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Antiviral effect of interferon lambda against West Nile virus

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ABSTRACT

Type III interferons (IFN), IFN-λ or IL-28/29, are new members of the IFN super-family. Except for using distinct receptors, type I and type III IFNs share the same major post receptor signaling components to activate the transcription of a similar set of IFN-stimulated genes (ISGs). To examine the antiviral effects of the new type IFNs against West Nile virus (WNV), we compared the antiviral effects of IFN-α and IFN-λ on WNV virus-like particle (VLP) infection and replicon replication in Huh7.5 and Hela cells. The results revealed that (i) both types of IFNs could efficiently prevent the WNV infection, but IFN-α demonstrated a stronger antiviral efficacy; (ii) WNV genome replication in VLP-infected cells and replicon-containing cell lines could only be inhibited by IFN-α, but not IFN-λ; (iii) in agreement with the observed antiviral effects, only IFN-λ-induced activation of JAK-STAT signaling pathway and induction of ISG expression were completely inhibited in WNV replicon-containing cell lines, but IFN-α signal transduction was either unaffected or only partially inhibited in Huh7.5 or Hela cells by the virus. Hence, the differential inhibition of WNV on IFN-α and IFN-λ signal transduction implies that the receptors of the two types of IFNs, but not the common post receptor signaling components, could be selectively targeted either directly by WNV nonstructural proteins or indirectly by the cellular responses induced by the virus infection to inhibit the signal transduction of the cytokines.

1. Introduction

West Nile virus (WNV) is a member of the flavivirus genus in the family *Flaviviridae* (Brinton, 2002). In addition to WNV, this genus contains several other important mosquito-borne human pathogens, including Japanese encephalitis virus (JEV), dengue virus (DENV) and yellow fever virus (YFV), and represents a significant public health problem worldwide (Mackenzie et al., 2004). Since its first incursion into New York City in 1999, WNV has rapidly spread throughout the continental United States and has recently reached South America (Lanciotti et al., 1999; Morales et al., 2006). In most cases, WNV infection of humans is characterized as asymptomatic or as a mild febrile illness termed West Nile fever. However, approximately 1% of infected individuals develop more severe neurological disorders, such as encephalitis and meningitis. Thus far, antiviral therapies and vaccines are not yet available to treat and prevent WNV infection (Kramer et al., 2007).

The interferon (IFN) family of cytokines is now recognized as a key component of the innate antiviral response and is classified into three types, based upon the usage of three distinct receptor complexes (Borden et al., 2007; Sen, 2001). Type III IFNs, IFN-λ or IL-28/29, are the recently identified members of IFN super-family (Kotenko et al., 2003; Sheppard et al., 2003). As with type I IFNs (IFN-α/β), IFN-λ is induced in many types of cells in response to virus infection, but it signals through a distinct receptor complex consisting of IL-10Rβ and IL-28Rα, rather than IFNAR1 and IFNAR2 for type I IFNs (Onoguchi et al., 2007; Osterlund et al., 2007). Interestingly, although neither the cytokines nor the receptors displays significant sequence similarity, type I and III IFNs share the same post receptor signaling components. As a consequence, binding of both types of IFNs to their cognate receptors on cell membrane activates the receptor-associated janus kinase 1 (JAK1) and tyrosine kinase 2 (Tyk2) through tyrosine phosphorylation, which in turn stimulates the tyrosine phosphorylation of STAT1 and STAT2. The phosphorylated STAT1 and STAT2, in combination with IRF9, form a trimeric ISGF3 complex that translocates into the nucleus and activates the expression of IFN-stimulated genes (ISGs), whose products can directly limit viral replication (Jiang et al., 2008; Sadler

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and Williams, 2008; Stark et al., 1998). Not surprisingly, it has been demonstrated that type III IFNs trigger a type I IFN-like ISG expression profile and inhibit the infection of a variety of viruses in vitro and in vivo (Ank et al., 2006; Bartlett et al., 2005; Marcello et al., 2006; Zhou et al., 2007).

