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# Hepatitis C virus impairs TLR3 signaling and inhibits IFN-λ 1 expression in human hepatoma cell line

**Yizhong Wang[1,2], Jieliang Li[2], Xu Wang[2], Li Ye[1], Yu Zhou[2], Rebecca M Thomas[2] and Wenzhe Ho[1,2]**

## Abstract

*Toll-like receptor 3 (TLR3)* activation plays an important role in the innate immune responses to viral infections. We show here that the activation of *TLR3* signaling pathway by poly I:C, a synthetic mimic of dsRNA, could induce high-level expression of *interferon (IFN)-λ1* in a hepatoma cell line. The induced *IFN-λ1* contributed to poly I:C-mediated inhibition of hepatitis C virus (HCV) Japanese fulminant hepatitis-1 (JFH-1) replication in Huh7 cells. This inhibitory effect of poly I:C on HCV replication, however, was compromised by HCV infection of Huh7 cells. Investigation of the mechanisms showed that HCV infection suppressed the expression of poly I:C-induced *IFN-λ1* and IFN-stimulated genes [*IFN-stimulated gene 56* (*ISG-56*), *myxovirus resistance A* (MxA) and *2′-5′-oligoadenylate synthetase 1* (OAS-1))], the key antiviral elements in IFN signaling pathway. Among the HCV nonstructural (NS) proteins tested, *NS3/4A*, *NS5A* and *NS5B* had the ability to inhibit poly I:C-induced *IFN-λ1* expression in Huh7 cells. These observations provide the experimental evidence that HCV and its proteins impair *TLR3* signaling and inhibit intracellular *IFN-λ1/ISG* expression in a hepatoma cell line, which may account for HCV persistence in the liver.

## Keywords

Hepatitis C virus, interferon-λ, IFN-stimulated genes, poly I:C, *Toll-like receptor 3*

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## Introduction

Hepatitis C virus (HCV) infection is a major cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma.[1] The clinical outcome of HCV infection and the degree of liver damage are the result of complicated interactions between the virus and host immune responses.[2] Although cellular and humoral immune responses are present during acute and chronic HCV infection, these immune responses appear to be ineffective in eradicating the virus.[3] The majority of HCV-infected patients develop chronic infection, suggesting that HCV has evolved strategies to overcome or evade host immune responses.[4–8]

Viral infections result in induction of interferons (IFNs) that are the key players in antiviral responses.[9] Induction of the IFN-based antiviral innate immunity depends on several *Toll-like receptors (TLRs)*, i.e. *TLR3, 7* and *9*.[10] *TLR3* is a major intracellular sensor that recognizes dsRNA and triggers the IFN signaling pathway. However, persistent HCV infection is associated with its ability to disrupt and evade TLR3-mediated immune responses. HCV nonstructural (NS)3/4A protease ablates TLR3 signaling by cleaving the *TLR3 adaptor protein Toll-IL-1 receptor domain-containing adaptor inducing IFN-β (TRIF)* to evade type I *IFN*-mediated innate immunity.[11] Recent studies have demonstrated that, in addition to *IFN-α/β*, *IFN-λ* also has the ability to inhibit HCV replication in both *in vitro* and in *in vivo* systems.[12–17] In the present study we examined whether *TLR3* signaling induces *IFN-λ* expression and whether HCV inhibits *IFN-λ* expression in hepatoma cell line.

[1]The Center for Animal Experiment/ABSL-3 Laboratory, Wuhan University, Hubei, People's Republic of China
[2]Department of Pathology and Laboratory Medicine, Temple University School of Medicine, Philadelphia, PA, USA

**Corresponding author:**
Wenzhe Ho, Department of Pathology and Laboratory Medicine, Temple University School of Medicine, 843 MERB, 3500 N. Broad Street, Philadelphia, PA 19140, USA.
Email: wenzheho@temple.edu

## Materials and methods

### Cells, plasmids and virus

A hepatoma cell line (Huh7) provided by Dr Charles Rice (The Rockefeller University, New York, NY, USA) was maintained in DMEM with 10% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml). HCV *NS3*, *NS3/4A, NS5A* and *NS5B* plasmids, and control plasmid (pcDNA 3.1) were obtained from Dr Michael Gale (University of Washington, Seattle, WA, USA).[5] The generation of infectious HCV JFH-1 and infection of Huh7 cells [multiplicity of infection (MOI) of 0.01] were carried out as described previously.[18,19] HCV JFH-1 infection of Huh7 was analyzed by the immunostaining with the mouse anti-HCV *core* Ab or by real time RT-PCR for HCV RNA.

