analog, poly(I-C), was introduced into cells using a liposome-mediated transfection procedure (the intracellular dsRNA, T-pIC), the other is to examine NS3-4A's inhibitory effects when poly(I-C) was added to the culture medium (the extracellular dsRNA, M-pIC). We observed that IFN-β gene promoter activity was strongly suppressed via the cleavage of Cardif by each NS3-4A when PH5CH8 cells were transfected with poly (I-C) (T-pIC) (Supplementary Fig. S1 in Electronic Supplementary Material). In contrast, IFN-β gene promoter activity was not significantly suppressed when poly (I-C) was added to the culture medium (M-pIC) (Fig. 4a). However, the promoter activity in cells expressing 1B-5-derived NS3-4A appeared to be slightly suppressed (Fig. 4a). Therefore, we next determined the levels of IFN-β mRNA by quantitative RT-PCR. The results revealed that IFN-β mRNA expression was not suppressed in cells expressing 1B-5-derived NS3-4A (Fig. 4b). We further showed that none of the NS3-4As examined cleaved the exogenously expressed TRIF (Fig. 4c). In addition, we showed that 1B-5, CH1, HCC1, or HCC2-derived NS3 interacted with endogenous TRIF, as was also observed with 1B-1-, O-, and JFH-1-derived NS3 (Figs. 2a, c, 4d). These results suggest that the suppressive effects of NS3-4As on dsRNA-induced IFN-β transcription and the interaction of NS3 with TRIF were not dependent on the HCV strain and genotype or associated with the stage or progression of hepatic disease.

Extracellular dsRNA-induced inflammatory cytokine production via the NF-κB signaling pathway was also not suppressed by NS3-4A

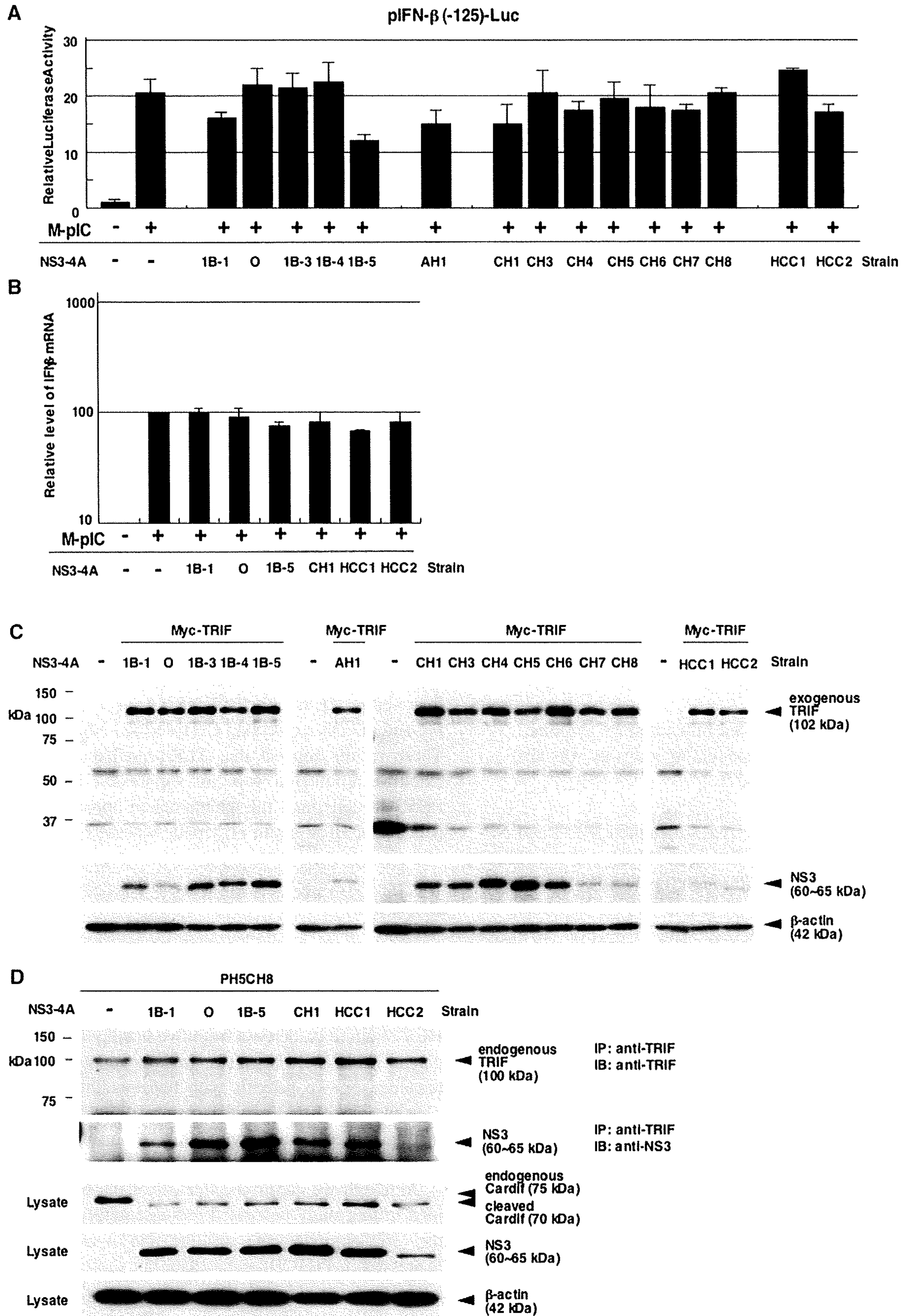
It was already known that TLR3-mediated IRF-3 and NF-κB activation pathways bifurcate at TRIF, and that TLR3 recruits TRAF6 via TRIF through the TRAF6-binding site of TRIF, resulting in NF-κB activation [17, 34]. Since we demonstrated that NS3 interacts with TRIF (Fig. 2a, c), we expected that NS3-4A might interfere with the recruitment of TRAF6 by TRIF (Fig. 5a). To examine this possibility, we considered whether or not NS3-4A affects M-pIC-induced NF-κB activation in PH5CH8 cells. Initially, we demonstrated that NF-κB-inducing promoter activity was also enhanced with M-pIC treatment and that this enhancement was mediated by TLR3 and TRIF, as promoter activity was found to be substantially suppressed by TLR3 or TRIF siRNA (Fig. 5b). The results revealed that none of the NS3-4As examined significantly suppressed M-pIC-induced NF-κB activation (Fig. 6a). However, the enhancement of promoter activity in cells expressing CH1-derived NS3-4A was slightly lower than that in cells expressing other strain-derived NS3-4As (Fig. 6a). Therefore, we performed quantitative RT-PCR analysis to examine the levels of IL-6 and IL-8 mRNAs, both of which were induced by NF-κB activation. The results revealed that neither IL-6 nor IL-8 mRNA expression was suppressed in cells expressing CH1-derived NS3-4A (Fig. 6b). These results suggest that TLR3-mediated inflammatory cytokine production was not suppressed by NS3-4A in PH5CH8 cells, and this phenomenon appears to be independent of HCV strain or hepatic disease type.

Fig. 4 None of the NS3-4As derived from patients with different hepatic disease diagnoses prevented M-pIC-induced IFN-β transcription via the TRIF-mediated pathway. **a** Effects of 15 NS3-4As on the activity of the IFN-β gene promoter. PH5CH8 cells transiently expressing NS3-4As from various HCV strains were subjected to M-pIC treatment. PH5CH8 cells transfected with pCX4bsr vector were used as a control (strain, –). The dual luciferase assay was performed as described in Materials and Methods. Data are expressed as the mean ± SD from three independent experiments, each of which was performed in triplicate. **b** Effect of NS3-4As on IFN-β mRNA induction by M-pIC treatment. PH5CH8 cells transiently expressing NS3-4As from several HCV strains containing 1B-5 were subjected to M-pIC treatment. PH5CH8 cells infected with pCX4bsr retrovirus were used as a control (strain, –). Quantitative RT-PCR for IFN-β mRNA was performed in triplicate. The IFN-β mRNA level was calculated relative to the level in control PH5CH8 cells, which was set at 100. **c** None of the NS3-4As cleaved exogenous TRIF. PH5CH8 cells were transfected with myc-TRIF and NS3-4A expression vectors. The production of myc-TRIF and NS3 was analyzed by immunoblot analysis using anti-myc and anti-NS3 antibody, respectively. PH5CH8 cells transfected with the pCX4bsr and pCX4pur vectors were used as a control (–). β-actin was used as a control for the amount of protein loaded per lane. **d** Endogenous TRIF interacts with NS3-4As from various HCV strains but not is cleaved by NS3-4As in PH5CH8 cells. The cell lysates were prepared and subjected to immunoprecipitation using anti-TRIF antibody, followed by immunoblot analysis using anti-TRIF or anti-NS3 antibody, as described in Fig. 2a. Cardif, NS3, and β-actin in the cell lysates were detected by anti-Cardif, anti-NS3, and anti-β-actin antibody, respectively. PH5CH8 cells infected with pCX4bsr retrovirus were used as a control (strain, –)

Discussion

In the present study, we demonstrated that neither IFN-β transcription nor NF-κB activation by extracellular dsRNA was suppressed by NS3-4A, regardless of the source of the HCV strain (e.g., derived from five healthy HCV carriers, a patient with acute hepatitis, seven patients with chronic hepatitis, or two patients with hepatocellular carcinoma). The findings of these studies using PH5CH8 cells suggest that the inhibitory activity of NS3-4A on antiviral signaling pathways is limited to the Cardif-mediated pathway.