IFNs have been evaluated for their potential therapeutic applications in clinic against the variety of virus infections, with the most noteworthy example being the successful treatment of chronic hepatitis C virus (HCV) infection with IFN-α (Manns et al., 2007). Consistent with its antiviral efficacy in vivo, IFN-α does not only prevent HCV infection of naïve cells, but is also able to cure the virus infected cells (Blight et al., 2002; Guo et al., 2001). In contrast, although pretreatment of cells with IFN-α could also inhibit WNV infection, it could not efficiently inhibit viral replication, if provided after cells were infected by the virus (Morrey et al., 2004). This is, at least in part, due to the inhibition of IFN-α signal transduction pathway by WNV nonstructural proteins (Guo et al., 2005; Ho et al., 2005; Lin et al., 2004; Liu et al., 2005; Munoz-Jordan et al., 2003). Consistent with these findings, it had been shown that a WNV strain deficient in blocking the signal transduction pathway of type I IFN was phenotypically attenuated in mice (Keller et al., 2006) and IFN-α based therapies were ineffective against WNV infections in humans (Chan-Tack and Forrest, 2005).

To examine the antiviral effects of the new type IFN and evaluate its potential for treatment of WNV infection, we compared the antiviral effects of IFN-α and IFN-λ on WNV virus-like particle (VLP) infection and replicon replication in Huh7.5 and Hela cells. Curiously, although IFN-α and IFN-λ use a common post receptor signal transduction system (s), IFN-α is a far more potent inhibitor of WNV. In addition, the signal transduction of IFN-λ, but not IFN-α, could be selectively inhibited by WNV in Huh7.5 cells. This observation favors a hypothesis that the receptors of type I and type III IFNs, but not the common post receptor signaling components, could be differentially targeted either directly by WNV nonstructural proteins or indirectly by the cellular factors induced or activated by the virus infection to inhibit the signal transduction of the cytokines.

2. Materials and methods

2.1. Cell culture and reagents

Huh7.5 and Hela cells were cultured in a complete Dulbecco's modified Eagle's medium (DMEM) that includes DMEM supplemented with 10% fetal bovine serum (FBS), penicillin G, streptomycin, non-essential amino acids, L-glutamine. HCV subgenomic replicon-containing Hela cell line (SL1) was described previously (Guo et al., 2001; Zhu et al., 2003). HCV subgenomic replicon-containing Huh7.5 (Huh7.5/HCVrep) cell line was derived from a single G-418-resistant colony of Huh7.5 transfected with in vitro transcribed HCV replicon RNA (Con1 strain). WNV replicon-containing Huh7.5 and Hela cell lines (Huh7.5/WNVrep and Hela/WNVrep) were derived from single G-418-resistant clones of the respective parent cell lines transfected with total RNA isolated from WNV-subgenomic replicon-replicating BHK cells (Rossi et al., 2005). Conditions for in vitro transcription, RNA electroporation and selection of G-418-resistant cell colonies are described previously (Guo et al., 2001). All replicon-containing cell lines were cultured with complete DMEM containing 500 μg/ml G-418. Recombinant IFN-α2b and IFN-λ1 were purchased from PBL Inc. and Proprotech (Rocky Hill, NJ), respectively.

2.2. WNV VLP packaging and infection

Packaging of virus-like particles containing Renilla luciferase reporter WNV replicons and titration of VLP titers were carried out as previously described (Puig-Basagoiti et al., 2005). To determine the effects of IFNs on WNV VLP infection, 4×10^4 Huh7.5 or Hela cells were seeded per well in a 96-well plate. At 16 h post seeding, the cells were either left untreated or treated with indicated concentrations of IFN-α or IFN-λ for 24 h. The cells were then infected with VLP at a multiplicity of infection (MOI) of 2 for 1 h and continued to be cultured with medium without or with the indicated concentrations of IFNs for 24 h. Cells were then lysed and luciferase activity in the cell lysates was quantified by using a Renilla luciferase assay kit (Promega). To examine the effects of the cytokines on WNV replicon replication in VLP-infected cells, 4×10^4 Huh7.5 or Hela cells per well in a 96-well plate were infected 24 h post seeding with the VLPs at a MOI of 2. Thirty hours after infection, cells were left untreated or treated with indicated concentrations of IFNs for 24 h. Cells were then lysed and luciferase activity was assayed as described above.