### Reagents

LyoVec transfection reagent, poly I:C (high molecular mass) were purchased from Invivogen (San Diego, CA, USA). ELISA kit for *IFN-λ1* was purchased from eBioscience Inc. (San Diego, CA, USA). Mouse Ab against HCV *core* antigen was purchased from ABR Affinity BioReagents, Thermo Scientific (Rockford, IL, USA). Rabbit Abs against *IFN-stimulated gene 56* (*ISG56*), *myxovirus resistance A* (*MxA*), *2′-5′-oligoadenylate synthetase 1*(*OAS-1*) and *actin* were purchased from Sigma-Aldrich (St Louis, MO, USA). Rabbit anti-*IFN stimulated transcription factor 3 gamma (ISGF3G)* Ab was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Hoechst 33342 was purchased from Molecular Probes (Carlsbad, CA, USA).

### Poly I:C stimulation

Huh7 cells were seeded at a density of $10^5$ each well in a 24 well-plate. After culturing for 24 h, the cells were stimulated with poly I:C (0.2–5 μg/ml) using LyoVec transfection reagent. Cells were collected for mRNA extraction after 24 h stimulation and supernatant (SN) was collected for ELISA assay after 24–48 h stimulation. For the anti-HCV experiment, the cells were stimulated with poly I:C for 8 h prior to HCV JFH-1 infection. Culture SN and cells were collected for mRNA extraction at JFH-1 72 h post-infection. For the poly I:C stimulation of HCV JFH-1-infected Huh7 cells experiment, JFH-1-infected Huh7 cells (72 h post-infection) were stimulated with poly I:C (0.2–5 μg/ml) using LyoVec transfection reagent. Culture SN and cells were collected for mRNA extraction after 48 h stimulation. As a negative control of the transfection experiment, cells were incubated with the LyoVec transfection reagent only. For the blocking experiments with Bafilomycin A1, a vacuolar $H^+$- ATPase inhibitor that inhibits the acidification of endosome,[20] Huh7 cells were treated with 100 nM of Bafilomycin A1 for 1 h prior to poly I:C stimulation.

### Plasmid transfection

Transfection of the plasmid DNA was carried out with LyoVec transfection reagent, as recommended by the manufacturer. The Huh7 cells were transfected with the plasmids (HCV *NS3, NS3/4A, NS5A* and *NS5B*) using LyoVec transfection reagent at a ratio of LyoVec: plasmid 50:1 (μl:μg) at a final plasmids concentration of 0.5 μg/ml of each cell culture well. The empty vector (pcDNA 3.1) for HCV NS genes containing constructs was used as the control and included in parallel for each transfection experiment.

### RNA extraction and real time RT-PCR

Total RNA from culture cells or SN was extracted with Tri-Reagent (Molecular Research Center, Cincinnati, OH, USA) as described previously.[21] Total RNA (1 μg) was subjected to RT using the RT system (Promega, Madison, WI, USA) with random primers for 1 h at 42°C. The reaction was terminated by incubating the reaction mixture at 99°C for 5 min, and the mixture was kept at 4°C. The resulting cDNA was used as a template for real time PCR quantification. Real time PCR was performed with 1/10 of the cDNA with the iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) as described previously.[22] The amplified products were visualized and analyzed using the software MyiQ provided with the thermocycler (iCycler iQ real time PCR detection system; Bio-Rad Laboratories). The oligonucleotide primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA, USA) and sequences will be available upon request. The cDNA was amplified by PCR and the products were measured using SYBR green I (Bio-Rad Laboratories). The data were normalized to GAPDH and presented as the change in induction relative to that of untreated control cells.

### ELISA

*IFN-λ1* gene expression analyzed by the real time RT-PCR was evaluated by ELISA for protein expression. SN collected from poly I:C-stimulated Huh7 cultures was tested directly for *IFN-λ1* protein levels by ELISA, which was performed according to the manufacturer's instructions.

### Statistical analysis

Student's *t*-test was used to evaluate the significance of difference between groups, and multiple comparisons

were performed by regression analysis and ANOVA. *P*-Values $< 0.05$ were considered significant. All data are presented as mean ± SD. Statistical analyses were performed with SPSS 11.5 for Windows (SPSS, Chicago, IL, USA). Statistical significance was defined as $P < 0.05$.

## Results

### *TLR3 signaling induces IFN-λ1 expression*

It is known that the activation of *TLR3* can induce type I IFNs in human hepatocytes.[23] However, it is unclear whether *TLR3* signaling has an effect on *IFN-λ* expression. We showed that poly I:C stimulation of Huh7 cells significantly induced the expression of *IFN-λ1* at both mRNA (Figure 1A) and protein (Figure 1B) levels, and this effect was dose dependent. To investigate whether *TLR3* activation is responsible for poly I:C-induced *IFN-λ1* expression, Bafilomycin A1, an inhibitor of the *TLR3* signaling pathway, was used to block the poly I:C action. As shown in Figure 1C, Bafilomycin A1 treatment compromised the poly I:C-induced *IFN-λ1* expression in Huh7 cells. To determine whether *retinoic acid-inducible gene I* (*RIG-I*) signaling is involved in poly I:C-mediated *IFN-λ* induction, we used Huh7.5 cells that have a defect in the *RIG-I* signaling pathway[18,24] in the experiments. We showed that poly I:C could induce *IFN-λ1* expression in Huh7.5 cells, which could be largely blocked by Bafilomycin A1 treatment (Figure 1D).