Although we confirmed that all of the NS3-4As examined in this study possessed protease activity that enabled the efficient cleavage of the NS5A-NS5B substrate expressed in PH5CH8 cells (data not shown), none of the NS3-4As were able to cleave either exogenous or endogenous TRIF in PH5CH8 cells, although all were able to cleave Cardif. These results suggest that both the non-


A
pIFN-β (-125)-Luc
Relative Luciferase Activity
30
20
10
0
M-pIC
NS3-4A
1B-1 O 1B-3 1B-4 1B-5 AH1 CH1 CH3 CH4 CH5 CH6 CH7 CH8 HCC1 HCC2 Strain
B
Relative level of IFN-β mRNA
1000
100
10
M-pIC
NS3-4A
1B-1 O 1B-5 CH1 HCC1 HCC2 Strain
C
Myc-TRIF
NS3-4A
1B-1 O 1B-3 1B-4 1B-5 AH1 CH1 CH3 CH4 CH5 CH6 CH7 CH8 HCC1 HCC2 Strain
150
kDa 100
75
50
37
exogenous TRIF (102 kDa)
NS3 (60~65 kDa)
β-actin (42 kDa)
D
PH5CH8
NS3-4A
1B-1 O 1B-5 CH1 HCC1 HCC2 Strain
150
kDa 100
75
endogenous TRIF (100 kDa)
IP: anti-TRIF
IB: anti-TRIF
NS3 (60~65 kDa)
IP: anti-TRIF
IB: anti-NS3
Lysate
endogenous Cardif (75 kDa)
cleaved Cardif (70 kDa)
Lysate
NS3 (60~65 kDa)
Lysate
β-actin (42 kDa)

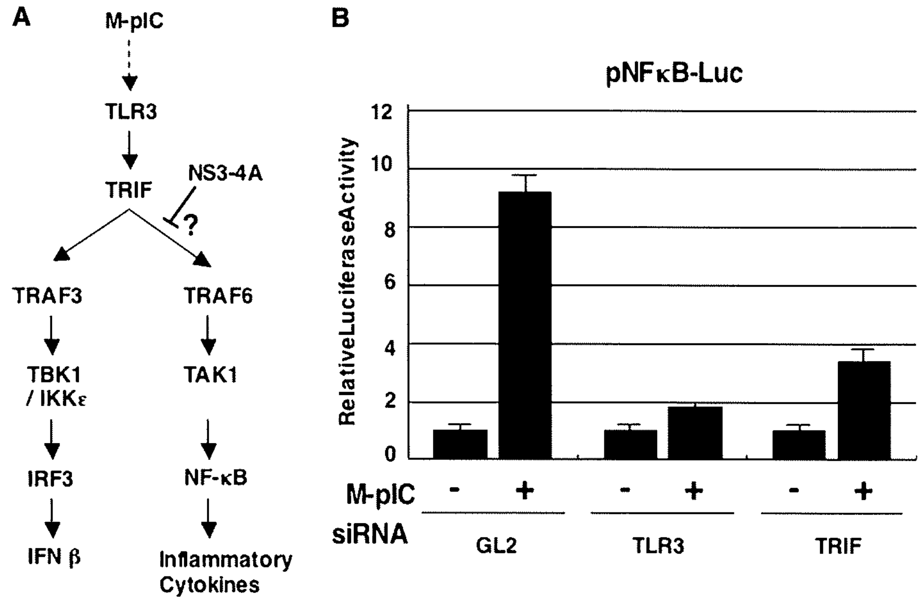


Fig. 5 TLR3-mediated activation of IRF-3 and NF-κB bifurcate at TRIF. **a** Model of TLR3-mediated signaling pathways. **b** Dual luciferase reporter assay of the NF-κB-inducing promoter using siRNA-transfected PH5CH8 cells treated with M-pIC

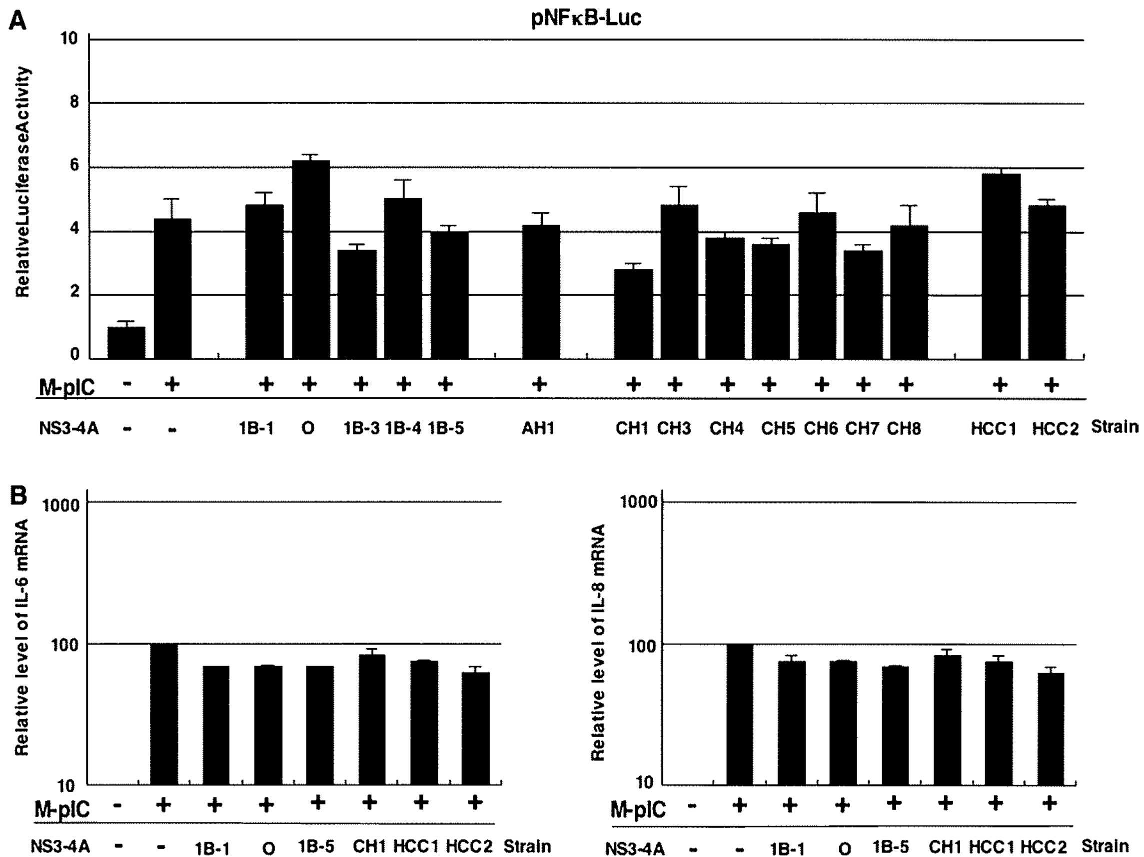


Fig. 6 None of the NS3-4As derived from patients with different hepatic disease diagnoses prevented M-pIC-induced NF-κB activation. **a** Effect of 15 NS3-4As on the activity of NF-κB-inducing promoter. PH5CH8 cells transiently expressing NS3-4As from various HCV strains were subjected to M-pIC treatment. PH5CH8 cells transfected with pCX4bsr vector were used as a control (strain, –). Data are expressed as the mean ± SD from three independent experiments, each of which was performed in triplicate. **b** Effect of NS3-4As on IL-6 or IL-8 mRNA induction by M-pIC treatment. PH5CH8 cells transiently expressing NS3-4A from several strains containing CH1 were subjected to M-pIC treatment. PH5CH8 cells infected with pCX4bsr retrovirus were used as a control (strain, –). Quantitative RT-PCR for IL-6 or IL8 mRNA was performed as described in Fig. 4b

cleavage of TRIF and the cleavage of Cardif by NS3-4A remain unaffected by the genetic diversity observed in NS3-4As derived from 15 different HCV strains (genotype 1b) derived from patients with different stages of hepatic disease as well as different genotypes (1b and 2a). However, other group [10, 27] previously reported that NS3-4A (N strain of genotype 1b) was able to inhibit IFN-β production via the cleavage of TRIF. Although we also observed the interaction of NS3 and TRIF in PH5CH8 and O cells as well as JFH-1-infected RSc cells, the reasons for conflicting results regarding the cleavage of TRIF by NS3-4A are still unclear. To clarify why TRIF is not cleaved by NS3-4A, further analysis will be necessary.

On the other hand, there appear to be some conflicting effects of different HCV proteins on IFN production, as we previously found that the NS5B protein induced IFN-β production in PH5CH8 cells [9, 31] and that the combination of NS5B with the core protein synergistically enhanced IFN-β production [9]. In that study, we showed that enhanced IFN-β production depended on the RNA-dependent RNA polymerase activity of NS5B and on aas 12 and 13 of the core protein, and we observed that NS3-4A significantly inhibited IFN-β production through a combination of the core and NS5B proteins [9]. However, in that case as well, IFN-β production was not completely suppressed by NS3-4A. This may be because NS3-4A is unable to suppress dsRNA-induced and TRIF-mediated IFN-β production, although Cardif-mediated IFN-β production has been shown to be completely suppressed under the same experimental conditions. To clarify the mechanisms underlying the conflicting effects of HCV proteins on IFN-β production mechanisms, an HCV proliferation system using PH5CH8 cells is still needed. However, a HuH-7-cell-based HCV proliferation system [42] would be unsuitable for such purposes due to the functional loss of TLR3 and/or the RIG-I signaling pathway(s) [26, 35]. Studies employing a cell system for HCV proliferation possessing functional TLR3 and/or RIG-I signaling pathways could enhance our understanding of the mechanisms of persistent HCV infection.

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Interleukin-27 Displays Interferon-γ–Like Functions in Human Hepatoma Cells and Hepatocytes

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Interleukin-27 (IL-27) is a cytokine belonging to the IL-6/IL-12 cytokine family. It is secreted by antigen-presenting cells, strongly acts on T cells, and also stimulates innate immune cells. In most studies, the effects of IL-27 on T cells were investigated; however, not much is known about possible effects of IL-27 on other cell types. IL-27 signals via the common IL-6–type cytokine receptor chain gp130 and the IL-27–specific chain WSX-1. Given the importance of gp130 in regulating liver responses such as the acute phase response or liver regeneration, we investigated whether IL-27 could also have a function in liver cells. We find that IL-27 stimulates hepatoma cells and hepatocytes by inducing a sustained signal transducer and activator of transcription (STAT)1 and STAT3 activation. Whereas the STAT3 mediated responses to IL-27 (γ-fibrinogen and hepcidin induction) are not detectable, we observe an interferon-gamma (IFN-γ)–like STAT1 response leading to the induction of interferon-regulated proteins such as STAT1, STAT2, interferon response factor (IRF)-1, IRF-9, myxovirus resistance A and guanylate binding protein 2. *Conclusion:* Our study provides evidence for a function of IL-27 in hepatoma cells and hepatocytes and shows that IL-27 responses are not restricted to the classical immune cells. Our results suggest that IL-27 exerts IFN-like functions in liver cells and that it can contribute to the antiviral response in these cells. (HEPATOLOGY 2009;50:585-591.)

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Interleukin-27 (IL-27) is a type I cytokine predominantly secreted by activated macrophages and dendritic cells. It can be allocated to the IL-6/IL-12 superfamily of cytokines. As a heterodimeric cytokine composed of the two subunits p28 and Epstein-Barr virus–induced gene 3,[1] IL-27 is a member of the IL-12 cytokine family, also encompassing IL-12 and IL-23. Like these cytokines, IL-27 has profound effects on T-cells and acts on innate immune cells.[2,3] Although IL-27 can have proinflammatory effects, most data point at the dominant role of IL-27 being immunosuppressive. Most studies have investigated the effects of IL-27 on CD4+ T-cells, and not much is known about possible effects of IL-27 on other cell types. IL-27 was shown to promote T helper 1 (TH1) responses through the induction of the transcription factors T-bet, up-regulation of IL-12Rβ2, and interferon-gamma (IFN-γ) production and suppression of the TH2 transcription factor GATA3.[1,4] However, IL-27 is also capable of suppressing both TH1 and TH2 responses during infection with a variety of pathogens.[5,6]

IL-27 signaling occurs via a receptor complex composed of the signal transducing receptor chains WSX-1 and glycoprotein (gp)130. Whereas WSX-1 is the IL-27–specific receptor chain,[7] gp130 is the common receptor subunit of IL-6–type cytokines.[8] Thus, IL-27 also belongs to this family. IL-6–type cytokines activate target genes involved in differentiation, survival, apoptosis, and proliferation. They can exert proinflammatory as well as anti-inflammatory properties and are major players in the acute phase response and the immune response of the organism. IL-6 is a major medi-

Abbreviations: FPV, fowl plague virus; GBP2, guanylate binding protein 2; IFN, interferon; IL, interleukin; IRF, interferon response factor; MxA, myxovirus resistance A; RIG-I, retinoic acid-inducible gene-I; STAT, signal transducer and activator of transcription; TH, T helper; OSM, oncostatin M.