2.3. RNA extraction and analyses

Cells were left untreated or treated with the indicated concentrations of cytokines. Upon the completion of treatment, total cellular RNA was extracted with TRIzol reagent (Invitrogen) by following the manufacturer's direction. Five micrograms of total RNA were fractionated on 1% agarose gel containing 2.2 M formaldehyde and transferred onto nylon membranes. Membranes were hybridized with riboprobes specific for plus-stranded HCV or WNV replicon RNA in the conditions described previously (Guo et al., 2003).

For real-time reverse transcriptase (RT)-PCR detection of WNV RNA, a TaqMan assay was used. Briefly, 100 ng of total RNA was combined with 50 pmol of forward prime (5′-TCAGCGATCTCTCCACCAAAG-3′) and reverse primer (5′-GGGTCAGCACGTTTGTCATTG-3′) and 10 pmol of the FAM- and TMARA-labeled probe (5′-TGCCCGACCATGGGAGAAGCTC-3′) in a 50 μl total reaction volume by using the TaqMan RT-PCR Ready-Mix Kit (PE Applied Biosystems). The real-time PCR condition as described previously (Lanciotti et al., 2000). Quantitation of WNV RNA in the samples was calculated by generating standard cureve with known amount of in vitro transcribed WNV RNA.

2.4. Western blot assay

Cells were left untreated or treated with the indicated concentrations of cytokines. Upon the completion of treatment, cells in 100-mm-diameter Petri dishes were washed once with ice-cold phosphate-buffered saline (PBS) and subsequently lysed with 800 μl of lysis buffer (20 mM Tris–HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA, 1% NP-40, 1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate [SDS]) supplemented with protease inhibitor cocktail (Roche) and 0.1 mM sodium orthovanadate. Lysates were centrifuged for 5 min at $10{,}000 \times g$ at 4 °C. Equal amounts of cell lysates were separated by SDS-polyacrylamide gel electrophoresis, transferred on Immuno-P membrane (Millipore), probed with antibodies for HCV NS5A protein (a gift of Dr. Chen Liu, Florida State University, Gainesville, FL), WNV NS3 protein (a gift of Dr. Peter W. Mason, University of Texas Medical Branch, Galveston, TX), ISG15 (Cell Signaling, Danvers, MA), STAT1αp91 (sc-417; Santa Cruz Biotechnology) and STAT2 (sc-1668; Santa Cruz Biotechnology), phosphotyrosine 701-STAT1 (9171; Cell Signaling Technologies), phosphotyrosine 689-STAT2 (07-224; Upstate Technology), Tyk2 (sc-169; Santa Cruz Biotechnology), phosphotyrosine 1022/1023-JAK1 (3331; Cell Signaling Technologies), phosphotyrosine 1054/1055-Tyk2 (9321; Cell Signaling Technologies), or β-actin (sc-1616; Santa Cruz Biotechnology). Bound antibodies were revealed by HPR-labeled secondary antibodies and visualized with an enhanced chemiluminescence detection system

(Amersham Pharmacia Biotech) according to the protocol of the manufacturer.

2.5. Reporter assay

Huh7.5 cells were transfected using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. For each transfection, 5×10^4 cells per well in 96-well plate were transfected with 0.1 μg of plasmid pISRE-Luc (Clontech). Twenty-four hours after transfection, transfected cells were left untreated or treated with 1000 IU/ml IFN-α2b or 500 ng/ml IFN-λ1 for 16 h. Cells were then harvested and lysed. Luciferase activities in cell lysates were determined using a dual luciferase assay system (Promega).

3. Results

3.1. Effects of IFN-α and IFN-λ on WNV VLP infection and replicon replication

As described previously (Puig-Basagoiti et al., 2005), when WNV structural proteins are supplied *in trans* into the cells that replicate the subgenomic replicons, the replicons can be packaged and secreted as virus-like particles which can infect cells as WNV virions do. However, because the replicon RNA contains a luciferase gene in lieu of viral structural genes, infection of cells with such VLPs is single cycled and incapable of spreading to neighboring cells. Instead, replication of replicons in the VLP-infected cells leads to the expression of luciferase, which can serve as a convenient and quantitative reporter of WNV infection and genome replication (Puig-Basagoiti et al., 2005; Scholle et al., 2004).