### *TLR3 signaling induces IFN-stimulated gene expression*

*ISGF3G* also known as *IFN regulatory factor 9* (*IRF-9*), plays a critical role in ISGs induction.[25] We observed that poly I:C induced *ISGF3G* expression in Huh7 cells at both mRNA (Figure 2A) and protein (Figure 3) levels. We next examined the effect of poly I:C on the

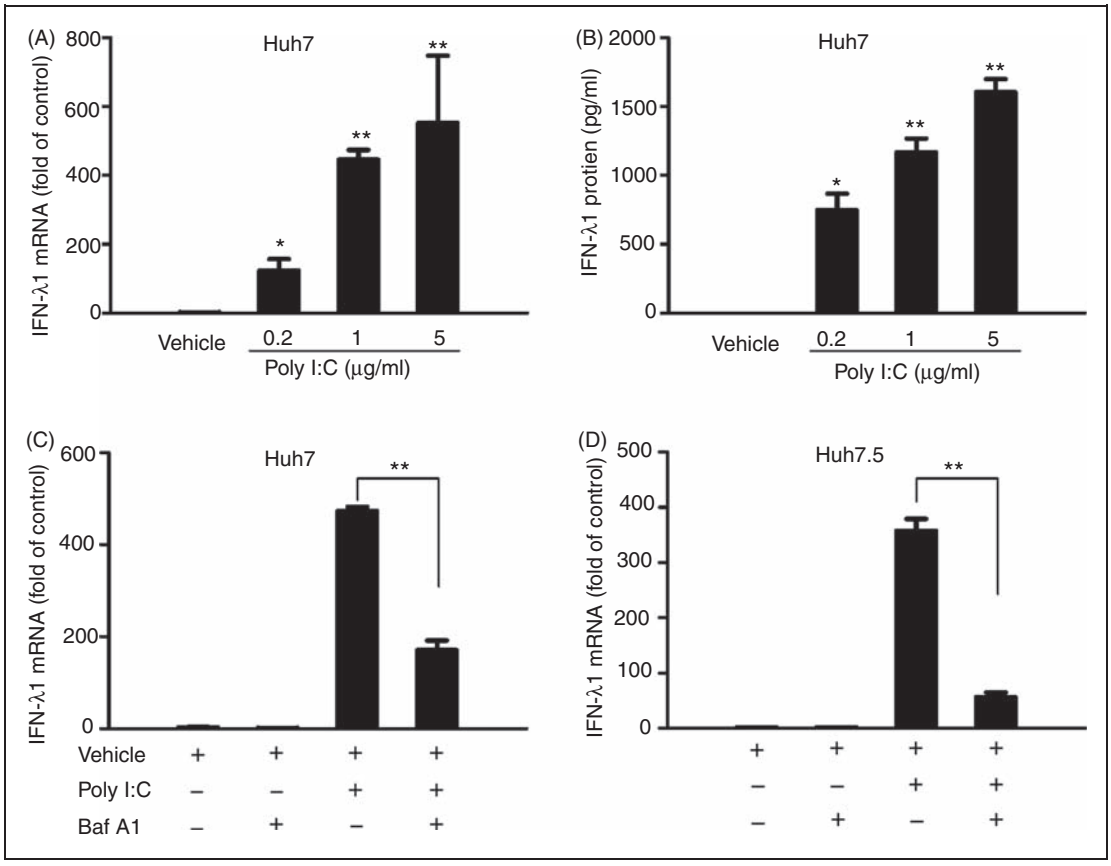



**Figure 1.** *TLR3* activation induces *IFN-λ1* expression. (A) Effect of poly I:C on *IFN-λ1* mRNA expression. Huh7 cells were stimulated with poly I:C at the indicated concentrations for 24 h. (B) Effect of poly I:C on *IFN-λ1* protein production. Huh7 cells were stimulated with poly I:C at the indicated concentrations for 48 h. SN was collected from the cell cultures for ELISA to measure the protein levels of *IFN-λ1*. (C, D) Blocking *TLR3* signaling compromises poly I:C-induced *IFN-λ1* expression. Huh7 (C) or Huh7.5 (D) cells were pretreated with or without Bafilomycin A1 (100 nM) for 1 h prior to poly I:C stimulation (24 h). Total RNA extracted from cells was subjected to the real time RT-PCR for the mRNA levels of *IFN-λ1* and *GAPDH*. The data are expressed *IFN-λ1* mRNA levels relative (fold) to the control (vehicle only, which defined as 1). The results shown are mean ± SD of triplicate cultures, representative of three experiments (poly I:C stimulated Huh7 cell vs vehicle only, $^{**}P < 0.01$, $^{*}P < 0.05$).

