	$1.10 \times 10^{-3}$	<i>CXCL10, CXCL9, CXCL11</i>
Activation of IRF by cytosolic pattern recognition receptors	$2.60 \times 10^{-3}$	<i>IFIH1, RIPK1, DDX58, STAT1, IFIT2, ISG15</i>
Dendritic cell maturation	$2.60 \times 10^{-3}$	<i>B2M, PIK3R3, TLR4, ICAM1, IL12A, IL1RN, IRF8, JAK2, TLR3, STAT1</i>
Interleukin 12 signaling and production in macrophages	$3.60 \times 10^{-3}$	<i>PIK3R3, TLR4, IL12A, IFNGR1, MAP3K8, IRF8, STAT1, IRF1</i>
Sphingosine-1-phosphate signaling	$3.60 \times 10^{-3}$	<i>PIK3R3, S1PR2, RND3, CASP1, RHOU, CASP4, CASP7, CASP5</i>
JAK-STAT signaling	$4.00 \times 10^{-3}$	<i>PIK3R3, SOCS1, SOCS6, SOCS2, JAK2, STAT1</i>
Growth hormone signaling	$4.70 \times 10^{-3}$	<i>PIK3R3, SOCS1, SOCS6, SOCS2, JAK2, STAT1</i>
Retinoic acid mediated apoptosis signaling	$8.50 \times 10^{-3}$	<i>TNFRSF10B, PARP8, TNFSF10, TIPARP, IRF1</i>

**NOTE.** *B2M*, beta-2-microglobulin; *CASP1*, caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase); *CASP4*, caspase 4, apoptosis-related cysteine peptidase; *CASP5*, caspase 5, apoptosis-related cysteine peptidase; *CASP7*, caspase 7, apoptosis-related cysteine peptidase; *CXCL9*, chemokine (C-X-C motif) ligand 9; *CXCL10*, chemokine (C-X-C motif) ligand 10; *CXCL11*, chemokine (C-X-C motif) ligand 11; *DDX58*, DEAD (Asp-Glu-Ala-Asp) box polypeptide 58; *GAD1*, glutamate decarboxylase 1 (brain, 67kDa); *ICAM1*, intercellular adhesion molecule 1; *IFIH1*, interferon induced with helicase C domain 1; *IFIT1*, interferon-induced protein with tetratricopeptide repeats 1; *IFIT2*, interferon-induced protein with tetratricopeptide repeats 2; *IFIT3*, interferon-induced protein with tetratricopeptide repeats 3; *IFNGR1*, interferon gamma receptor 1; *IL1RN*, interleukin 1 receptor antagonist; *IL12A*, interleukin 12A (natural killer cell stimulatory factor 1, cytotoxic lymphocyte maturation factor 1, p35); *IRF*, interferon regulatory factor; *IRF1*, interferon regulatory factor 1; *IRF8*, interferon regulatory factor 8; *ISG15*, ISG15 ubiquitin-like modifier; *JAK2*, Janus kinase 2; *MAP3K8*, mitogen-activated protein kinase kinase kinase 8; *MX1*, myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse); *NMI*, N-myc (and STAT) interactor; *NOD1*, nucleotide-binding oligomerization domain containing 1; *NOD2*, nucleotide-binding oligomerization domain containing 2; *OAS2*, 2'-5'-oligoadenylate synthetase 2, 69/71kDa; *OAS3*, 2'-5'-oligoadenylate synthetase 3, 100kDa; *PARP8*, poly (ADP-ribose) polymerase family, member 8; *PIK3R3*, phosphoinositide-3-kinase, regulatory subunit 3 (gamma); *PPM1J*, protein phosphatase, Mg<sup>2+</sup>/Mn<sup>2+</sup> dependent, 1J; *PPP2R2A*, protein phosphatase 2, regulatory subunit B, alpha; *RHOU*, ras homolog gene family, member U; *RIPK1*, receptor (TNFRSF)-interacting serine-threonine kinase 1; *RIPK2*, receptor-interacting serine-threonine kinase 2; *RND3*, Rho family GTPase 3; *S1PR2*, sphingosine-1-phosphate receptor 2; *SOCS1*, suppressor of cytokine signaling 1; *SOCS2*, suppressor of cytokine signaling 2; *SOCS6*, suppressor of cytokine signaling 6; *STAT1*, signal transducer and activator of transcription 1, 91kDa; *TAP1*, transporter 1, ATP-binding cassette, sub-family B (MDR/TAP); *TICAM1*, Toll-like receptor adaptor molecule 1; *TIPARP*, TCDD-inducible poly(ADP-ribose) polymerase; *TLR3*, Toll-like receptor 3; *TLR4*, Toll-like receptor 4; *TNFRSF10B*, tumor necrosis factor receptor superfamily, member 10b; *TNFSF10*, tumor necrosis factor (ligand) superfamily, member 10; *TREM1*, triggering receptor expressed on myeloid cells 1.

without HBV infection, focusing on the 525 upregulated genes with IFN treatment and using all obtained gene expression profiles. Interestingly, 61.3% of the extracted genes maintained an IFN response, but in 34.9% of those genes, IFN responses were attenuated by HBV infection (Supplementary data 8A). Genes corresponding to interferon signaling, including suppressor of cytokine signaling 1 (*SOCS1*) and interferon regulatory factor 1, and those corresponding to pattern recognition of bacteria and viruses, including nucleotide-binding oligomerization domain containing 1 (*NOD1*) and receptor-interacting serine-threonine kinase 2 (*RIPK2*), were statistically significantly associated with HBV-mediated attenuation to IFN response ( $P = 1.0 \times 10^{-8}$  and  $P = 1.2 \times 10^{-8}$ , respectively). According to these results, HBV infection significantly up-regulated *SOCS1* expression and reduced the IFN responsiveness of *SOCS1*. Thus, *SOCS1* might

support chronic infection of HBV in escaping the effects of innate immunity or IFN therapy. On the other hand, genes involved in recognition of viral infection were also inhibited following HBV infection. Both *NOD1* and *RIPK2* are related to innate and adaptive immune responses [14, 15]. We speculated that inhibition of *NOD1* or *RIPK2* expression facilitates HBV survival. Although further study is needed, these results may have important implications for the mechanisms of viral escape from innate immunity.