To determine the effects of IFN-α and IFN-λ on WNV infection, Huh7.5 and Hela cells were left untreated or pretreated with indicated amount of cytokines for 24 h, followed by infection with WNV VLPs for 1 h. The infected cells were continued to be cultured in medium without or with the indicated concentrations of IFNs. Cells were lysed 24 h post infection and VLP infection was quantified by the luciferase assay. The results were expressed as the ratios of light units obtained from wells treated with IFN over that obtained in untreated control wells. As shown in Fig. 1A and B, both IFN-α and IFN-λ inhibited the VLP infection in Huh7.5 and Hela cells in a dose-dependent manner. However, IFN-α demonstrated a much stronger antiviral potency in comparison with IFN-λ in both cell types. Interestingly, although treatment of cells with IFN-α at 30 h after the VLP infection for 24 h reduced the levels of luciferase expression less dramatically as compared with IFN-α pretreatment, the cytokine did induce a dose-dependent and significant reduction of luciferase activity, presumably due to the inhibition of WNV replicon replication in VLP-infected Huh7.5 and Hela cells (Fig. 1C and D). On the contrary, post-infection treatment of IFN-λ did not significantly reduce the luciferase activity in either Huh7.5 or Hela cells, suggesting that the replicon replication is completely resistant to the cytokine, once its replication has become established.

3.2. IFN-λ fails to induce ISG expression and inhibit replicon replication in WNV replicon-containing cell lines

To further investigate the effects of IFNs on WNV genome replication in established cell lines and determine the effects of the viral replication on the signal transduction of the cytokines, we compared the abilities of IFN-α and IFN-λ1 to induce the expression of ISG56 and inhibit the replication of WNV replicons in Huh7.5 and Hela cells stably transfected with WNV replicon RNA. Cell lines that support HCV replicon replication were included as positive controls, since the IFNs have been shown to be effective in inhibition of HCV genome replication (Guo et al., 2001; Guo et al., 2003). As shown in Fig. 2, while both IFN-α and IFN-λ treatment induced the expression of ISG56 mRNA in parental and HCV replicon-containing Huh7.5 and Hela cells in a dose-dependent manner, only IFN-α, but not IFN-λ, was able to induce ISG56 mRNA in both WNV replicon-replicating Huh7.5 and Hela cells. Compared to that in parental and HCV replicon-containing cells, ISG56 induction by IFN-α was partially compromised in Huh7.5 and Hela cells in the presence of WNV replicons. Consistent with the profile of ISG56 induction, HCV replicon replication can be efficiently inhibited by both IFN-α and IFN-λ, but WNV replicon replication could only be inhibited by IFN-α, but not IFN-λ in both cell types.

To confirm the above observations, we further determined the effects of the IFNs on the induction of ISG15 protein expression and steady-state levels of HCV and WNV nonstructural proteins in Huh7.5 and Hela cells. As shown in Fig. 3, both types of IFNs dose-dependently induced ISG15 expression in the parental and HCV replicon-containing Huh7.5 and Hela cells (Fig. 3A, lanes 1–6 and 7–12; Fig. 3B, lanes 1–6 and 13–18). As a consequence, dose-dependent reductions of HCV NS5A protein could be observed in HCV replicon-containing Huh7.5 and Hela cells in response to both IFN-α and IFN-λ treatment (Fig, 3A, lanes 7–12 and data not shown). Interestingly, while the expression of ISG15 could be induced by IFN-α in Huh7.5/WNVrep cells with a similar efficiency as observed in its parental Huh7.5 cells (Fig. 3A, lanes 13–18), replication of WNV replicons in Hela cells not only dramatically inhibited the ISG15 induction by IFN-α (Fig. 3B, lanes 7–12), but also reduced the basal level expression of ISG15 (Fig. 3B, comparing lane 7 with lanes 1 and 13). As observed with ISG56, induction of ISG15 by IFN-λ in both Huh7.5/WNVrep and Hela/WNVrep cells were completely abrogated (Fig. 3A, lanes 13–18; Fig. 3B, lanes 7–12). Consistently, WNV nonstructural protein expression was not affected by IFN-λ1 in both cell lines.