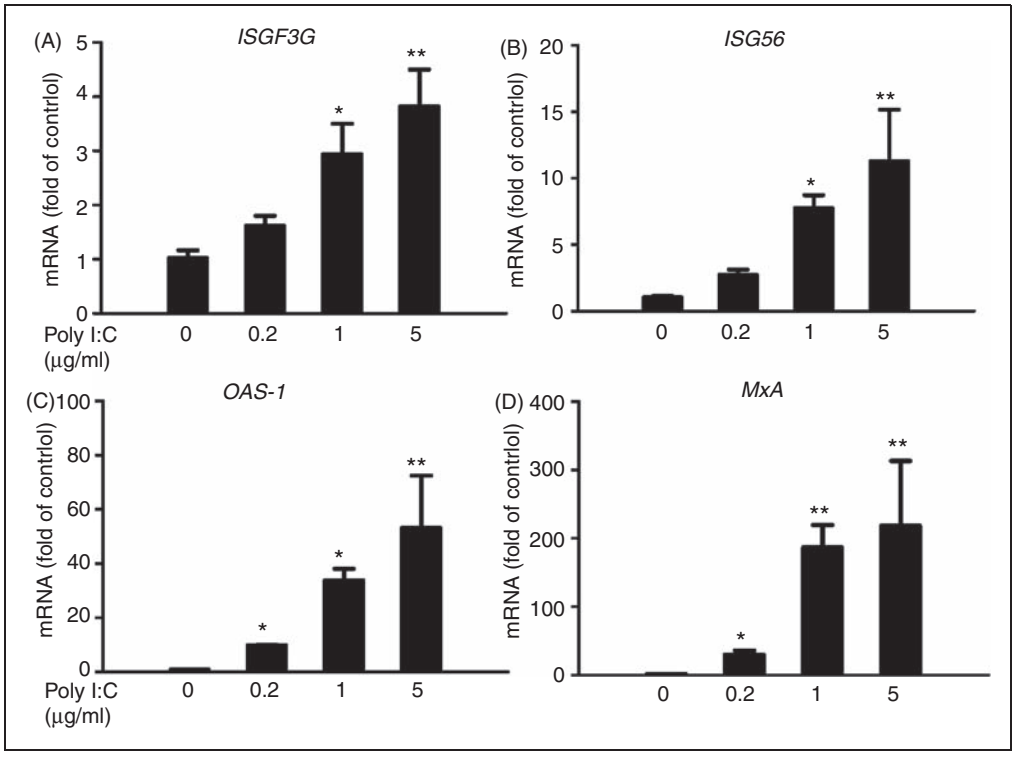



**Figure 2.** Effect of poly I:C on the mRNA expression of ISGs. Huh7 cells were stimulated with poly I:C at the indicated concentrations for 24 h. Total RNA extracted from cells was subjected to the real time RT-PCR for the mRNA levels of *ISGF3G* (A), *ISG56* (B), *OAS-1* (C), *MxA* (D) and *GAPDH*. The data are expressed as mRNA levels relative (fold) to the control (vehicle only, which defined as 1). The results shown are mean ± SD of triplicate cultures, representative of three experiments (poly I:C stimulated Huh7 cell vs vehicle only, $^{**}P < 0.01$, $^{*}P < 0.05$).

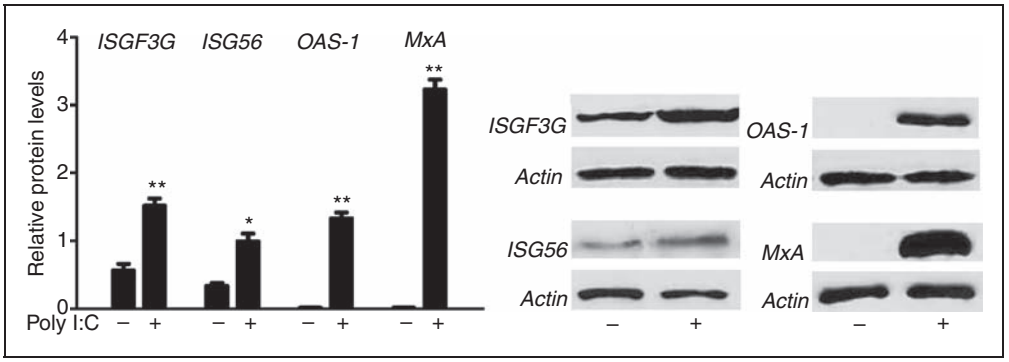



**Figure 3.** Poly I:C induces ISGs protein expression. Huh7 cells were stimulated with poly I:C (1 μg/ml) for 48 h. Total proteins extracted from cell cultures were subjected to Western blot assay using the Abs against *ISGF3G*, *ISG56*, *OAS-1*, *MxA* and *β-actin*. Representative blots from three independent experiments were shown. Densitometry analysis of the blot was performed using ImageJ1.44 software (National Institutes of Health, Bethesda, MD, USA) and plotted into graphs from data collected from triplicate experiments (poly I:C stimulated Huh7 cell vs vehicle only, $^{**}P < 0.01$, $^{*}P < 0.05$).