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ator for the acute phase response of the liver as well as in liver regeneration.[9,10]

IL-6–type cytokines stimulate tyrosine phosphorylation of signal transducer and activator of transcription (STAT)1 and STAT3, which can form STAT3 and STAT1 homodimers as well as STAT3/STAT1 heterodimers. However, the importance of the detected STAT1 phosphorylation by IL-6–type cytokines remains elusive. For example, IL-6 and oncostatinM (OSM) only seem to induce an IFN-γ–like response in STAT3 knockout cells.[11,12] There are multiple reasons for this inefficient STAT1 response.[13] Not only is STAT1 tyrosine phosphorylation after IL-6–type cytokine stimulation very transient,[11,14] but additionally, most of the phosphorylated STAT1 seems to be trapped in STAT1/STAT3 heterodimers.[13]

We describe a function of IL-27 in hepatoma cells and hepatocytes. We show that IL-27 elicits an efficient STAT1 response and leads to the expression of IFN-γ–regulated genes in these cells.

Materials and Methods

Cell Culture. The human hepatoma cell line HepG2 (DSMZ) was maintained in Dulbecco's modified Eagle medium/Nut. MixF-12 medium with Glutamax supplemented with 10% fetal bovine serum, 100 mg/L streptomycin, and 60 mg/L penicillin. The human hepatocyte cell line PH5CH8 was described previously.[15]

Isolation and Cultivation of Rat Hepatocytes. Hepatocytes were isolated from adult male Sprague-Dawley rats as described before.[16] Details are provided as Supporting Information.

Cell Lysis, Preparation of Nuclear Extracts for Electrophoretic Mobility Shift Assay, Western Blot Analysis, and Antibodies. All of these procedures were performed as previously described.[13] The antibodies used are listed as Supporting Information.

Viral Infections and Plaque Assay. Fowl plague virus (FPV) was propagated and used as described previously.[17] For infection, 7×10^5 HepG2 cells were left untreated or were pretreated with 50 ng/mL IL-27 for 18 hours. Cells were then washed with phosphate-buffered saline followed by incubation with FPV (0.001 multiplicity of infection) diluted in phosphate-buffered saline/BA (phosphate-buffered saline containing 0.2% bovine serum albumin, 1 mM $MgCl_2$, 0.9 mM $CaCl_2$, 100 U/mL penicillin, and 0.1 mg/mL streptomycin) for 30 minutes at 37°C. The inoculum was aspirated, and cells were incubated for 24 hours with infection medium containing 0.2% bovine serum albumin and antibiotics supplemented either with or without 50 ng/mL IL-27. As a positive control for antiviral activity, infections were performed in the presence of 1000 U/mL interferon-alpha (IFN-α) for 24 hours. Plaque assays were performed as described previously.[18] Results are given as plaque-forming units per milliliter, and standard deviations are represented as error bars.

Statistical Analysis. The statistical analysis was performed using a Student t test. $P < 0.05$ was regarded as being statistically significant.

Results

IL-27 Acts on Human Hepatoma Cells and Cultured Human Hepatocytes. By screening different cell lines for their response to IL-27, we observed that human hepatoma cells are sensitive to IL-27. HepG2 cells were stimulated with increasing amounts of IL-27, and tyrosine phosphorylation of STAT3 (pY705) and STAT1 (pY701) was assessed by western blot analysis. Stimulation of HepG2 cells for 15 minutes with IL-27 leads to a phosphorylation of both STAT3 and STAT1 in a dose-dependent manner (Fig. 1A). As a control, HepG2 cells were also stimulated with IL-6. STAT1 as well as STAT3 tyrosine phosphorylation occurred on treatment of these cells with IL-6 for 15 minutes. The levels of phosphorylated STAT1 and STAT3 were higher in IL-6–stimulated HepG2 cells than in those treated with IL-27. We next investigated the kinetics of STAT phosphorylation on IL-27 stimulation in HepG2 cells and in the human hepatocyte cell line PH5CH8. Both of these cell lines express the IL-27 receptor WSX-1 on their surface (Supporting Fig. 1). As a control, the cells were also stimulated with IL-6, IFN-γ, and IFN-α. IL-27 induces a sustained phosphorylation of STAT1 and STAT3 (Fig. 1B,C; lanes 2-5) in both HepG2 and PH5CH8 cells, whereas IL-6 only leads to a sustained, albeit more pronounced, STAT3 phosphorylation (lanes 10-13). STAT1 activation after IL-6 stimulation was very transient (Supporting Fig. 2). However, a prominent STAT1 phosphorylation was observed on stimulation of the cells with interferons (lanes 6-9 and lanes 14-17), and STAT2 phosphorylation was only observed on treatment with IFN-α. Of note, up-regulation of both STAT1 and STAT2 was observed when the cells were stimulated with IL-27, IFN-γ, or IFN-α, an indication for an efficient STAT1 activation.

IL-27 Leads to a Prolonged STAT1 and STAT3 Activation in Liver Cells. As we previously reported, the STAT1 phosphorylation observed on treatment of hepatoma cells and primary human macrophages with IL-6–type cytokines such as IL-6 and OSM does not necessarily lead to the formation of active STAT1 ho-

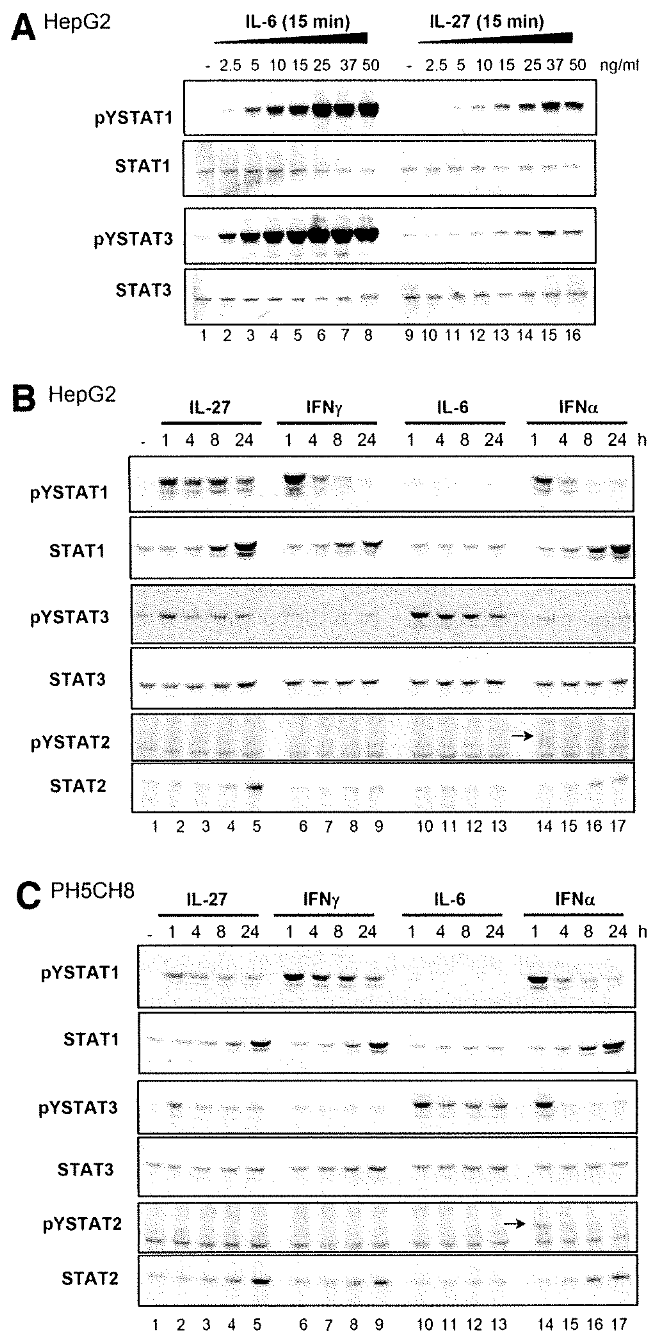

Fig. 1. IL-27 phosphorylates STAT1 and STAT3 in hepatoma cells and hepatocytes. (A) HepG2 cells were stimulated with the indicated amounts of IL-6 and IL-27. After 15 minutes, the cells were lysed, proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis and tyrosine phosphorylation of STAT1, and STAT3 was detected by western blot analysis using phospho-specific antibodies for pY701-STAT1 and pY705-STAT3. Equal loading of the samples was assessed by stripping and reprobing the blot with antibodies recognizing STAT1 and STAT3. (B, C) Western blot analysis showing STAT1, STAT2, and STAT3 phosphorylation on stimulation of HepG2 hepatoma cells (B) or the cultured hepatocyte cell line PH5CH8 (C) with 20 ng/mL IL-27, IFN-γ, IL-6, or IFN-α for up to 24 hours. Western blot analysis was performed as described above.

modimers. Most of the phosphorylated STAT1 is rather trapped in STAT1/STAT3 heterodimeric complexes.[13] Thus, we performed electrophoretic mobility shift assays to examine whether phosphorylated STAT1 is forming homodimers on treatment of liver cells with IL-27 (Fig. 2). As controls, we used cells stimulated with IL-6, IFN-γ, or IFN-α. On stimulation of HepG2 or PH5CH8 cells with IL-27, the sustained formation of STAT1/STAT1 (lanes 2-5) complexes shows that IL-27 induces a persistent STAT1 activation. Although STAT3 homodimers also can be detected, STAT3 activation is weak if compared with IL-6. Of note, STAT1/STAT1 dimers are hardly observed on stimulation of the cells with IL-6 at these time points, indicating that the prominent but transient STAT1 phosphorylation observed 15 minutes after stimulation (Fig. 1A) is not translated into a STAT1 response. IL-27, however, should be capable of inducing STAT3 as well as STAT1 responses, because both factors are activated in a sustained manner and bind DNA in their homodimeric form. As expected, IFN-γ and IFN-α mainly induced the formation of STAT1 homodimers. Overall, both interferons show a more sustained STAT1 activation in PH5CH8 cells than in HepG2 cells (Figs. 1B,C, 2).

Compared with IL-6, IL-27 Does Not Induce the STAT3-Dependent Genes γ-Fibrinogen and Hepcidin. To assess whether IL-27 induces STAT3-dependent genes, we investigated the induction of the acute-phase protein genes γ-fibrinogen and hepcidin in both HepG2− and PH5CH8 cells (Supporting Fig. 3). In contrast to IL-6, neither IL-27, IFN-γ, nor IFN-α seem to induce significant levels of these genes.