Taken together, the results presented in Figs. 2 and 3 suggested that WNV infection of cells only partially and cell type-specifically (only in Hela, but not Huh7.5) disrupted the signal transduction pathway of IFN-α, but completely inhibited the signal transduction of IFN-λ. In agreement with this observation, only IFN-α, but not IFN-λ, could at least partially inhibit WNV replication in cells that viral replication is established.

3.3. Replication of WNV replicons inhibits the signal transduction of IFN-λ in both Huh7 and Hela cells

To confirm this explanation, we first compared the transcription activity of ISRE promoter in response to IFN-α and IFN-λ in parental and HCV or WNV replicon-containing Huh7.5 cells by a luciferase reporter assay. As shown in Fig. 4A, while IFN-α induced similar levels of luciferase activity in all three cell lines, the ability of IFN-λ1 to induce ISRE-driven luciferase expression was impaired in WNV, but not HCV replicon-containing Huh7.5 cells. We next examined the STAT1 activation in parental and HCV or WNV replicon-containing Hela cells in response to IFN-α and IFN-λ1 treatment, respectively. As shown in Fig. 4B and consistent with our previous report (Guo et al., 2005), IFN-α-induced STAT1 tyrosine phosphorylation was not affected by HCV replicons, but significantly reduced in WNV replicon-containing Hela cells. Interestingly, IFN-λ1-induced STAT1 phosphorylation was completely abolished in WNV replicon-containing cells.

While the above result suggested that IFN-λ signal transduction was impaired in WNV replicon-containing Huh7.5 and Hela cells, it was not yet clear if this was due to the presence of the replicons or the specific cell clones obtained during G-418 selection. To resolve this issue, the replicon-containing Huh7.5 cells were cultured in 500 IU/ml IFN-α, with multiple passages, in the absence of G-418 for two weeks. Elimination of WNV replicon from the cells by the cytokine treatment is confirmed by an undetectable level of WNV RNA with a real-time RT-PCR assay and inability to