In conclusion, we performed complementary DNA microarray analysis using human hepatocyte chimeric mice. With this system, we could analyze the direct effects of IFN treatment and HBV infection without the confounding effects of the lymphocyte immunological response and obtained evidence that HBV infection attenuated the virus recognition and IFN response in



hepatocytes, by which means HBV could evade innate immune detection and response.

## Supplementary Data

Supplementary data are available at *The Journal of Infectious Diseases* online.

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# In Vivo Adaptation of Hepatitis C Virus in Chimpanzees for Efficient Virus Production and Evasion of Apoptosis

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Hepatitis C virus (HCV) employs various strategies to establish persistent infection that can cause chronic liver disease. Our previous study showed that both the original patient serum from which the HCV JFH-1 strain was isolated and the cell culture-generated JFH-1 virus (JFH-1cc) established infection in chimpanzees, and that infected JFH-1 strains accumulated mutations after passage through chimpanzees. The aim of this study was to compare the *in vitro* characteristics of JFH-1 strains emerged in each chimpanzee at early and late stages of infection, as it could provide an insight into the phenomenon of viral persistence. We generated full-genome JFH-1 constructs with the mutations detected in patient serum-infected (JFH-1/S1 and S2) and JFH-1cc-infected (JFH-1/C) chimpanzees, and assessed their effect on replication, infectious virus production, and regulation of apoptosis in cell culture. The extracellular HCV core antigen secreted from JFH-1/S1-, S2-, and C-transfected HuH-7 cells was 2.5, 8.9, and 2.1 times higher than that from JFH-1 wild-type (JFH-1/wt) transfected cells, respectively. Single cycle virus production assay with a CD81-negative cell line revealed that the strain JFH-1/S2, isolated from the patient serum-infected chimpanzee at a later time point of infection, showed lower replication and higher capacity to assemble infectious virus particles. This strain also showed productive infection in human hepatocyte-transplanted mice. Furthermore, the cells harboring this strain displayed lower susceptibility to the apoptosis induced by tumor necrosis factor  $\alpha$  or Fas ligand compared with the cells replicating JFH-1/wt. **Conclusion:** The ability of lower replication, higher virus production, and less susceptibility to cytokine-induced apoptosis may be important for prolonged infection *in vivo*. Such control of viral functions by specific mutations may be a key strategy for establishing persistent infection. (HEPATOLOGY 2011;54:425-433)

Currently, approximately 200 million people are infected with hepatitis C virus (HCV) and are at continuous risk of developing chronic liver diseases such as chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma.<sup>1,2</sup> Although acute HCV infection elicits innate and adaptive immune responses, the virus successfully evades clearance in approximately 75% of infected individuals.<sup>3,4</sup> The mechanisms by

Abbreviations: Ag, antigen; CTL, cytotoxic T lymphocytes; FasL, Fas ligand; HCV, hepatitis C virus; JFH-1cc, cell culture-generated JFH-1 virus; JFH-1/wt, JFH-1 wild-type; MFI, mean fluorescence intensity; NK, natural killer; NS, nonstructural; PARR, poly(adenosine diphosphate ribose) polymerase; TNF- $\alpha$ , tumor necrosis factor  $\alpha$ ; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling.

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which HCV leads to persistent infection at a high frequency are not yet fully understood. Lack of appropriate animal models, except chimpanzees, has rendered such studies difficult. Human hepatocyte-transplanted mice,<sup>5,6</sup> a useful small animal model to study HCV infection, are unsuitable to study the mechanisms of virus persistence because of a lack of B and T cell-mediated immunity.

HCV is a noncytopathic positive-stranded RNA virus of the *Flaviviridae* family. It primarily infects hepatocytes of humans and chimpanzees, where, thanks to error-prone RNA-dependent RNA polymerase, the infected virus accumulates a high number of mutations rapidly, thus providing opportunity for selection of viruses that have the ability to escape the immune system and establish persistent infection. Deciphering the strategies employed by HCV to establish persistence can be helpful in the development of new strategies to eradicate the virus and to stop disease progression. Until recently, the lack of an HCV strain having the ability to establish infection *in vivo* and *in vitro* was a substantial hindrance in studying the molecular mechanisms of virus persistence. This problem was solved by the identification of an HCV strain, JFH-1, that was isolated from a fulminant hepatitis patient and found to be capable of replicating and assembling infectious virus particles in chimpanzees as well as in cell culture.<sup>7-10</sup> This clone can be used to study the molecular mechanisms by which HCV evades the host immune system and causes chronic infection.