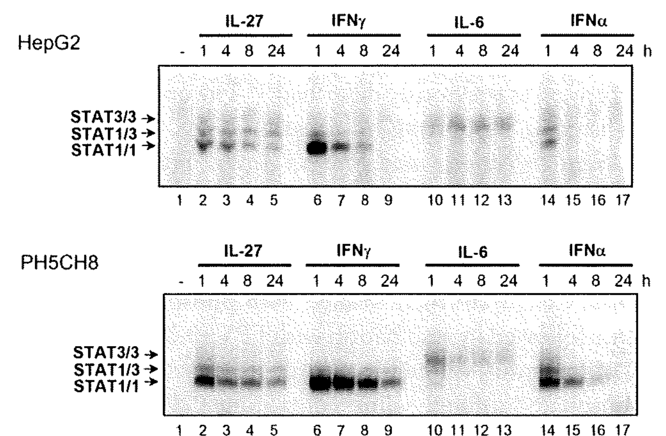

Fig. 2. IL-27 leads to a sustained STAT1 and STAT3 activation. HepG2 cells and PH5CH8 cells were stimulated with 20 ng/mL IL-27, IFN-γ, IL-6, or IFN-α for the times indicated, and nuclear extracts were prepared. These were analyzed by electrophoretic mobility shift assays, and STAT3/3, STAT3/1 and STAT1/1 dimer species were visualized by autoradiography.

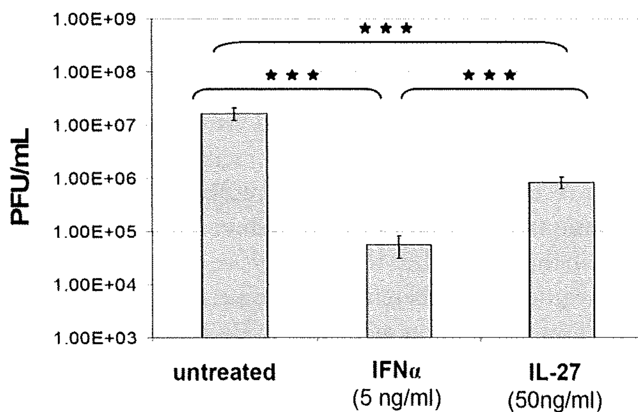

Fig. 3. IL-27 displays antiviral activity. HepG2 cells were pretreated with 50 ng/mL IL-27 for 18 hours before infection with FPV for 24 hours. The infection medium also contained 50 ng/mL IL-27. Antiviral activity was monitored by plaque assay. As a positive control, cells were infected in the presence of 5 ng/mL (1000 U/mL) IFN-α for 24 hours. Results are given in plaque-forming units per milliliter, and standard deviations are represented as error bars ($n = 4$; ***$P < 0.001$).

IL-27 Displays Antiviral Activity. Because IL-27 leads to a sustained STAT1 activation, we investigated possible antiviral activities of IL-27 by performing infection assays with HepG2 cells using the fowl plague virus FPV. HepG2 cells were pretreated with IL-27 for 18 hours before infection. Cells were then infected with FPV for 24 hours in the presence or absence of IL-27. As a positive control, infection assays were also performed in the presence of IFN-α, a cytokine that is well known to suppress virus replication. Figure 3 shows that treatment of HepG2 cells with IL-27 reduces the amount of progeny viruses after 24 hours of infection. Similar albeit less pronounced effects could be observed with a human influenza virus A7Puerto-Rico/8/34 isolate (data not shown).

IL-27 Mediates IFN-γ–like, STAT1-Dependent Responses in Liver Cells. To further investigate whether IL-27 induces an efficient STAT1 response, we investigated whether IL-27 would regulate STAT1-dependent gene transcription and thereby mediate interferon-like responses. We performed reporter gene assays in HepG2 and PH5CH8 cells and found IL-27 to prominently induce an interferon response factor (IRF)-1 promotor luciferase construct (Supporting Fig. 4). We then performed western blot analyses in these cells to monitor STAT1-dependent protein expression on treatment of these cells with IL-27, IL-6, IFN-γ, and IFN-α for different times. Figure 4A (HepG2) and 4B (PH5CH8) show that IL-27 up-regulates the STAT1-dependent genes STAT1 (lanes 4, 5), STAT2 (lanes 4, 5), and IRF-1 (lanes 2-5). Up-regulation of these genes also can be observed on treatment of the cells with IFN-γ or IFN-α, although the STAT2 up-regulation is barely detectable in HepG2 cells stimulated with IFN-γ (lane 9). In contrast, IL-6 fails to up-regulate any of the investigated STAT1-dependent genes. We further checked whether IL-27 would induce other interferon-regulated genes such as guanylate binding protein 2 (GBP2) and myxovirus resistance A (MxA), which are regulated by IFN-γ and IFN-α, respectively. Both genes are implicated in the antiviral response after interferon treatment of cells.[19] IL-27 up-regulates GBP2 in a similar manner to IFN-γ (lanes 5 and 9), whereas IFN-α does not induce GPB2. In contrast, IFN-α leads to a prominent up-regulation of MxA protein expression (lanes 15-17), whereas IL-27 only shows a weak induction (lane 5). IFN-γ only induces MxA expression in PH5CH8 cells (lane 9). Because MxA is known to be regulated by type I interferons, we investigated whether its IL-27–mediated induction could be attributable to the up-regulation of IFN-α or IFN-β by IL-27 (Supporting Fig. 5). However, stimulation of HepG2 cells with IL-27 in the presence of neutralizing antibodies directed against IFN-α or IFN-β did not affect IL-27–mediated induction of MxA, suggesting a direct induction by IL-27.

Because the IL-27–regulated transcription factor IRF-1 was recently reported to play a central role in the regulation of the antiviral protein RNA helicase retinoic acid–inducible gene-I (RIG-I),[20] we investigated whether IL-27 can induce RIG-I expression. We therefore monitored RIG-I induction in HepG2 and PH5CH8 cells by real-time polymerase chain reaction and western blot analysis. We detected a relatively weak twofold to sixfold increase in RIG-I messenger RNA expression (Supporting Fig. 6), whereas RIG-I protein up-regulation was hardly detectable after 24 hours by western blot (Fig. 4A,B; lane 5). In comparison, RIG-I protein was clearly induced on treatment of both cell lines with IFN-α (lanes 15-17).

Because we found both STAT1 and STAT2 to be up-regulated on stimulation with IL-27 (Fig. 4) and phosphorylation of these factors after treatment with IFN-α, we investigated whether prestimulation with IL-27 could enhance subsequent IFN-α–mediated signaling in PH5CH8 cells. We found that pretreatment with IL-27 enhances subsequent STAT1 and STAT2 phosphorylation on IFN-α treatment (Supporting Fig. 7A) and also induces the expression of IRF-9 (Supporting Fig. 7B), which forms the transcription factor complex interferon-stimulated gene factor 3 together with pYSTAT1 and pYSTAT2. However, we did not detect increased expression of the IFN-α–regulated genes RIG-I and MxA (Supporting Fig. 7C).

IL-27 Acts on Primary Rat Hepatocytes. To verify whether IL-27 also acts on hepatocytes in primary culture, we isolated primary rat hepatocytes and stimulated these cells with IL-27, IFN-γ, or IL-6 for different times. Treatment of

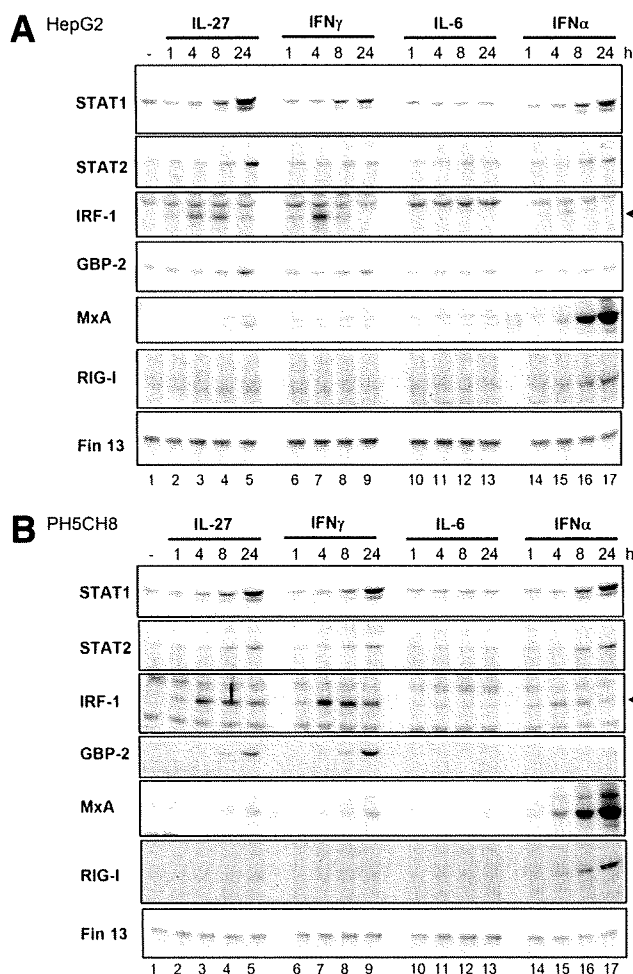

Fig. 4. IL-27 mediates STAT1 responses in hepatoma cells and cultured hepatocytes. (A, B) Western blot analysis monitoring up-regulation of STAT1, STAT2, IRF-1, GBP2, MxA, and RIG-I protein expression on stimulation of HepG2 cells (A) or PH5CH8 cells (B) with 20 ng/mL IL-27, IFN-γ, IL-6, or IFN-α for up to 24 hours. Expression levels of Fin13 are provided to compare the protein amount in the samples.

these cells with IL-27 induces a sustained phosphorylation of both STAT1 and STAT3 (Fig. 5), showing that primary hepatocytes respond to IL-27. Whereas the IL-27–mediated STAT1 phosphorylation is comparable to the one obtained after treatment with IFN-γ, the STAT3 response is much weaker than the one initiated by IL-6, confirming the results we obtained in the cell lines.

Discussion

Within the gastrointestinal system, a role for IL-27 was reported in the context of concanavalin-induced hepatitis,[21] Crohn disease,[22] as well as colon carcinoma.[23] However, all of these studies highlight IL-27 functions in infiltrating immune cells such as T cells and natural killer cells. IL-27 also acts on liver cells, namely, human hepatoma cells, cultured human hepatocytes, and primary rat hepatocytes. We find IL-27 to induce a sustained activation of STAT1 and STAT3 in these cells (Figs. 1 and 5).

IL-6–type cytokine signaling is characterized by a sustained STAT3 activation mediated via the different contributing receptor chains gp130, leukemia inhibitory factor receptor, and oncostatinM receptor. This STAT3 activation is of primordial importance for various functions in the liver such as the acute-phase response and liver regeneration. We therefore investigated the potency of the STAT3 activation mediated by IL-27, the new member of the IL-6–type cytokine family. For this, we selected γ-fibrinogen and hepcidin, two type II acute-phase proteins that are synthesized by hepatocytes in response to IL-6. In our experiments, IL-27 (as well as IFN-γ) was not able to lead to a significant induction of these genes (Supporting Fig. 3). These results suggest that IL-27 at most very weakly contributes to the acute-phase response of the liver and that it is in general a quite weak initiator of STAT3 responses in parenchymal liver cells. Because of the weak but sustained STAT3 activation that we observe after IL-27 stimulation (Figs. 1 and 2), it may be of interest to investigate a larger panel of acute-phase proteins to further dissect the potential contribution of IL-27 to the expression of type II acute-phase response genes.