ISGs that have antiviral effects. As shown in Figure 2B–D, poly I:C significantly induced *ISG56*, *MxA* and *OAS-1* mRNA expression in a dose-dependent manner. We also examined the protein levels of these ISGs; as shown in Figure 3, poly I:C could increase the protein levels of *ISG 56*, *OAS-1* and *MxA*, the key antiviral elements in the IFN signaling pathway.[26–29]

## *TLR3 signaling inhibits HCV replication*

Knowing that *TLR3* signaling could induce the expression of *IFN-λ1* and the antiviral ISGs, we then examined whether *TLR3* signaling of Huh7 cells can inhibit HCV replication. As shown in Figure 4A and B, poly I:C significantly inhibited HCV JFH-1 infection of

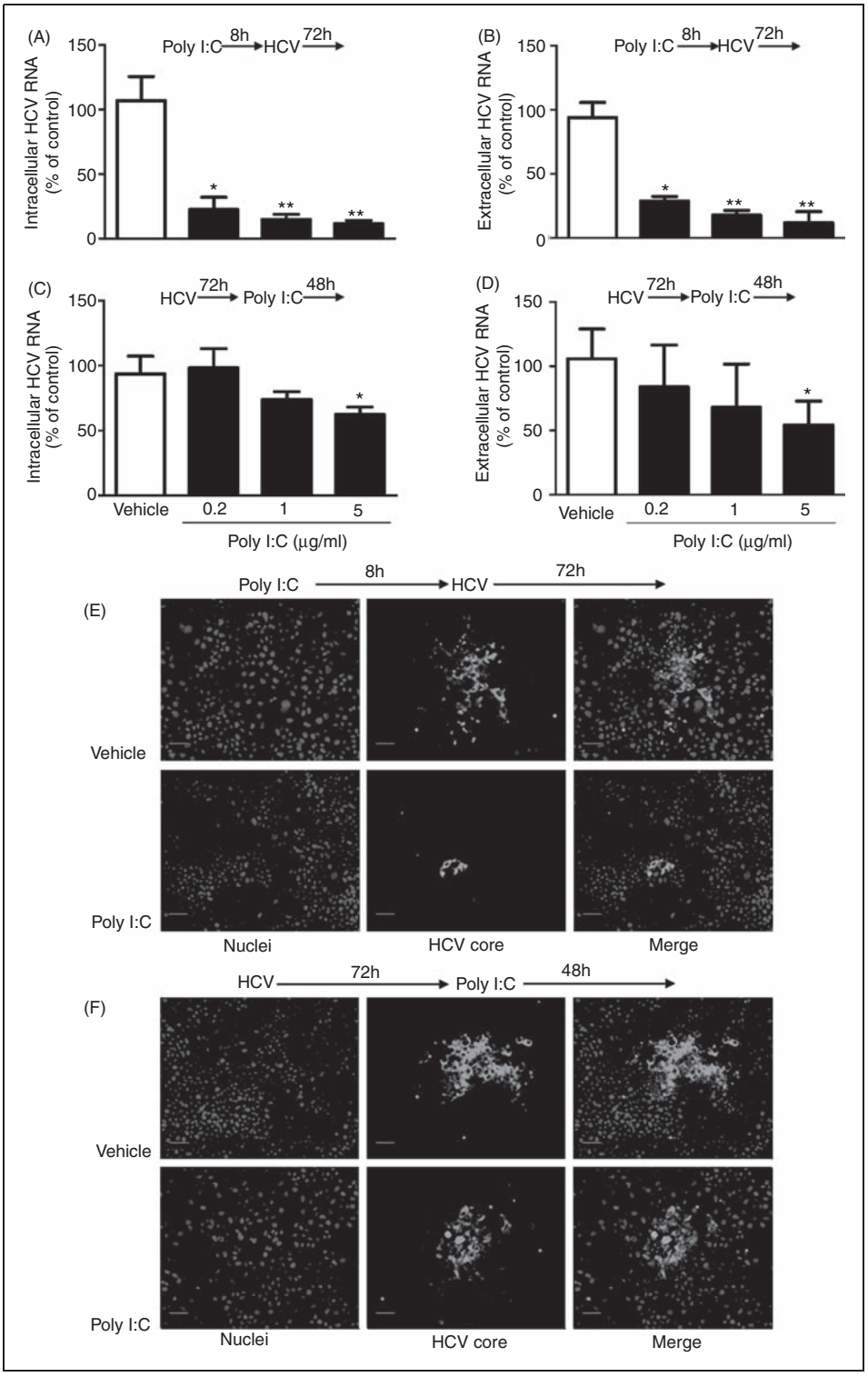



**Figure 4.** Effect of poly I:C on HCV replication. (A–D) Effect of Poly I:C on HCV RNA replication. Huh7 cells were stimulated with poly I:C at indicated concentration for 8 h prior to HCV JFH-1 infection (A, B) or stimulated with poly I:C at 72 h post-infection (C, D). Intracellular (A, C) and extracellular (B, D) RNA extracted from Huh7 cells or culture SN was subjected to the real time RT-PCR for HCV and *GAPDH* RNA quantification. HCV RNA level is expressed as HCV RNA levels relative (%) to the control (vehicle only, which are defined as 100%). The results shown are mean ± SD of triplicate cultures, representative of three experiments (poly I:C-stimulated Huh7 cell vs vehicle-treated cells only: $^{**}P < 0.01$, $^{*}P < 0.05$). (E, F) Effect of poly I:C on HCV core protein expression. Huh7 cells were stimulated with or without poly I:C (1 μg/ml) for 8 h prior to HCV JFH-1 infection (E) or 72 h post-infection for 48 h (F). HCV *core* protein expression was determined by immunofluorsence staining with Ab against HCV *core* protein (green). The nuclei were stained with Hoechst 33342 (blue). One representative experiment is shown (original magnification 200×, scale bar: 20 μM).

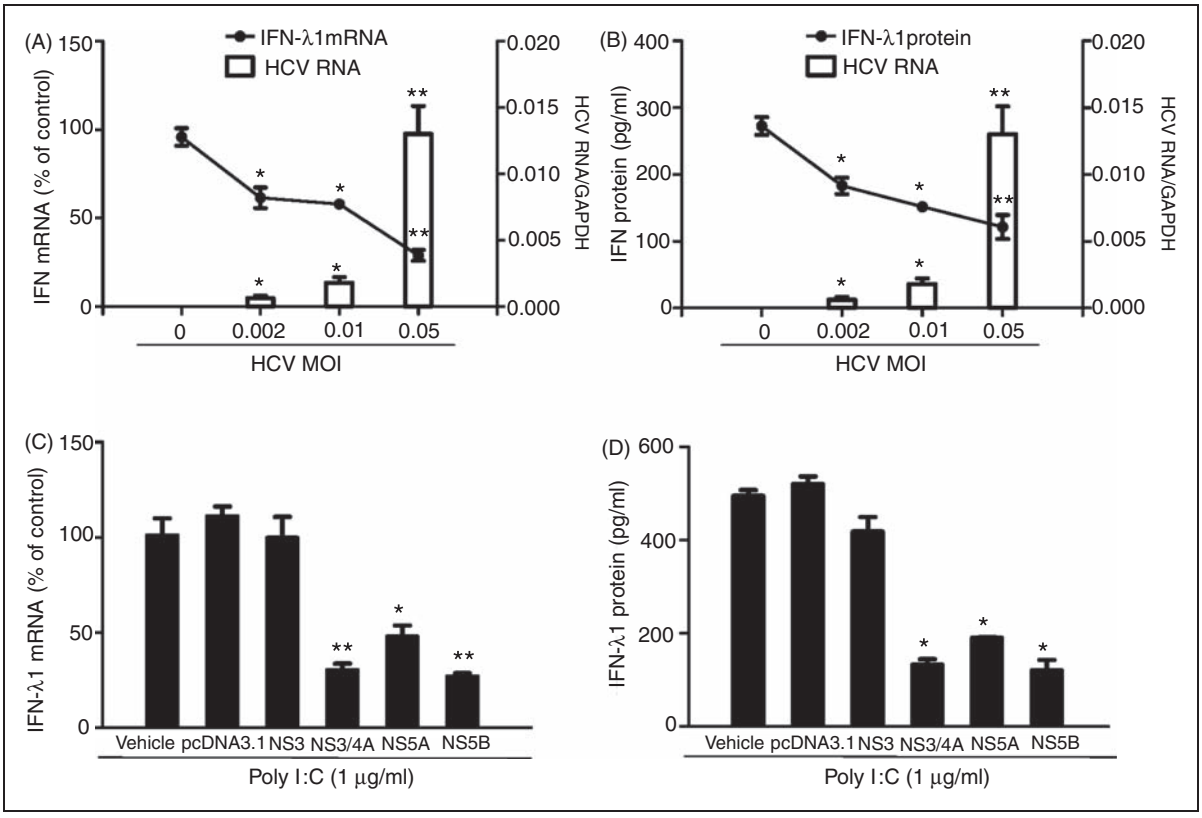



**Figure 5.** HCV inhibits poly I:C-induced *IFN-λ1* expression. (A, B) Huh7 cells were infected with HCV JFH-1 at indicated MOI for 72 h, then stimulated with poly I:C (1 μg/ml) for 24 h. (C, D) Effect of HCV NS proteins on poly I:C induced *IFN-λ1* expression. Huh7 cells were stimulated with the plasmids containing HCV NS genes for 18 h prior to poly I:C (1 μg/ml) stimulation for 24 h. Total RNA extracted from cells was subjected to real time RT-PCR for the mRNA levels of *IFN-λ1* and *GAPDH*. The data are expressed as *IFN-λ1* mRNA (A, B) levels relative (fold) to the control (without HCV infection or without plasmids transfection, which defined as 100%). SN collected from cell cultures was assayed by ELISA to measure the *IFN-λ1* protein (B, D). The results shown are mean ± SD of triplicate cultures, representative of three independent experiments (poly I:C-stimulated Huh7 cell versus vehicle only: $^{**}P < 0.01$, $^{*}P < 0.05$).