In a previous report, we inoculated patient serum from which the JFH-1 strain was originally isolated and cell culture-generated JFH-1 virus (JFH-1cc) into two different chimpanzees.<sup>11</sup> HCV established infection in both animals within 3 days of inoculation. In the JFH-1cc-infected chimpanzee, genome sequence of predominant infecting virus at week 2 was identical to JFH-1 wild-type (JFH-1/wt [in this study, this abbreviation was used instead of JFH-1 to distinguish it from other variant strains]), and the infecting virus has four synonymous and seven nonsynonymous mutations at week 7. In the JFH-1 patient serum-infected chimpanzee, 19 synonymous and six nonsynonymous mutations were observed in predominantly circulating virus at week 2, and this number increased to 35 synonymous and 17 nonsynonymous mutations at the later stage of infection course (week 23).<sup>11</sup> From these observations, we presumed that the isolates evolved in each chimpanzee at later stages of infection might have some advantage over the viruses isolated at earlier time points for survival in infected animals. Thus, in this study, we generated JFH-1 variants con-

taining the mutations observed in these animals and assessed their effect on replication and infectious virus production in cell culture. Furthermore, we examined the effects of infection of these strains to tumor necrosis factor  $\alpha$  (TNF- $\alpha$ )- or Fas ligand (FasL)-mediated apoptosis.

## Materials and Methods

The complete Materials and Methods are provided in the Supporting Information.

## Results

**Effects of Mutations Identified in Chimpanzees.** To investigate the effect of mutations on virus phenotype, we generated constructs containing the mutations observed in JFH-1 patient serum-infected chimpanzee and JFH-1cc-infected chimpanzee at various time points. The JFH-1 variants JFH-1/S1 and JFH-1/S2 contain the mutations observed in the patient serum-infected chimpanzee at week 2 and week 23, respectively, and JFH-1/C contains the mutations observed in the JFH-1cc-infected chimpanzee at week 7 (Supporting Table 1). The replication and virus production capacity of these variants in HuH-7 cells was compared with that of JFH-1/wt. After electroporation of *in vitro*-synthesized full-genome RNA of JFH-1/wt and variant strains, extracellular and intracellular HCV RNA and core antigen (Ag) were measured (Fig. 1). At day 5 posttransfection, all constructs displayed similar intracellular HCV RNA levels. However, extracellular HCV RNA level of JFH-1/C was 1.6 times higher than that of JFH-1/wt. Likewise, extracellular HCV RNA level of JFH-1/S2 was 3.4 times higher than that of JFH-1/S1 (Fig. 1A). Intracellular HCV core Ag levels of JFH-1/S2 and C were  $240.9 \pm 58.2$  and  $189.8 \pm 42.1$  fmol/mg protein, respectively, and were significantly lower ( $P < 0.005$ ) than that of JFH-1/S1 ( $526.1 \pm 58.2$  fmol/mg protein) and JFH-1/wt ( $511.7 \pm 32.9$  fmol/mg protein) at day 1, but reached comparable levels at day 5 posttransfection. On the other hand, extracellular HCV core Ag level of JFH-1/C was 2.2 times higher than that of JFH-1/wt, and that of JFH-1/S2 was 3.6 times higher than that of JFH-1/S1 at day 5 posttransfection (Fig. 1B). Transfection efficiency of these strains, indicated by intracellular HCV core Ag levels at 4 hours posttransfection, was almost identical (data not shown).

**Single Cycle Virus Production Assay.** For detailed analysis of the effects of these mutations on different stages of the virus lifecycle, we used a Huh7-25 cell

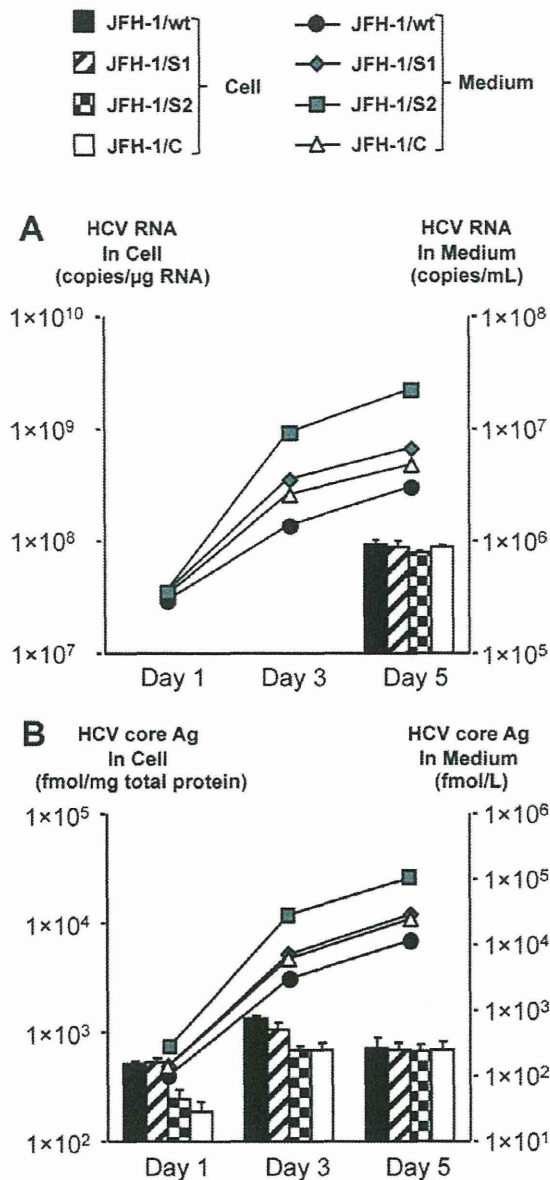


Fig. 1. Effects of *in vivo* adaptive mutations on virus production in HuH-7 cells. One million cells were transfected with 10  $\mu$ g *in vitro*-transcribed RNA of JFH-1/wt, JFH-1/S1, JFH-1/S2, and JFH-1/C. (A) HCV RNA and (B) core Ag levels in cell lysates and medium were measured at the indicated time points. Assays were performed in triplicate, and data are presented as the mean  $\pm$  SD.