However, the observed activation of STAT1 on stimulation of hepatoma cells and hepatocytes with IL-27 was of special interest because IL-6–type cytokines such as IL-6 and OSM fail to induce an efficient STAT1 response despite the fact that STAT1 phosphorylation is observed.[11-13] Thus, the detection of mere STAT1 phosphorylation (as detected after a 15-minute stimulation in Fig. 1A) does not allow drawing conclusions about STAT1 activity. Together with the very

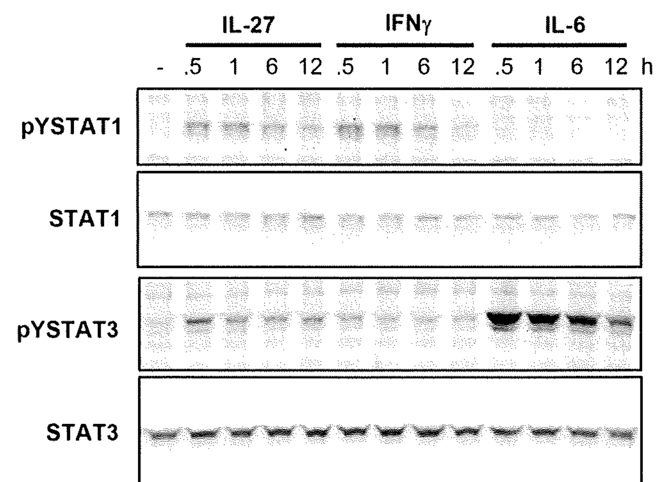

Fig. 5. IL-27 induces STAT1 and STAT3 tyrosine phosphorylation in primary hepatocytes. Primary rat hepatocytes were treated with 20 ng/mL IL-27, IL-6, or IFN-γ for the times indicated. Phosphorylation of STAT1 and STAT3 was monitored by western blot analysis.

transient phosphorylation of STAT1 after stimulation of hepatoma cells with IL-6, the fact that most of the phosphorylated STAT1 is found in STAT1/STAT3 heterodimers contributes to the lack of STAT1-dependent gene induction after treatment of cells with IL-6.[13] It also provides an explanation for the fact that IL-6 and OSM induce an interferon-like response in STAT3 knockout cells,[11,12] because the lack of STAT3 prevents the formation of heterodimers and thereby favors STAT1 homodimer formation. Here we show that IL-27 leads to a sustained STAT1 activation characterized by the formation of STAT1 homodimers (Fig. 2). Investigating whether the observed STAT1 activation translates to the induction of STAT1 target genes, we show that IL-27 up-regulates STAT1, STAT2, IRF-1, and IRF-9 protein expression (Fig. 4; Supporting Fig. 7B). This induction is comparable to the up-regulation after stimulation of these cells with IFN-γ or IFN-α, with the exception of an impaired STAT2 up-regulation by IFN-γ in HepG2 cells (Fig. 4A). This shows that IL-27 mounts an efficient STAT1 response and can mediate interferon-like responses in liver cells. This result corroborates previous data obtained in CD4+T cells and macrophages that highlight the importance of STAT1 for distinct biological activities mediated by IL-27.[4,24,25]

It is an interesting thought that the extent of STAT1 and STAT3 activation may be differently regulated as STAT3 responses are mediated via the gp130 receptor chain, whereas STAT1 responses will most likely only efficiently be mediated via the IL-27–specific WSX-1 receptor chain. This may lead to case-sensitive STAT1 or STAT3 responses. For example, it was recently reported that IL-27 activates both STAT1 and STAT3 in early activated T cells, whereas it displays a preferential activation of STAT3 in fully activated CD4+T cells.[26] The reasons for this may be manifold and could involve regulatory proteins or may even solely be attributable to different expression levels of STAT1 and STAT3 and thus to different distributions of STAT-dimer species.

The prolonged activation of STAT1 as well as the up-regulation of STAT1-dependent genes led us to investigate whether IL-27 possesses antiviral activity. We performed a plaque assay in hepatoma cells to assess the antiviral potency of IL-27 and show that, similarly to IFN-α, IL-27 reduces the amount of progeny viruses in HepG2 cells (Fig. 3). This result suggests that IL-27 can mediate antiviral effects in the liver.

To further assess the IL-27–mediated regulation of proteins involved in host resistance to pathogens, we then investigated the regulation of RIG-I, MxA, and GBP2 on stimulation of HepG2 and PH5CH8 cells with IL-27. The RNA helicase RIG-I is induced by retinoic acid as well as interferons and constitutes the first line of defense against viral infections by sensing viral double-stranded RNA.[27] Because it was recently shown that IRF-1 plays a central role in the regulation of RIG-I expression, we investigated whether IL-27 would induce this sensor for viral double-stranded RNA. Although we detected an increase in RIG-I messenger RNA levels on IL-27 stimulation in both human hepatoma cells and cultured human hepatocytes (Supporting Fig. 6), RIG-I protein was barely detectable. This shows that although IRF-1 expression may be necessary for RIG-I induction,[20] its expression alone is not sufficient. One may speculate that additional cellular signals may lead to an up-regulation of RIG-I protein expression by IL-27 and IFN-γ. Investigating the induction of the IFN-γ–induced protein GBP2 and the IFN-α–regulated MxA protein, we found IL-27 to up-regulate both proteins. IL-27 regulated these genes in a manner comparable to that of IFN-γ treatment. Because MxA is a gene regulated by type I interferons, we tested whether its induction after IL-27 treatment could be mediated through the induction of type I interferons. Experiments with neutralizing antibodies against IFN-α and IFN-β1 did not affect MxA nor GPB2 and STAT1 induction, suggesting that the observed regulation by IL-27 does not involve type I interferon production (Supporting Fig. 5).

Furthermore, we tested whether IL-27 could prime cells for a subsequent IFN-α stimulation (Supporting Fig. 7). Most interestingly, we found that prestimulation with IL-27 enhances subsequent IFN-α–mediated STAT1 and STAT2 phosphorylation and also up-regulates IRF-9. However, we did not detect increased expression of the IFN-α–regulated proteins RIG-I and MxA at the different doses of IFN-α tested. Nevertheless, it would be of interest to investigate other genes induced by the transcription factor complex interferon-stimulated gene factor 3 composed of pYSTAT1, pYSTAT2, and IRF-9.

Recent reports suggest that IL-27 can have antiviral activities in peripheral blood mononuclear cells, CD4+T cells, and macrophages and can inhibit human immunodeficiency virus 1 replication.[25,28] It was shown that IL-27 significantly induces interferon-inducible antiviral genes such as myxovirus protein 1, 2′-5′-oligoadenylate synthetase 2 and RNA-dependent protein kinase in macrophages, suggesting that IL-27 inhibits human immunodeficiency virus replication by eliciting an interferon-like response.[25] Together with our data, this suggests that IL-27 can elicit a multifaceted antiviral response.

The results presented in this study suggest that IL-27 may be a potential candidate for studies on combination therapies against hepatitis C. The standard care for a chronic hepatitis C infection is a combination therapy of IFN-α plus ribavirin. For a standard treatment, the response rate is approximately 50% for patients with hepatitis C virus genotype 1 and abut 80% for genotypes 2 and 3.[29] For the development of future

therapies, interests are focusing on combination therapies with different classes of anti–hepatitis C virus drugs such as protease or polymerase inhibitors. Furthermore, novel IFN-based products are being developed.[29] Our results that IL-27 acts on hepatocytes and hepatoma cells and displays IFN-like signaling in these cells as well as the antiviral effects of IL-27 observed in immune cells[25,28] indicate that treatment with IL-27 could be used in the therapy of hepatitis C virus infection.

Taken together, we present data showing that IL-27 acts on hepatocytes and hepatoma cells, elicits IFN-γ–like STAT1-mediated responses in these cells, and is able to regulate genes involved in host resistance to pathogens.

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Original Article

Development of a hepatitis C virus relapse model using genome-length hepatitis C virus ribonucleic acid-harboring cells possessing the interferon-α-resistance phenotype

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Aim: The cure rate of current interferon (IFN) therapy is limited to approximately 50% and most of the relapses after therapy are caused by genotype-1. To develop a relapse model in cell culture, we attempted to obtain genome-length hepatitis C virus ribonucleic acid (HCV RNA) harboring cells possessing the IFN-α-resistance phenotype from previously established OR6 cells, which enabled the luciferase reporter assay for monitoring of HCV RNA replication.

Methods: The IFN-α-resistant HCV RNA-harboring cells and control cells were obtained by the treatment of OR6 cells with and without IFN-α, respectively. Then, we examined the relapse of HCV in IFN-α-resistant HCV RNA-harboring cells.

Results: Only type I IFN (α and β) showed significantly different anti-HCV activity between IFN-α-resistant HCV RNA-harboring cells and control cells. There was no significant difference in the anti-HCV activity of IFN-γ, fluvastatin, or cyclosporine A between the two types of cells. Furthermore, we showed that fluvastatin or cyclosporine A in combination with IFN-α could prevent the relapse after therapy in the IFN-α-resistant HCV RNA-harboring cells.

Conclusion: We developed a HCV relapse model in cell culture using IFN-α-resistant HCV RNA-harboring cells. Thus anti-HCV reagents, which have a mechanism different from IFN-α, were shown to be useful for preventing a relapse of IFN-α-resistant HCV.

Key words: cyclosporine A, hepatitis C virus, interferon-α, interferon-γ, statin

INTRODUCTION

HEPATITIS C VIRUS (HCV) is a major cause of chronic liver disease, which progresses to liver cirrhosis and hepatocellular carcinoma.[1] Because approximately 170 million individuals are estimated to be infected with HCV worldwide, this infection is a major global health problem. HCV belongs to the family *Flaviviridae*, whose genome consists of a positive-stranded 9.6 kilobase ribonucleic acid (RNA) and encodes a large polyprotein precursor of about 3000 amino acid residues.[2,3] This polyprotein is processed by a combination of the host and viral proteases into at least ten proteins: the core, envelope 1 (E1), E2, p7, nonstructural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B.[4–6]

The present standard therapy of pegylated interferon (PEG IFN) with ribavirin achieves a sustained virological response (SVR) in approximately 50% of patients with chronic hepatitis C (CHC). The relapse after IFN therapy is a serious problem, especially in patients with genotype-1 HCV infection. The most significant event in HCV research has been the development of a cell culture system. The subgenomic replicon system enables robust HCV RNA replication in hepatoma cells.[7–14] Recently, the complete life cycle of HCV has been achieved using a genotype-2a strain, JFH1.[15] These HCV cell culture systems have promoted the discovery and development of anti-HCV reagents. Previously, we developed a genome-length HCV RNA (strain O of genotype-1b) replication system (OR6) with renilla luciferase (RL) as

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a reporter.[8] Using this OR6 assay system, we found that mizoribine,[16] as an immunosuppressant, and fluvastatin (FLV)[17,18] and pitavastatin,[19,20] as the reagents for hypercholesterolemia, suppressed genome-length HCV RNA replication.