Huh7 cells and the degree of the suppression was correlated positively with the doses of poly I:C. This inhibitory effect of poly I:C on HCV could be observed before or after HCV infection (Figure 4). However, *TLR3* signaling by poly I:C prior to HCV infection (Figure 4A, C, E) was much more potent in HCV inhibition than that after HCV infection (Figure 4B, D, F).

### *HCV suppresses* IFN-λ1 *and ISG expression*

We next examined whether HCV has the ability to inhibit *TLR3* activation-induced *IFN-λ1* and ISG expression. We infected Huh7 cells with HCV JFH-1 at different doses (MOI 0.002, 0.01 and 0.05) for 72 h prior to poly I:C stimulation. We observed that the poly I:C-induced *IFN-λ1* expression was decreased at both mRNA (Figure 5A) and protein (Figure 5B) levels in HCV-infected cells. Similarly, HCV infection inhibited the expression of the ISG induced by poly I:C (Figure 6A–C). To determine which HCV protein(s) play a major role in HCV-mediated down-regulation of intracellular *IFN-λ1* expression induced by poly I:C in Huh7 cells, we transfected Huh7 cells with the plasmids containing HCV genes that encode NS proteins for 18 h prior to poly I:C stimulation. The expression of the HCV *NS3* protein had little effect on poly I:C-induced *IFN-λ1* expression. In contrast, the expression of HCV *NS3/4A, NS5A* and *NS5B* proteins in Huh7 cells resulted in the significant suppression of poly I:C-induced *IFN-λ1* expression at both mRNA (Figure 5C) and protein (Figure 5D) levels.

## Discussion

The interaction of HCV and host immune responses is an important determinant of the outcome of HCV infection. Innate immunity is the first line of defense against viral infections. IFNs are the key players in host innate immunity, as they possess antiviral activity against a number of viruses,[9] including HCV.[30,31] In addition to type I and type II IFNs, which have been known for decades as the antiviral cytokines, type III IFNs (*IFN-λ*) are also shown to have a strong antiviral function.[32] Although *IFN-λ1* and *IFN-λ2/3* exert

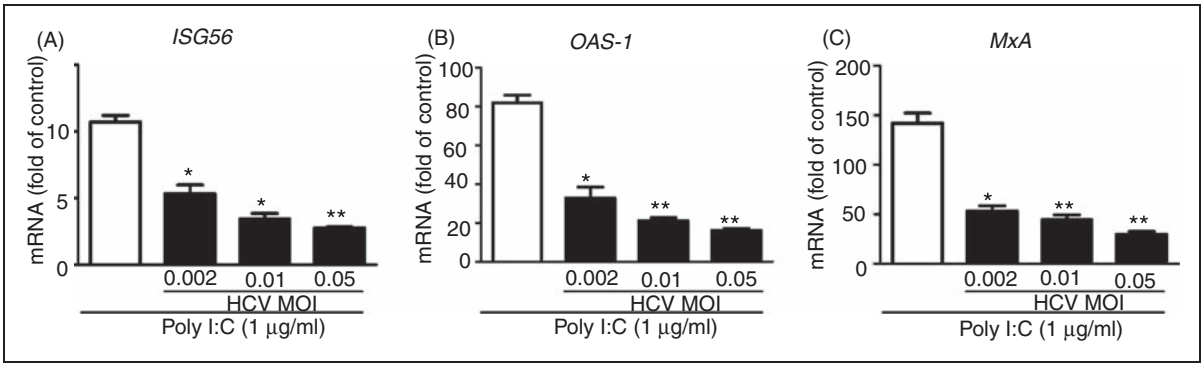



**Figure 6.** HCV suppresses poly I:C-induced ISGs expression. Huh7 cells were infected with HCV JFH-1 at the indicated MOI for 72 h, then stimulated with poly I:C for 24 h. Total RNA extracted from cells was subjected to real time RT-PCR for the mRNA levels of *ISG56*, *OAS-1*, *MxA* and *GAPDH*. The data are expressed as *ISG56* (A), *OAS*-1 (B) and *MxA* (C) mRNA level relative (fold) to the control (vehicle only, which defined as 1). The results shown are mean ± SD of triplicate cultures, representative of three experiments (poly I:C-stimulated infected Huh7 cells vs poly I:C-stimulated uninfected Huh7 cells, $^{**}P < 0.01$, $^{*}P < 0.05$).