line that lacks the surface expression of CD81, one of the cellular receptors for HCV entry. Three days after transfection with full-genome RNA of JFH-1/wt, JFH-1/S1, JFH-1/S2, and JFH-1/C, HCV RNA levels and infectivity titer were measured, and the specific infectivity was calculated (Table 1). Intracellular HCV RNA levels of JFH-1/C and JFH-1/S2 were lower than those of JFH-1/wt and S1, suggesting lower repli-

cation efficiency of these strains. However, the intracellular infectivity titers of JFH-1/C and JFH-1/S2 were 2.03 and 11.0 times higher than those of JFH-1/wt and JFH-1/S1, respectively ( $P < 0.005$ ). Intracellular-specific infectivities (infectivity titer/HCV RNA copy number) of JFH-1/C and JFH-1/S2 showed more pronounced difference from those of JFH-1/wt and JFH-1/S1 (3.92 times and 12.9 times higher, respectively;  $P < 0.005$ ). The infectious virus secretion rate (extracellular infectivity titer/intracellular infectivity titer) was not significantly different between JFH-1/wt and variant strains. These data indicate that mutations identified in chimpanzees at the later time point of infection led to reduced viral replication and increased assembly of infectious virus particles without any effect on viral release in cell culture.

**Subgenomic Replicon Assay.** To further confirm the replication efficiencies of strains observed in chimpanzees, we generated subgenomic replicons of JFH-1/wt, JFH-1/S1, JFH-1/S2, and JFH-1/C carrying the firefly luciferase reporter gene (SGR-JFH-1/Luc/wt, SGR-JFH-1/Luc/S1, SGR-JFH-1/Luc/S2, and SGR-JFH-1/Luc/C). *In vitro*-transcribed RNAs of these constructs were transfected into HuH-7 cells, and luciferase activity was measured to assess their replication capacity. The luciferase activities of SGR-JFH-1/Luc/C and SGR-JFH-1/Luc/S2 replicons were 7.30 and 7.33 times lower than those of SGR-JFH-1/Luc/wt and SGR-JFH-1/Luc/S1, respectively, at day 1 ( $P < 0.00005$ ), suggesting attenuated replication capacities of variant replicons isolated from each animal at later time points of infection (Supporting Fig. 1A). The luciferase activity 4 hours after transfection was comparable, indicating similar levels of transfection efficiency (data not shown). Based on these data, we found that the mutations that emerged in nonstructural (NS)3-NS5B of JFH-1/S2 and JFH-1/C reduced the replication efficiency in cell culture.

**Genomic Regions Responsible for Lower Replication and Higher Assembly of JFH-1/S2.** To further clarify the genomic region responsible for lower replication efficiency and higher assembly rate of JFH-1/S2, we generated the chimeric constructs JFH-1/S2-wt and JFH-1/wt-S2 as described in the Supporting Materials and Methods. *In vitro*-transcribed RNAs of JFH-1/wt, JFH-1/S2, JFH-1/S2-wt, and JFH-1/wt-S2 were introduced into HuH-7 cells by electroporation and intracellular and extracellular HCV RNA and core Ag were measured. At day 5 posttransfection, all constructs displayed comparable intracellular HCV RNA levels (Fig. 2). However, extracellular HCV RNA levels of JFH-1/S2 and JFH-1/S2-wt were significantly



**Table 1. Infectious Virus Production and Release of JFH-1/wt and Variants in Huh7-25 Cells**

Strain	Intracellular			Extracellular		Secretion Ratio (Extracellular/ Intracellular)
	HCV RNA (copies/ $\mu$ g RNA)	Infectivity Titer (ffu/well)	Specific Infectivity (ffu/copies)	Infectivity Titer (ffu/well)		
JFH-1/wt	$7.75 \times 10^8 \pm 1.04 \times 10^8$	$4.21 \times 10^2 \pm 4.32 \times 10^1$	$2.09 \times 10^{-7} \pm 7.06 \times 10^{-8}$	$1.94 \times 10^3 \pm 3.76 \times 10^1$		$4.6 \pm 1.3$
JFH-1/S1	$7.04 \times 10^8 \pm 8.49 \times 10^7$	$4.72 \times 10^2 \pm 5.63 \times 10^1$	$2.91 \times 10^{-7} \pm 6.00 \times 10^{-8}$	$3.02 \times 10^3 \pm 2.77 \times 10^2$		$5.4 \pm 2.0$
JFH-1/S2	$4.16 \times 10^{8**} \pm 7.47 \times 10^6$	$5.19 \times 10^{3**} \pm 8.24 \times 10^1$	$3.76 \times 10^{-6**} \pm 7.01 \times 10^{-7}$	$3.23 \times 10^{4**} \pm 3.52 \times 10^3$		$6.2 \pm 3.0$
JFH-1/C	$3.15 \times 10^{8*} \pm 5.02 \times 10^7$	$8.59 \times 10^{2*} \pm 4.81 \times 10^1$	$8.19 \times 10^{-7*} \pm 5.68 \times 10^{-8}$	$3.68 \times 10^3 \pm 3.02 \times 10^3$		$4.3 \pm 1.4$
JFH-1/ S2-wt	$7.07 \times 10^8 \pm 8.43 \times 10^7$	$4.40 \times 10^{3*} \pm 9.5 \times 10^1$	$2.73 \times 10^{-6*} \pm 2.35 \times 10^{-7}$	$3.0 \times 10^{4*} \pm 1.1 \times 10^3$		$6.7 \pm 0.7$
JFH-1/ wt-S2	$4.21 \times 10^{8*} \pm 1.97 \times 10^7$	$2.7 \times 10^2 \pm 2.9 \times 10^1$	$2.02 \times 10^{-7} \pm 4.0 \times 10^{-8}$	$1.7 \times 10^3 \pm 1.3 \times 10^2$		$4.5 \pm 0.4$

Abbreviation: ffu, focus-forming units.