We previously established an IFN-resistant cell culture model from the genotype-1b 1B-1 strain,[21] while Noguchi *et al.*[22] established a similar model from the genotype-1b Con1 strain. Both cell culture models were developed using subgenomic HCV replicons. Recently, it was reported in an epidemiological study that the 70th amino acid (AA) and 91st AA in core is significant for IFN resistance.[23] Therefore, we developed IFN-α-resistant genome-length HCV RNA-harboring cells using OR6 cells.

In the present study, we developed a relapse cell culture model using IFN-α-resistant genome-length HCV RNA-harboring cells. We also showed that anti-HCV reagents, which exhibited a mechanism different from IFN-α, efficiently prevented the relapse of IFN-α-resistant HCV.

MATERIALS AND METHODS

Reagents

HUMAN IFN-α AND human IFN-γ were purchased from Sigma–Aldrich (St. Louis, MO, USA), and cyclosporine A (CsA) and FLV were purchased from Calbiochem (Los Angeles, CA, USA). IFN-β was kindly provided by Toray Industries, Inc. (Tokyo, Japan).

Cell culture

OR6 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin, streptomycin, and 0.3 mg/mL of G418 (Geneticin; Invitrogen, Carlsbad, CA, USA) and passaged twice a week at a 5:1 split ratio. OR6c cells are cured OR6 cells from which genome-length HCV RNA has been eliminated by IFN-α treatment (500 IU/mL for 2 weeks) without G418, as previously described.[8]

Luciferase reporter assay

For the RL assay, 2×10^4 OR6 cells were plated in 24-well plates at least in triplicate for each assay and were cultured for 24 h. The cells were then treated with reagents for 72 h, harvested with renilla lysis reagent (Promega, Madison, WI, USA), and subjected to the RL assay according to the manufacturer's protocol.

Immunofluorescence analysis

Immunofluorescence analysis was carried out according to a method described previously.[9] The OR6 cells were treated with the reagents for 96 h as described above. The primary antibody used to detect core was anti-core (CP11; Institute of Immunology, Tokyo, Japan) and the secondary antibody was Cy3-conjugated anti-mouse antibody (Jackson ImmunoResearch). The nucleus was stained with 4′, 6-diamidine-2′-phenylindole (Sigma). The cells were photographed under a confocal laser scanning microscope (LSM510; Carl Zeiss Inc., Tokyo).

Dual-luciferase reporter assay

For the dual-luciferase assay, firefly luciferase vector, pISRE-Luc (Stratagene, La Jolla, CA) was used. The reporter assay was carried out as previously described.[24] The experiments were carried out in at least triplicate.

RNA transfection and selection of G418-resistant cells

For electroporation, OR6c cells were washed twice with ice-cold phosphate buffered saline (PBS) and resuspended at 10^7 cells/mL in PBS. RNA was mixed with 500 μL of the cell suspension in a cuvette with a gap width of 0.2 cm (Bio-Rad, Hercules, CA, USA). The mixture was immediately subjected to two pulses of current at 1.2 kV, 25 μF and maximum resistance. After 10 min of incubation at room temperature, the cells were seeded into 10-cm dishes and selected in complete DMEM with 0.3 mg/mL of G418.

Reverse transcription polymerase chain reaction and direct sequencing

Sequence analysis of HCV RNA was carried out by the direct sequencing method. Total RNA from each cell lines were extracted by using a Qiagen RNeasy® Mini kit (Tokyo, Japan). Two primers (O-9559R: 5′-TCTGCAG AGAGGCCAGTATCAGC-3′; O-6215R: 5′-TCATTGATC CACTGGTGGAGTCTC-3′) were used for reverse transcription (RT). The three parts of HCV were amplified by polymerase chain reaction (PCR) using proofreading KOD-plus DNA polymerase (Toyobo, Osaka, Japan): the first part covered the core to the NS3 region, the second part covered the NS3 region to the NS4B region, and the third part covered the NS5A region to the NS5B region. PCR was carried out using the following three pairs of primers: EMCV: 5′-AGGTACCCCATTGTATGG GATCTG-3′ and O-3540R: 5′-ATGCAGGTCGCCAAGAA GGACTG-3′ for the first part; O-3427: 5′-TCACG

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GCCTATTCCCAACAGACG-3′ and O-6215R: 5′-TCATTGATCCACTGGTGGAGTCTC-3′ for the second part; and O-5968: 5′-CCGAGGACCTAGTCAACTTGCTC-3′ and O-9382R: 5′-CCTATTGGCCTGGAGTGTTTAGC-3′ for the third part. The lengths of the three PCR products were 3356 bp, 2812 bp, and 3437 bp, respectively. The PCR products were sequenced in both the sense and antisense directions using Big Dye terminator cycle sequencing on an ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA).

Western blot analysis

Preparation of the cell lysate, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and immunoblotting were carried out as previously described.[10] The antibodies used in the present study were those against core (Institute of Immunology, Tokyo, Japan) and β-actin (Sigma). Immunocomplexes on the membranes were detected by an enhanced chemiluminescence assay (Renaissance; Perkin Elmer Life Science, Wellesley, MA, USA).

Statistical analysis

Statistical comparison of luciferase activities among the various treatment groups was carried out using Student's *t*-test. *P*-values of less than 0.05 were considered statistically significant.

RESULTS

The relapse of HCV RNA replication after IFN-α treatment

WE FIRST EXAMINED the dose and duration of IFN-α treatment required to eliminate HCV. OR6 cells were treated with IFN-α (1, 10, and 100 IU/mL) for 2 or 4 weeks and subjected to a luciferase assay as shown in the experimental design (Fig. 1a).

After 2-week IFN-α treatment, 1 IU/mL of IFN-α failed to reduce HCV to the background level, which was designated as the luciferase activity of cured OR6c cells (less than 1000 luciferase units; Fig. 1b, left panel). Two-week IFN-α treatment at 10 IU/mL decreased HCV to below the background level, but HCV RNA was increased after the cessation of the therapy, in a manner corresponding to a relapse in clinical therapy (Fig. 1b). Finally, 2-week IFN-α treatment at 100 IU/mL decreased HCV to below the background level, and there was no relapse after therapy (Fig. 1b).

In regard to the 4-week treatment duration, 1 IU/mL of IFN-α failed to reduce HCV to the background level (Fig. 1b, right panel). IFN treatment at 10 or 100 IU/mL for 4 weeks reduced HCV to below the background level and prevented a relapse after therapy (Fig. 1b, right panel). Together, these results indicate that longer treatment duration is more effective for preventing the relapse after treatment. As shown in Table 1, the luciferase value ratios (72 h/0 h) less than 1.1 are required to prevent relapse. Interestingly, at the dose of 1 IU/mL IFN-α, 4-week treatment resulted in a smaller luciferase value ratio than did 2 week-treatment (5.6 *vs* 19.8). These results indicate that longer treatment is more effective at preventing HCV RNA replication, even if the IFN-α cannot reduce HCV to an undetectable level.

We further characterized the effects of dose and duration in IFN-α treatment using a more sensitive colony formation assay. The colony formation assay could detect the remaining HCV RNA even within individual cells. As shown in Fig. 1c, IFN-α-treated cells were subjected to G418 selection after 1 week of recovery culture. At a dose of 100 IU/mL IFN-α, neither 2- nor 4-week treatments produced any colonies after 3 weeks of G418 selection (Fig. 1d). In contrast, under the 10 IU/mL IFN-α condition, both 2- and 4-week treatments produced G418-resistant colonies (Fig. 1d). Although the relapse assay 4 week-treatment with IFN-α at 10 IU/mL prevented relapse, latent HCV RNA produced G418-resistant colonies. Therefore, at least 100 IU/mL of IFN-α treatment for 2 weeks is needed to achieve the complete elimination of HCV RNA. The production rates of G418-resistant colonies are summarized in

Figure 1 The relapse and complete hepatitis C virus ribonucleic acid (HCV RNA) elimination after interferon (IFN)-α treatment in cell culture. (a) Schema of the experimental design for the relapse cell culture model in IFN-α treatment. OR6 cells were treated with IFN-α (1, 10, and 100 IU/mL) for 2 or 4 weeks without G418. At the end of the treatment, cells were cultured without IFN-α for 0, 24, 48, and 72 h and subjected to luciferase assay. (b) Transient luciferase assay for the cells at the end of the 2-week treatment (left panel) and 4-week treatment (right panel). (c) Schema of the experimental design for the complete HCV elimination by colony formation. The OR6 cells were treated with IFN-α (1, 10, and 100 IU/mL) for 2 or 4 weeks without G418. At the end of the treatment, cells were cultured without IFN-α for 1 week as the recovery culture. Then the cells were treated with 0.3 mg/mL of G418 for 3 weeks. (d) At the end of the G418 selection in (c), the colonies were stained by trypan blue.

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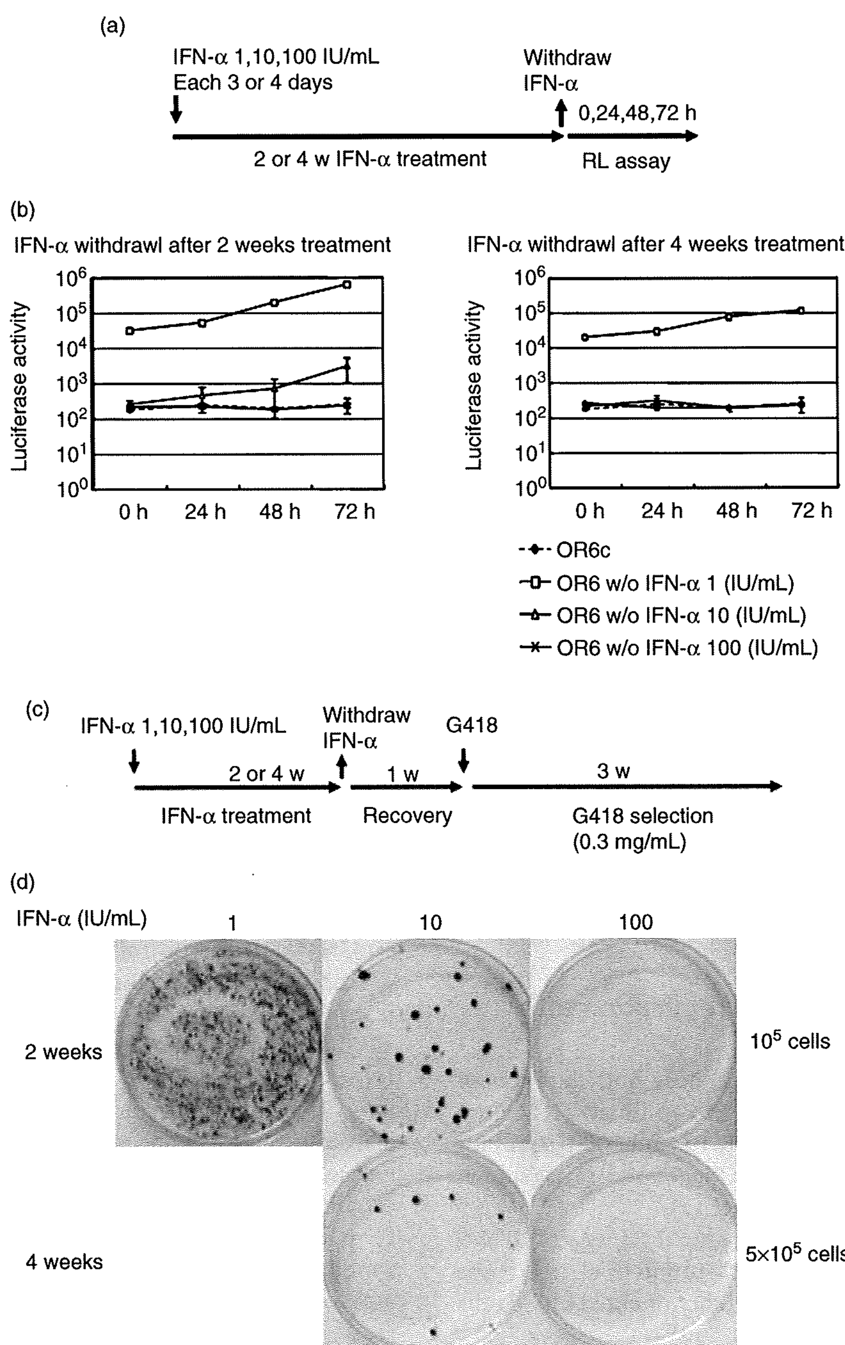

Table 2. Inhibition of the G418-resistant colony might correspond to SVR in the clinical therapy of CHC. These results suggest that the IFN-α-resistant HCV RNA-harboring cells were useful as a SVR model as well as a relapse model.