biological activities similar to type I IFNs, they appear to have a more specialized role in innate antiviral defense.[32] *IFN-λ* exhibits antiviral action on HCV replication in both replicon and cell culture infectious virus model systems.[12–14] *TLR3* plays a crucial role in the innate immunity against viral infection, as its activation induces type I IFN-based antiviral immunity.[33–35] However, little is known about whether *TLR3* signaling of hepatocytes can induce *IFN-λ* expression against HCV infection/replication. We demonstrated that poly I:C could induce intracellular *IFN-λ1* expression in Huh7 cells at both mRNA and protein levels. This induction of *IFN-λ* was associated with the decrease of HCV RNA and core protein expression in Huh7 cells activated by poly I:C. This finding is supported by a recent report that HCV could induce the expression of *IFN-λ1* and ISGs in primary liver cultures, which limited the growth and spread of the virus.[36]

Poly I:C, a synthetic analog of dsRNA to mimic viral infection, has been used widely in *TLR3* signaling related studies for years. There are two commercially available poly I:Cs [high (HMM) or low molecular mass (LMM)]. Studies have shown that stimulation of cells with HMM or LMM poly I:C resulted in different cytokine profile.[37,38] We demonstrated recently that the efficiency of *TLR3* signaling of Huh7 cells by HMM poly I:C was significantly higher than that by LMM poly I:C.[39] In addition to being recognized by *TLR3*, poly I:C could be recognized by *RIG-I* or *melanoma differentiation-associated protein 5* (*MDA-5*). To determine whether the *TLR3* signaling is critical in HMM poly I:C-mediated IFN induction, we used Bafilomycin A1, an inhibitor of the *TLR3* signaling pathway to block the poly I:C effect. We found that Bafilomycin A1 could largely block poly I:C-induced *IFN-λ1* expression in Huh7 cells. Furthermore, to determine the role of *RIG-I* in poly I:C-mediated *IFN-λ1* induction, we performed the experiments with Huh7.5 cells that have a defect in the *RIG-I* signaling pathway.[18,24] We observed that poly I:C could induce *IFN-λ1* expression in Huh7.5 cells, suggesting that poly I:C induces *IFN-λ1* via a *RIG-I* independent mechanism. With regard to the role of *MDA-5* in poly I:C-mediated action on *IFN-λ1*, our previous study[39] showed that the knock down of *MDA-5* by siRNA had little effect on poly I:C-mediated IFN induction in Huh7.5 cells. Taken together, these results indicate clearly that *TLR3* is the major sensor for the poly I:C action on *IFN-λ1* induction in Huh7 cells.

In order to establish a persistent infection in the liver, HCV has evolved several strategies to evade host immune response, including inhibition of intracellular innate immunity.[40] Our previous study[22] demonstrated that HCV inhibits intracellular *IFN-α* and *IRF-7* expression in human hepatic cell lines. Others have documented that HCV *NS3/4A* protease is able to impair *TLR3* by cleaving the *TRIF* adaptor protein and block *RIG-I* signaling by cleaving the mitochondrial antiviral signaling protein off the mitochondria to inhibit *IRF-3* activation and *IFN-β* expression.[11,41,42] In addition, HCV infection could impair *IRF-7* translocation and *IFN-α* synthesis in immortalized human hepatocytes.[43] In agreement with these findings, we showed that HCV replication impaired poly I:C-activated *TLR3* signaling of Huh7 cells, which is evidenced by the observation that poly I:C-induced intracellular *IFN-λ1* and ISG expression was inhibited by HCV in infected Huh7 cells.

HCV-encoded proteins, including structural proteins (*E2, core*) and NS proteins (*NS3/4A, NS5A* and *NS5B*), are immunosuppressive.[22,40,44–46] Among these HCV immunosuppressive proteins, *NS3/4A* has the ability to impair *TLR3* and *RIG-I* signaling pathways and inhibits *IFN-β* expression.[5,11,41,42] In agreement with these findings, we showed that *NS3/4A* had an important role in HCV-mediated suppression of intracellular *IFN-λ1* expression. In addition, HCV *NS5A* and *NS5B* were also involved in the HCV-mediated suppression of

intracellular *IFN-λ1* expression in hepatic cells. *NS5B* has been identified as a RNA-dependent RNA polymerase responsible for the synthesis of negative-strand HCV RNA.[47] Inhibition of *NS5B* has long been considered an attractive target for therapeutic intervention in HCV-infected patients.[47,48] Our finding that there was a negative effect of *NS5B* on *IFN-λ*-mediated host cell innate immunity in Huh7 cells further highlights the important role of *NS5B* in HCV-mediated evasion of host cell immunity.

Taken together, we have provided further evidence to demonstrate the ability of HCV to evade the antiviral mechanisms in the host cells. Our data indicate that HCV not only inhibits intracellular type I IFN (*IFN-α*) expression,[22] but also suppresses *TLR3* activation-induced type III IFN (*IFN-λ1*) expression in hepatoma cell line. Thus, HCV and its proteins use the complex mechanisms to target multiple steps of the *TLR3*/IFN signaling pathway in hepatocytes, compromising the intracellular innate immunity. Further studies are needed to understand the *in vivo* interplays between HCV and host cell innate immunity in the context of persistent infection in the liver.

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## Conflict of interest

The authors do not have any potential conflicts of interest to declare.

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