\* $P < 0.005$  versus JFH-1/wt.\*\* $P < 0.005$  versus JFH-1/S1.

higher ( $P < 0.0005$ ) than that of JFH-1/wt. On the other hand, extracellular RNA level of JFH-1/wt-S2 chimeric construct was lower than that of JFH-1/S2 and JFH-1/S2-wt and similar to that of JFH-1/wt. Likewise, extracellular core Ag levels of JFH-1/S2 and JFH-1/S2-wt were also significantly higher than that of JFH-1/wt. Intracellular HCV core Ag levels of JFH-1/S2 and JFH-1/wt-S2 on day 1 posttransfection were  $240.9 \pm 58.2$  and  $134.3 \pm 17.1$  fmol/mg protein, respectively, and were significantly lower ( $P < 0.005$ ) than that of JFH-1/wt ( $526.1 \pm 58.2$  fmol/mg protein), whereas intracellular HCV core Ag level of JFH-1/S2-wt was comparable to that of JFH-1/wt. Transfection efficiency of these strains, indicated by intracellular HCV core Ag levels at 4 hours posttransfection, was almost identical (data not shown).

To further elucidate, we transfected Huh7-25 cells with *in vitro*-transcribed RNA of JFH-1/wt, JFH-1/S2, JFH-1/S2-wt, and JFH-1/wt-S2 and measured HCV RNA, core Ag, and infectivity titer in the cells and culture medium. Intracellular HCV RNA levels of JFH-1/S2 and JFH-1/wt-S2 were similar and lower than those of JFH-1/wt and JFH-1/S2-wt, suggesting mutations in NS3-NS5B were responsible for lower replication efficiency of JFH-1/S2 (Table 1). Intracellular infectivity titer of JFH-1/S2 and JFH-1/S2-wt was 12.3 and 10.4 times higher, respectively, than that of JFH-1/wt ( $P < 0.005$ ) on day 3 posttransfection. The intracellular specific infectivities of JFH-1/S2 and JFH-1/S2-wt were significantly higher than that of JFH-1/wt (18 times and 13.1 times higher, respectively;  $P < 0.005$ ). On the other hand, intracellular specific infectivity of JFH-1/wt-S2 was comparable to that of JFH-1/wt. The infectious virus secretion rate was not significantly different among all the constructs (Table 1). These data indicate that mutations emerged in the core-NS2 region of JFH-1/S2 are responsible

for the enhanced assembly of infectious virus particles compared with JFH-1/wt.

**Mapping Study for JFH-1/S2 Strain.** Because our experiments with JFH-1/S2 subgenomic replicon and JFH-1/wt-S2 chimeric construct showed that mutations emerged in the NS3-NS5B region are responsible for reduced replication efficiency of JFH-1/S2, we performed mapping studies by generating various JFH-1 subgenomic replicons, each containing the mutations observed in individual nonstructural protein. Although mutations in NS4B and NS5A were associated with attenuated replication capacity of JFH-1, the most significant decrease in replication was observed with NS5B mutations (Supporting Fig. 1B).

For detailed analysis of mutations responsible for higher assembly, *in vitro*-transcribed RNAs of JFH-1/wt, JFH-1/S2, JFH-1/S2-wt, JFH-1/N397S, JFH-1/L752V, JFH-1/S2-NS2 (containing mutations G838R, A878V, and V881A), JFH-1/G838R, and JFH-1/A878V were transfected into Huh7-25 cells, and intracellular-specific infectivities were compared (Supporting Table 2). As reported previously, JFH1/G838R showed higher intracellular specific infectivity than that of JFH-1/wt, but could not reach the level of JFH-1/S2 or JFH-1/S2-wt. Among the mutants, intracellular specific infectivities of JFH1/L752V, JFH1/NS2, and JFH1/G838R were 4.02, 5.42, and 3.07 times higher than that of JFH-1/wt, but those of JFH1/N397S and JFH1/A878V were similar to that of JFH-1/wt. Thus, the combination of mutations in P7 and NS2 was found to contribute to the higher assembly of the JFH-1/S2 strain.

**Human Hepatocyte-Transplanted Mouse Assay.** To assess the *in vivo* infectivity of these strains, we inoculated culture medium containing  $10^7$  copies (HCV RNA titer measured by RTD-PCR) of JFH-1/wt, JFH-1/S1, JFH-1/S2, and C viruses into human