Characterization of IFN-α-resistant, HCV RNA-harboring cells

Based on the conditions regarding IFN-α resistance, we designed an experiment for the characterization of

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Table 1 72 h/0 h Luciferase value ratio

		IFN-α (IU/mL) withdrawal					
		2 weeks			4 weeks		
OR6c	OR6	1	10	100	1	10	100
1.4		19.8	11.7	1.1	5.6	0.9	1.1

IFN, interferon.

IFN-α-resistant HCV RNA-harboring cells, as shown in Figure 2a and b. For the production of control cells (OR6(C)) and IFN-α-resistant HCV RNA-harboring cells (OR6(R)), OR6 cells were untreated or treated with 5 IU/mL of IFN-α for 2 weeks. The cells were then treated with G418 (0.3 mg/mL) for 3 weeks after 1 week of recovery culture. These cells were the first generation of control cells (OR6(C)) and IFN-α-resistant HCV RNA-harboring cells (OR6(R)). Then, we introduced total RNA from OR6(C) cells or OR6(R) cells into naïve cured OR6c cells for the development of the second-generation control cells (OR6(2C)) and IFN-α-resistant cells (OR6(2R)) (Fig. 2a,b).

The first generations of OR6(C) cells and OR6(R) cells were treated with IFN-α (0, 1, 2, 4, 8, and 16 IU/mL) for 72 h. OR6(R) cells were significantly less sensitive to IFN-α than OR6(C) cells (Fig. 2c). Then, the second generations of OR6(2C) cells and OR6(2R) cells were treated with IFN-α. OR6(2R) cells were less sensitive to IFN-α than OR6(2C) cells, although the difference was not significant (Fig. 2c). These results suggest that cellular factors might play a major role in IFN-α resistance.

We next examined the sequences of HCV in relation to IFN-α resistance. The nucleotide sequences of the open reading frames (ORF) were analyzed by RT–PCR direct sequencing. The AA mutations of HCV in OR6(C) cells and OR6(R) cells were compared with the sequence in the original OR6 cells (Fig. 3a). By the sequence analysis of HCV from OR6(C) cells, we identified one

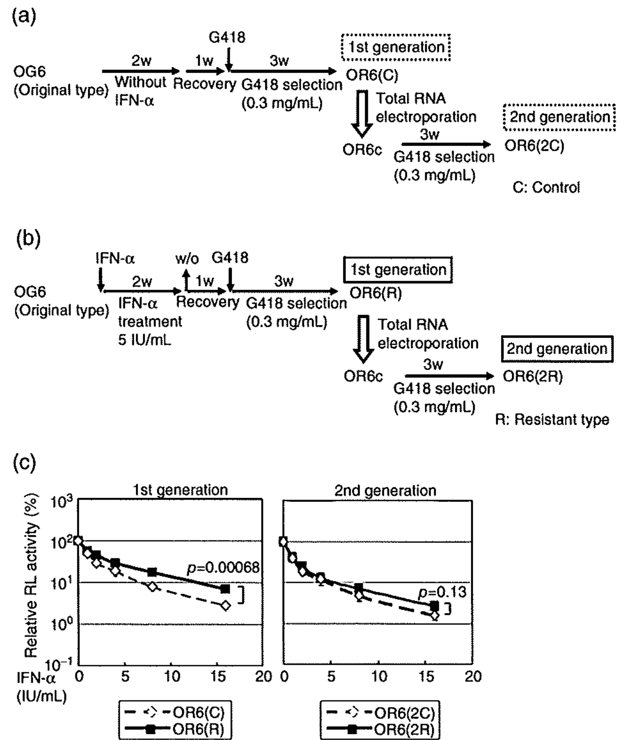

Figure 2 Establishment of the relapse cell culture model. (a and b) Schema for the establishment of the control cells (a) and interferon (IFN)-α-resistant cells (b). OR6 cells were treated without or with IFN-α (5 IU/mL) for 2 weeks. After 1 week of recovery culture without IFN-α, the cells were subjected to G418 selection for 3 weeks. The established cell lines were designated as the first generation of the control cells (OR6(C)) (a) and the IFN-α-resistant cells (OR6(R)) (b). The total ribose nucleic acid (RNA) from OR6(C) or OR6(R) were transfected into naïve OR6c for the development of second generation control cells (OR6(2C)) and IFN-α-resistant cells (OR6(2R)) by 3 week G418 treatment. (c) The sensitivity of the control cells and the IFN-α-resistant cells to IFN-α. The control cells and the IFN-α-resistant cells were treated with IFN-α (0, 1, 2, 4, 8, and 16 IU/mL) for 72 h using first generation cells (left panel) and second generation cells (right panel).

mutation, I65M, in the core; one mutation, Q501R, in E2; two mutations, P1115L and T1286A, in NS3; and two mutations, A1821G and G1926, in NS4B (Fig. 3b). In the HCV sequence from OR6(R) cells, we identified two mutations, Y276H and M324V, in E1, and one mutation, E1202G, in NS3 (Fig. 3c). Although the core and NS5A were intensively investigated in relation to IFN resistance, we did not identify any mutations in these regions of HCV from IFN-resistant HCV RNA-harboring cells.

Table 2 Numbers of colonies by G418 selection after interferon-α treatment

	Numbers of colonies (/10^5 Cells)	
	IFN-α (IU/mL)	
	10	100
2 weeks	27.6 ± 6.7	0
4 weeks	0.9 ± 0.3	0

IFN, interferon.

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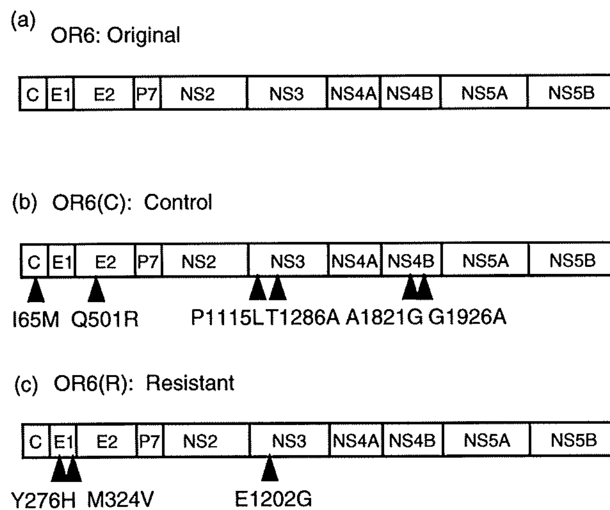

Figure 3 The sequence analysis for hepatitis C virus (HCV) in interferon (IFN)-α-resistant cells. HCV open reading frames were determined by the RT–PCR direct sequencing methods from the genome-length HCV RNA derived from the original OR6, the control, and IFN-α-resistant cells. The arrows indicate the AA substitutions in the control cells and IFN-α-resistant cells compared with original OR6.

IFN-α-resistant HCV RNA-harboring cells are resistant to IFN-β but not to IFN-γ, CsA (cyclosporine A), or FLV (fluvastatin)

We next examined the sensitivities of the established IFN-α-resistant HCV-harboring cells to anti-HCV reagents. IFN-β belongs to the type I IFN family and shares a common JAK-STAT signaling pathway with IFN-α. Therefore, we examined the sensitivity of HCV to IFN-β in OR6(C) cells and OR6(R) cells. OR6(C) cells and OR6(R) cells were treated with IFN-β (0, 0.125, 0.25, 0.5, 1, 2, and 4 IU/mL) as well as IFN-α (0, 0.25, 0.5, 1, 2, and 4 IU/mL) for 72 h. HCV in OR6(R) cells was significantly less sensitive to both IFN-α and IFN-β than that in OR6(C) cells (Fig. 4a). These results suggest that IFN-α-resistant HCV-harboring cells are also resistant to IFN-β, which shares a similar anti-HCV mechanism with IFN-α.

Next, we investigated the sensitivity of HCV in OR6(R) cells to the type II IFN, IFN-γ. IFN-γ exhibits its anti-HCV activity via a signaling pathway different from that of the type I IFN and induces unique IFN-stimulated genes. OR6(C) cells and OR6(R) cells were treated with IFN-γ (0, 0.125, 0.25, 0.5, 1, 2, and 4 IU/mL) for 72 h. There was no significant difference in the sensitivity to IFN-γ between these cells (Fig. 4b, left panel).

Then, we investigated the other well-characterized anti-HCV reagents, FLV and CsA, for their sensitivity using IFN-α-resistant HCV RNA-harboring cells.[17,25–29] OR6(C) cells and OR6(R) cells were treated with FLV (0, 0.25, 0.5, 1, 2, 4, 8, and 16 μM) or CsA (0, 0.031, 0.062, 0.125, 0.25, 0.5, and 1 μg/mL) for 72 h. There were no significant differences in the sensitivity to FLV or CsA between these cells (Fig. 4b, middle and right panel). We also evaluated and compared the EC_{50} values of the anti-HCV reagents between OR6(C) cells and OR6(R) cells (Table 3). The averages and standard deviations were calculated from the three independent experiments and subjected to Student's *t*-test. The EC_{50} of type I IFN in OR6(R) cells was approximately twice as high as that in OR6(C) cells, whereas there were no significant differences between the two types of cells in sensitivity to IFN-γ, FLV and CsA. Collectively, these results suggest that anti-HCV reagents that have inhibitory mechanisms different from that of IFN-α are useful for the treatment of IFN-α-resistant HCV-RNA harboring cells.

Using the immunofluoresence analysis, we also showed the effect of anti-HCV reagents on core expression in OR6(C) and OR6(R) cells (Fig. 4c). The expression of core in OR6(C) cells were relatively lower than that in OR6(R) after treatment with IFN-α (4 IU/mL) or IFN-β (2 IU/mL). In contrast, there were no differences between OR6(C) and OR6(R) cells after treatment with IFN-γ (4 IU/mL), FLV (10 μM) or CsA (1 μg/mL).

To clarify whether the different effects of type I IFN observed between OR6(C) and OR6 (R) cells are dependent on the cellular potentials of the type I IFN signaling pathway, we carried out a dual-luciferase reporter assay using a type I IFN-inducible ISRE promoter. The response of OR6(C) cells to IFN-α (0, 1, 10 and 100 IU/mL) was stronger than that of OR6(R) cells (Fig. 4d). This result suggests that low sensitivity of OR6(R) cells to IFN-α might be caused by the weak induction of interferon stimulated genes.

Development and characterization of a relapse model using IFN-α-resistant HCV RNA-harboring cells

We next attempted to develop a relapse model of IFN treatment using IFN-α-resistant HCV RNA-harboring cells. The experimental design is shown in Figure 5a. OR6 cells were treated with IFN-α alone or in combination with other anti-HCV reagents for 72 h. After the withdrawal of the reagents, HCV RNA levels were monitored for 72 h by luciferase activity. Western blot analysis was also carried out using the cell lysate 120 h after withdrawal of the reagents. First, we examined the concentration of IFN-α required to prevent the relapse after treatment of OR6(C) cells and OR6(R) cells for 72 h.

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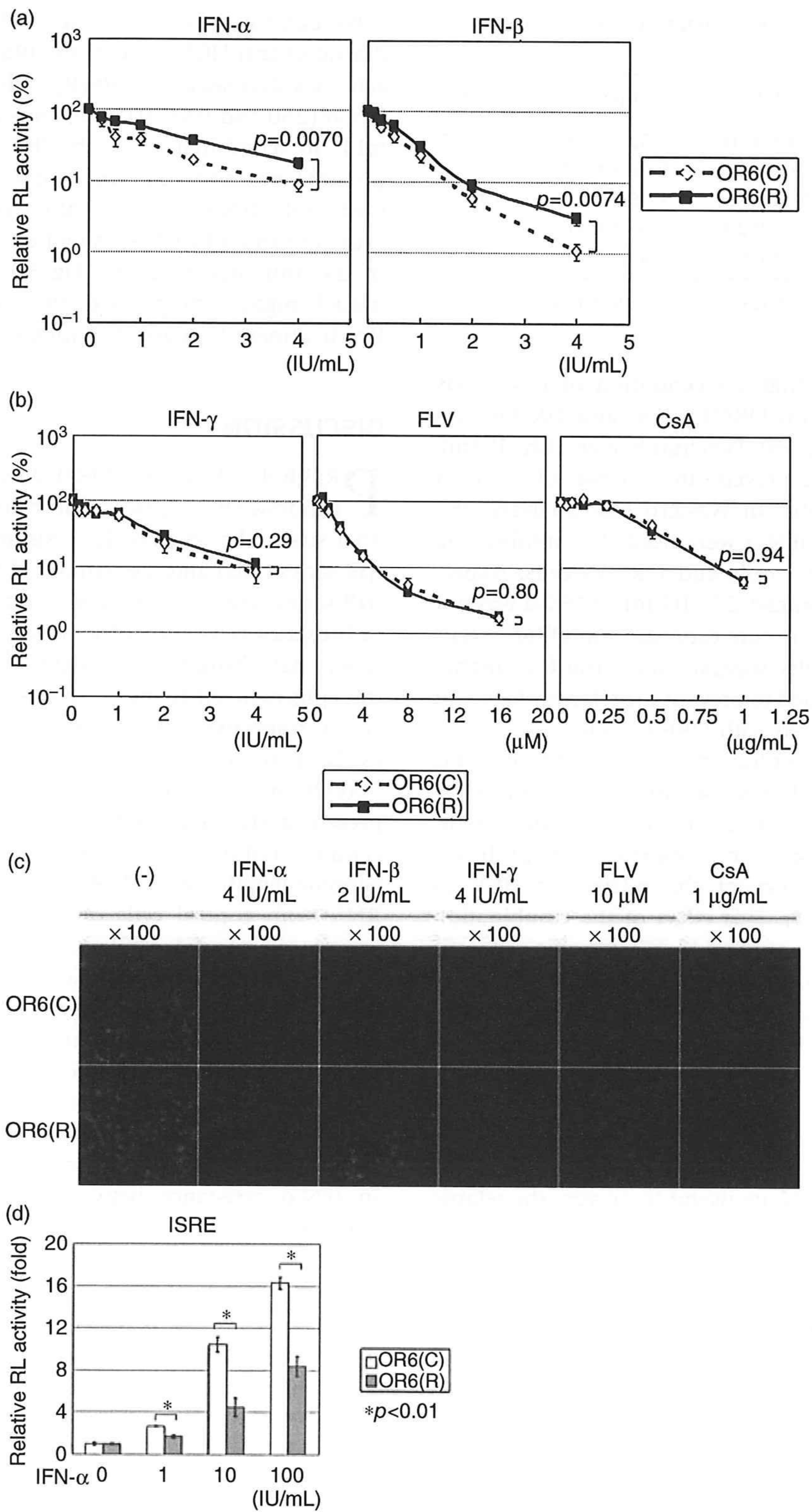

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Table 3 EC_{50} values of anti-hepatitis C virus reagents in OR6(C) and OR6(R) cells

	OR6(C)	OR6(R)	*P*-value
IFN-α (IU/mL)	0.60 ± 0.20	1.32 ± 0.16	0.0077
IFN-β (IU/mL)	0.38 ± 0.12	0.66 ± 0.11	0.047
IFN-γ (IU/mL)	1.21 ± 0.16	1.30 ± 0.13	0.46
FLV (μM)	1.50 ± 0.20	1.65 ± 0.10	0.44
CsA (μg/mL)	0.46 ± 0.05	0.42 ± 0.06	0.50

CsA, cyclosporine A; FLV, fluvastatin; IFN, interferon.

For this purpose, a high concentration of IFN-α was needed: 250 IU/mL for OR6(C) cells and 1000 IU/mL for OR6(R) cells (Fig. 5b). Two hundred and fifty IU/mL of IFN-α could not prevent the relapse of HCV in OR6(R) cells (Fig. 5b). In Western blot analysis, 250 and 1000 IU/mL of IFN-α were needed to inhibit core expression in OR6(C) cells and OR6(R) cells, respectively (Fig. 5c). In contrast, 250 IU/mL of IFN-α was not sufficient to inhibit the core expression in OR6(R) cells (Fig. 5c). These results suggest that a fourfold higher dose of IFN-α is needed to prevent the relapse of HCV in OR6(R) cells compared with OR6(C) cells.

We adopted a concentration of 250 IU/mL of IFN-α for the relapse model as a cotreatment with anti-HCV reagents to prevent the relapse of HCV in OR6(R) cells. IFN-γ (4 and 8 IU/mL) in combination with IFN-α (250 IU/mL) could prevent the relapse of HCV in OR6(R) cells (Fig. 5d). This effect of the combination treatment was also confirmed by Western blot analysis (Fig. 5e). FLV (5 and 10 μM) or CsA (0.5 and 1 μg/mL) in combination with IFN-α (250 IU/mL) was also sufficient to prevent the relapse of HCV in OR6(R) cells in luciferase assay (Fig. 5f,h). These effects of the combination treatment were also confirmed by Western blot analysis (Fig. 5g,i). These results suggest that anti-HCV reagents that have an inhibitory mechanism different from that of type I IFN are useful to prevent the relapse of IFN-α-resistant HCV.

We examined the time course before and after withdrawal of anti-HCV reagents in OR6(R) cells. Luciferase activities decreased in OR6(R) cells after treatment of IFN-α (250 and 1000 IU/mL) alone and IFN-α (250 IU/mL) in combination with IFN-γ (4 IU/mL), FLV (10 μM), or CsA (0.5 μg/mL) for 72 h (Fig. 5j). In contrast, only IFN-α (250 IU/mL) treatment in its own caused relapse of HCV RNA replication after withdrawal of the anti-HCV reagents (Fig. 5j). This result in our model might correspond to the transient response in IFN treatment for patients with CHC.

DISCUSSION

PREVIOUSLY, WE REPORTED two IFN-resistant subgenomic HCV replicon-harboring cell lines from the 1B-1 strain: the severely IFN-resistant replicon cell lines (βR-series cells) and the partially IFN-resistant cell lines (αR-series cells).[30,31] The severe and mild IFN resistance of βR-series cells and αR-series cells are caused by the functional disruption of type I IFN receptors and the epigenetic silencing of interferon stimulated genes (ISG), respectively. These IFN-resistant cell lines were established with a high concentration of IFN-α (100–2000 IU/mL) and/or IFN-β (400–1000 IU/mL). In the present study, we treated the OR6 cells with a low concentration of IFN-α (5 IU/mL) to establish IFN-resistant genome-length HCV RNA-harboring cells. The total RNA from control cells or IFN-α-resistant cells were introduced into naïve OR6c cells to establish the second generation of control cells (OR6(2C)) or IFN-resistant cells (OR6(2R)). OR6(2R) cells were less sensitive to IFN-α than OR6(2C), although the difference was not significant. More recently, Noguchi *et al.*[22] reported that IFN-α-resistant HCV replicon-harboring cells were isolated by treatment with a relatively low concentration of IFN-α (10 and 30 IU/mL). In that study, the difference in IFN-α resistance between the second generation control and IFN-α-resistant cells decreased compared

Figure 4 The sensitivities of hepatitis C virus (HCV) in the interferon (IFN)-α-resistant cells to anti-HCV reagents. (a) The sensitivity of HCV in the IFN-α-resistant cells to type I IFNs. The control cells and the IFN-α-resistant cells were treated with IFN-α (0, 0.25, 0.5, 1, 2, and 4 IU/mL) (left panel) and IFN-β (0, 0.125, 0.25, 0.5, 1, 2, and 4 IU/mL) (right panel), respectively, for 72 h. Then these cells were subjected to luciferase assay. (b) The sensitivity of HCV in the IFN-α-resistant cells to IFN-γ, FLV, and CsA. The control cells and the IFN-α-resistant cells were treated with IFN-γ (0, 0.125, 0.25, 0.5, 1, 2, and 4 IU/mL; left panel) and FLV (0, 0.25, 0.5, 1, 2, 4, 8, and 16 μM; middle panel), and CsA (0, 0.031, 0.062, 0.125, 0.25, 0.5, and 1.0 μg/mL; right panel), respectively, for 72 h. Then these cells were subjected to luciferase assay. (c) Expression of core in the control cells and the IFN-α-resistant cells. The control cells and the IFN-α-resistant cells were treated with IFN-α (0 and 4 IU/mL), IFN-β (2 IU/mL), IFN-γ (4 IU/mL), FLV (10 μM), and CsA (1 μg/mL) for 72 h and subjected to immunofluorescence analysis. (d) Dual-luciferase reporter assay of IFN-α. The control cells and the IFN-α-resistant cells were treated with IFN-α (0, 1, 10, and 100 IU/mL) for 6 h and subjected to ISRE reporter assay.

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