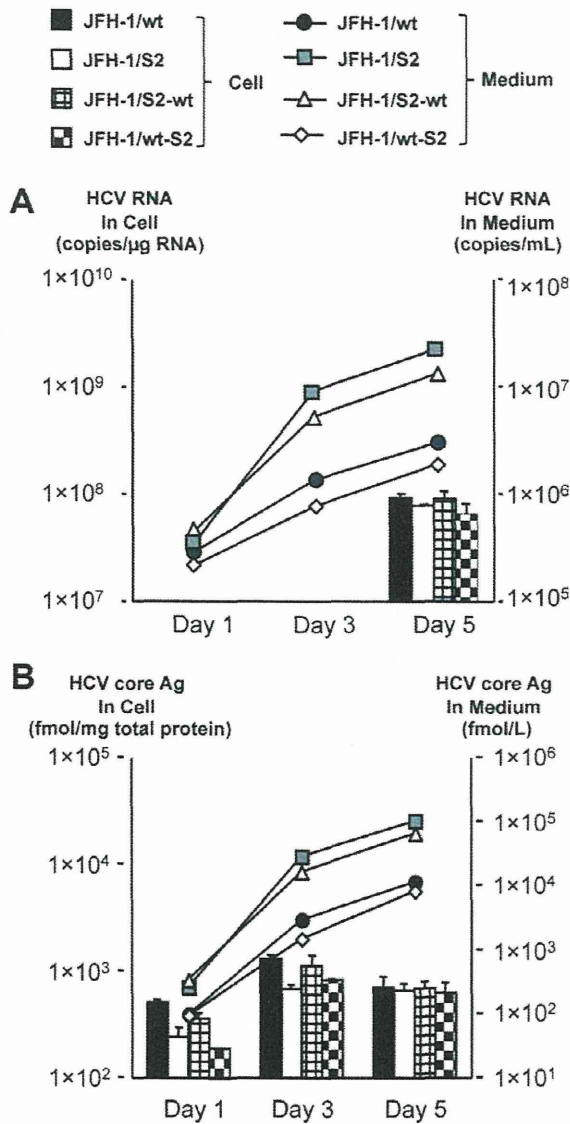


Fig. 2. Virus production of JFH-1/S2 chimeric constructs in HuH-7 cells. One million cells were transfected with 10  $\mu$ g *in vitro*-transcribed RNA of JFH-1/wt, JFH-1/S2, JFH-1/S2-wt, and JFH-1/wt-S2. (A) HCV RNA and (B) core Ag levels in cell lysates and medium were measured at the indicated time points. Assays were performed in triplicate, and data are presented as the mean  $\pm$  SD.

hepatocyte-transplanted mice. Two mice were used for each virus. Two weeks after intravascular inoculation, all mice but one became HCV RNA-positive (Fig. 3). Two mice died 3 weeks after inoculation; one was inoculated with JFH-1/wt and had developed infection, and the other was inoculated with JFH-1/C and died without developing infection. HCV RNA levels in infected mice fluctuated, ranging from  $10^6$  to  $10^9$  copies/mL. We could not observe much difference of

infected HCV RNA titer among these inoculated mice. Sequence analyses of the complete open reading frames revealed that infecting JFH-1/wt virus and variant strains had no nonsynonymous mutations at the time of development of infection. From these data, we concluded that not only JFH-1/wt virus but also JFH-1/S1, JFH-1/S2, and JFH-1/C viruses were able to establish productive infection in human hepatocyte-transplanted mice.

**Apoptosis Induction Assay.** To investigate the survival strategy against the host defense system, we examined the susceptibility of JFH-1/wt and variant strains to TNF- $\alpha$ -mediated apoptosis induction. After transfection with *in vitro*-transcribed RNA of JFH-1/wt, JFH-1/S1, JFH-1/S2, and JFH-1/C, Huh-7.5.1 cells were exposed to TNF- $\alpha$  plus actinomycin D. Without exposure, apoptosis was observed in a limited number of HCV-positive cells (Supporting Fig. 2A). Forty-eight hours later, cells were harvested, fixed, and

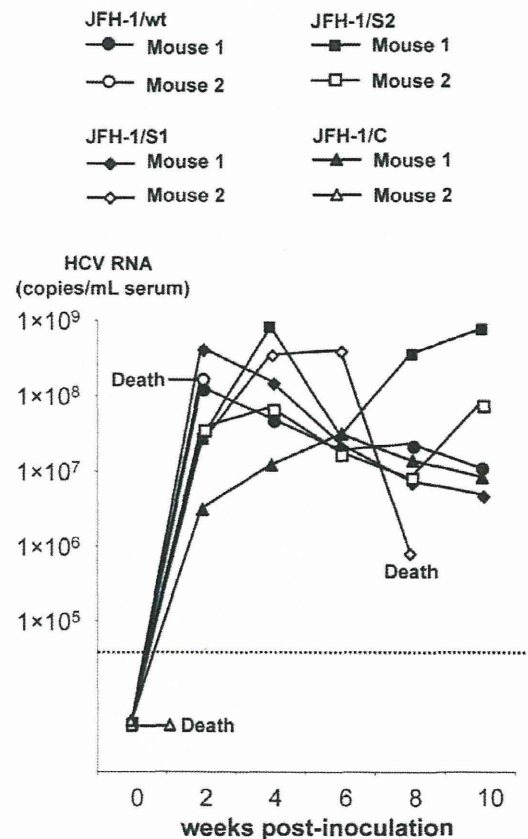


Fig. 3. *In vivo* infection study of JFH-1/wt and its variants in human hepatocyte-transplanted mice. Cell culture medium containing  $1 \times 10^7$  HCV RNA copies of JFH-1/wt, JFH-1/S1, JFH-1/S2, and JFH-1/C were inoculated into human hepatocyte-transplanted mice, and HCV RNA levels in mice serum were monitored.

subjected to terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay and anti-HCV NS5A staining. The effects of JFH-1/wt, JFH-1/S1, JFH-1/S2, and JFH-1/C transfection on apoptosis induction were determined by calculating the ratio of apoptosis between HCV-positive and HCV-negative populations and expressed as an apoptosis induction index. After treatment of JFH-1/wt-transfected cells with TNF- $\alpha$ , apoptosis was observed in 36.8% of the HCV-positive population and in 19.3% of the HCV-negative population, and the apoptosis induction index was  $1.85 \pm 0.06$  (Fig. 4). The apoptosis induction indexes of JFH-1/S1-transfected and JFH-1/C-transfected cells were  $1.23 \pm 0.06$  and  $1.16 \pm 0.10$ , respectively, suggesting lower susceptibility to apoptosis induction compared with JFH-1/wt. On the other hand, the apoptosis induction index of JFH-1/S2 was  $0.74 \pm 0.17$ , which was substantially lower than that of JFH-1/wt, demonstrating the more reduced apoptosis in the cells harboring this strain. Similar results were obtained by treatment with FasL plus actinomycin D (Supporting Fig. 2B). To confirm the lower susceptibility of JFH-1/S2-transfected cells, apoptosis was also detected by staining with anticlaved poly(adenosine diphosphate ribose) polymerase (PARP) antibody. The apoptosis induction indexes of JFH-1/wt and JFH-1/S2-transfected cells were  $2.28 \pm 0.24$  and  $1.15 \pm 0.14$ , respectively, and were consistent with TUNEL assay (Fig. 5). Although the HCV NS5A-positive rate in JFH-1/S2-transfected cells was higher than that in JFH-1/wt, the mean fluorescence intensity of the NS5A-positive population in JFH-1/S2-transfected cells was significantly lower ( $185.0 \pm 8.7$ ) than that in JFH-1/wt-transfected cells ( $395.0 \pm 98.0$ ), corresponding to the observed phenotype of the JFH-1/S2 strain in the single cycle virus production assay (i.e., lower replication efficiency and rapid spread to surrounding cells).

To clarify the genomic region responsible for lower susceptibility of JFH-1/S2 to cytokine-induced apoptosis, we examined the effect of TNF- $\alpha$  on the cells carrying subgenomic reporter replicons. The apoptosis induction index of SGR-JFH1/Luc/S2-transfected cells was lower than that of SGR-JFH1/Luc/wt-transfected cells (Supporting Fig. 2C); however, the difference was not as pronounced as with full-genome constructs, indicating that mutations in the NS3-NS5B region contribute to lower susceptibility of JFH-1/S2 to cytokine-induced apoptosis, but they are not sufficient to explain the difference between JFH-1/wt and JFH-1/S2. We confirmed these results by use of the chimeric

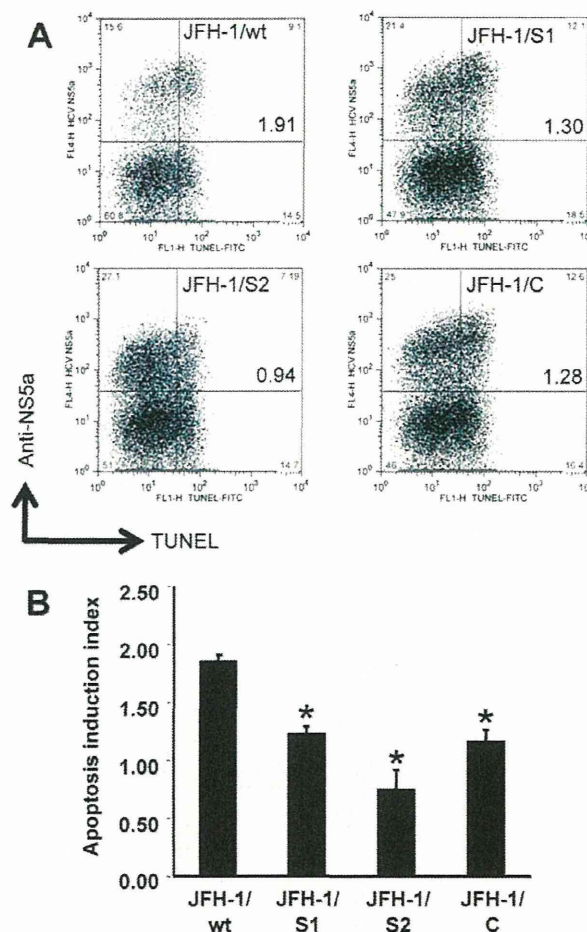


Fig. 4. Apoptosis induction in Huh-7.5.1 cells transfected with JFH-1/wt and its variants. (A) Three million cells were transfected with 3  $\mu$ g *in vitro*-transcribed full-genome RNA of JFH-1/wt, JFH-1/S1, JFH-1/S2, and JFH-1/C. Forty-eight hours later, apoptosis was induced by exposing cells to 20 ng/mL TNF- $\alpha$  plus 50 ng/mL actinomycin D. Cells were harvested after 48 hours of treatment and subjected to TUNEL and anti-HCV NS5A staining. Dot plots show HCV replication and apoptosis at the single cell level. Quadrant gates were determined using unstained and a terminal deoxynucleotidyltransferase-untreated control in each culture condition. The clone names and apoptosis induction indexes are indicated in the upper right box. (B) Apoptosis induction indexes of JFH-1/wt-, JFH-1/S1-, JFH-1/S2-, and JFH-1/C-transfected cells. The mean  $\pm$  SD of three independent experiments is shown. \* $P < 0.005$  versus JFH-1/wt.

constructs JFH-1/S2-wt and JFH-1/wt-S2. The apoptosis induction indexes of JFH-1/S2-wt-transfected and JFH-1/wt-S2-transfected cells were  $1.42 \pm 0.13$  and  $1.71 \pm 0.08$ , respectively (Fig. 5). These data indicate that both structural and nonstructural regions of JFH-1/S2 were associated with lower susceptibility to cytokine-induced apoptosis, although mutations in core-NS2 seemed to have higher contribution toward this phenotype. Together, these results indicate that the JFH-1/S2 strain, which was selected after passage in



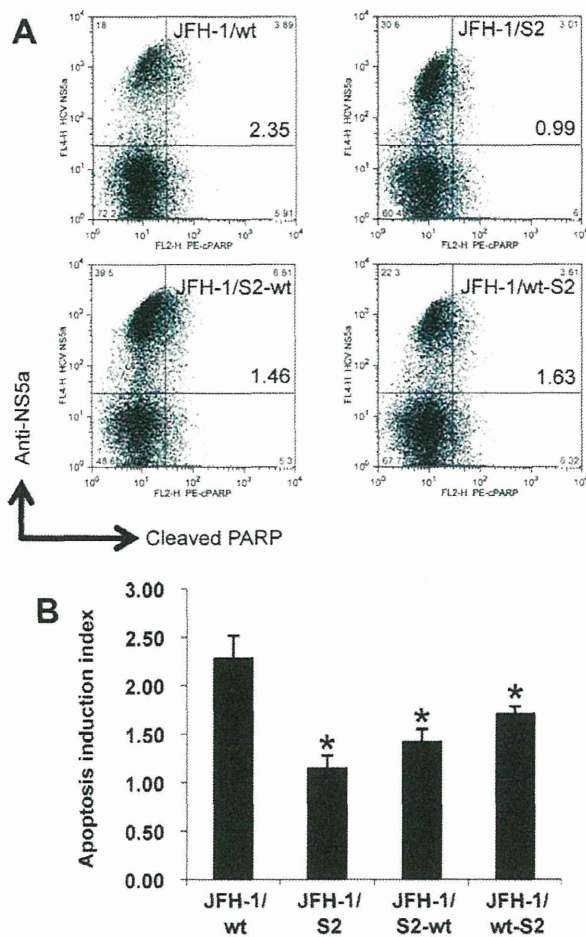


Fig. 5. Apoptosis induction in Huh-7.5.1 cells transfected with JFH-1/wt, JFH-1/S2, and their chimeric constructs. (A) Three million cells were transfected with 3  $\mu$ g *in vitro*-transcribed full-genome RNA of JFH-1/wt, JFH-1/S2, JFH-1/S2-wt, and JFH-1/wt-S2. Apoptosis was induced by exposing cells to 20 ng/mL TNF- $\alpha$  plus 50 ng/mL actinomycin D and detected by anticlaved PARP staining. The clone names and apoptosis induction indexes are indicated in the upper right box. (B) Apoptosis induction indexes of JFH-1/wt-, JFH-1/S2-, JFH-1/S2-wt-, and JFH-1/wt-S2-transfected cells. The mean  $\pm$  SD of three independent experiments is shown. \* $P < 0.05$  versus JFH-1/wt.

the patient serum-infected chimpanzee, acquired less susceptibility to the cytokine-induced apoptosis.

## Discussion

HCV develops chronic infection in the vast majority of infected patients<sup>1</sup>; however, the mechanisms of its persistence are still under investigation. Many viruses have evolved different strategies to cope with host immune systems, thus causing the development of persistent infection. For example, some viruses interfere with the major histocompatibility complex class I presentation of viral antigens, whereas others modulate

lymphocyte and macrophage functions, including cytokine production.<sup>12-16</sup> In our previous study, we detected an increasing number of mutations in the HCV genome isolated from JFH-1 patient serum-infected chimpanzees. Thus, we reasoned that these detected mutations might have imparted some advantage to this virus for long-time survival. To examine this hypothesis, we compared the phenotypes of JFH-1 variant strains emerged at early and late stages of infection in JFH-1 patient serum-infected and JFH-1cc-infected chimpanzees and found that the JFH-1/S2 strain isolated from the patient serum-infected chimpanzee at a later time point of infection replicated slowly, produced more infectious viruses, and displayed reduced susceptibility to cytokine-induced apoptosis.

The JFH-1 variant strain JFH-1/C, which contains seven nonsynonymous mutations identified in the JFH-1cc-infected chimpanzee at week 7, showed comparatively slower replication kinetics and slightly enhanced infectious virus production in cell culture. The intracellular specific infectivity of this strain in Huh7-25 cells was 3.9 times higher than that of JFH-1/wt (Table 1). These characteristics might have imparted some advantage to this strain for establishing productive infection in the chimpanzee. The other JFH-1 variant strains, JFH-1/S1 and JFH-1/S2, contain 6 and 17 nonsynonymous mutations identified in the JFH-1 patient serum-infected chimpanzee at weeks 2 and 23 postinfection, respectively. Replication kinetics and infectious virus production of the JFH-1/S1 strain were comparable to that of JFH-1/wt in cultured cells (Fig. 1, Table 1). In contrast, the JFH-1/S2 strain showed lower replication efficiency. Although the intracellular HCV RNA level of this strain in Huh7-25 cells was lower than that of JFH-1/wt and JFH-1/S1, and almost the same as that of JFH-1/C (Table 1), intracellular specific infectivity was 18.0 and 12.9 times higher than that of JFH-1/wt and JFH-1/S1, respectively, suggesting a significant increase in the assembly of infectious virus particles ( $P < 0.005$ , Table 1). The enhanced capacity of this strain to assemble infectious virus particles resulted in a higher extracellular infectivity titer that contributed to the rapid spread of virus to surrounding cells. Flow cytometry analyses of cells transfected with JFH-1/wt and variant strains revealed that the percentage of the HCV NS5A-positive population in JFH-1/S2-transfected cells was higher, but the mean fluorescence intensity of the anti-NS5A signal was lower than that in JFH-1/wt-transfected cells, thus confirming higher spread and lower replication of this strain. Taken together, both JFH-1/C and JFH-1/S2 exhibited a tendency toward