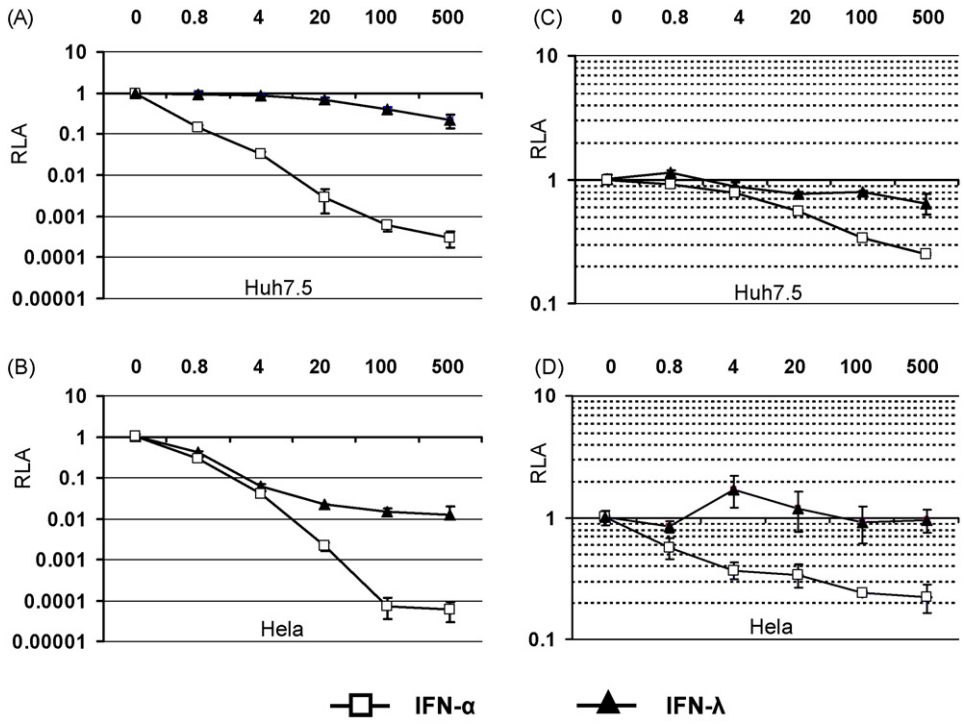

Fig. 1. Effects of IFN-α and IFN-λ on WNV VLP infection and genome replication. (A and B) To determine the effects of IFN-α and IFN-λ on WNV infection, 4×10^4 Huh7.5 (A) or Hela (B) cells were seeded per well in a 96-well plate. At 16 h post seeding, the cells were either left untreated or treated with indicated concentrations of IFN-α (IU/ml) or IFN-λ (ng/ml) for 24 h. The cells were then infected with VLP at a multiplicity of infection (MOI) of 2 for 1 h and followed by continuing to culture in medium without or with the indicated concentrations of IFNs for 24 h. Cells were lysed and luciferase activity in the cell lysates was quantified by using a Renilla luciferase assay kit (Promega). The relative luciferase activity (RLA) represents the means ± standard derivations ($n = 3$) of the ratios of light units obtained from wells treated with IFNs over that obtained from the untreated control wells. (C and D) To examine the effects of the cytokines on WNV replicon replication in VLP-infected cells, 4×10^4 Huh7.5 (C) or Hela (D) cells seeded per well in a 96-well plate were infected 24 h post seeding with the VLPs at a MOI of 2. Thirty hours after infection, cells were left untreated or treated with indicated concentrations of IFNs for 24 h. Cells were then lysed and luciferase activity was assayed as described above.

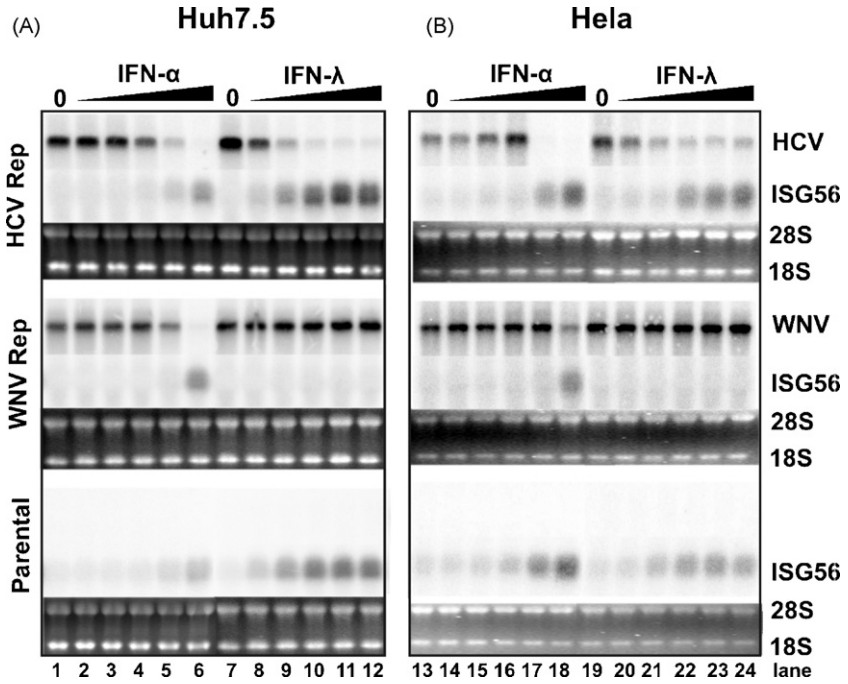

Fig. 2. Effects of IFNs on induction of ISG56 and replication of WNV and HCV replicons in Huh7.5 (A) and Hela cells (B). Parental cells and cells containing HCV and WNV subgenomic replicons (HCVrep and WNVrep, labeled on the left side of the figure) were mock-treated (lanes 1, 7, 13, 19) or treated with 0.01, 0.1, 1, 10, and 100 IU/ml of IFN-α (lanes 2–6 and 14–18) and 0.05, 0.5, 5, 50, and 500 ng/ml of IFN-λ1 (lanes 8–12 and 20–24), respectively, for 48 h. Total cellular RNA was extracted and 5 μg of total RNA were resolved in 1% agarose gel containing 2.2 M formaldehyde and transferred onto nylon membrane. HCV and WNV replicon RNA were detected by Northern blot hybridization with a-^{32}P-UTP-labeled riboprobe complementary with the plus-strand of replicon RNA in neomycin phosphotransferase coding region. The same membrane was re-hybridized with a-^{32}P-UTP-labeled riboprobe specific to ISG56 mRNA. Ribosomal RNAs served as loading controls.

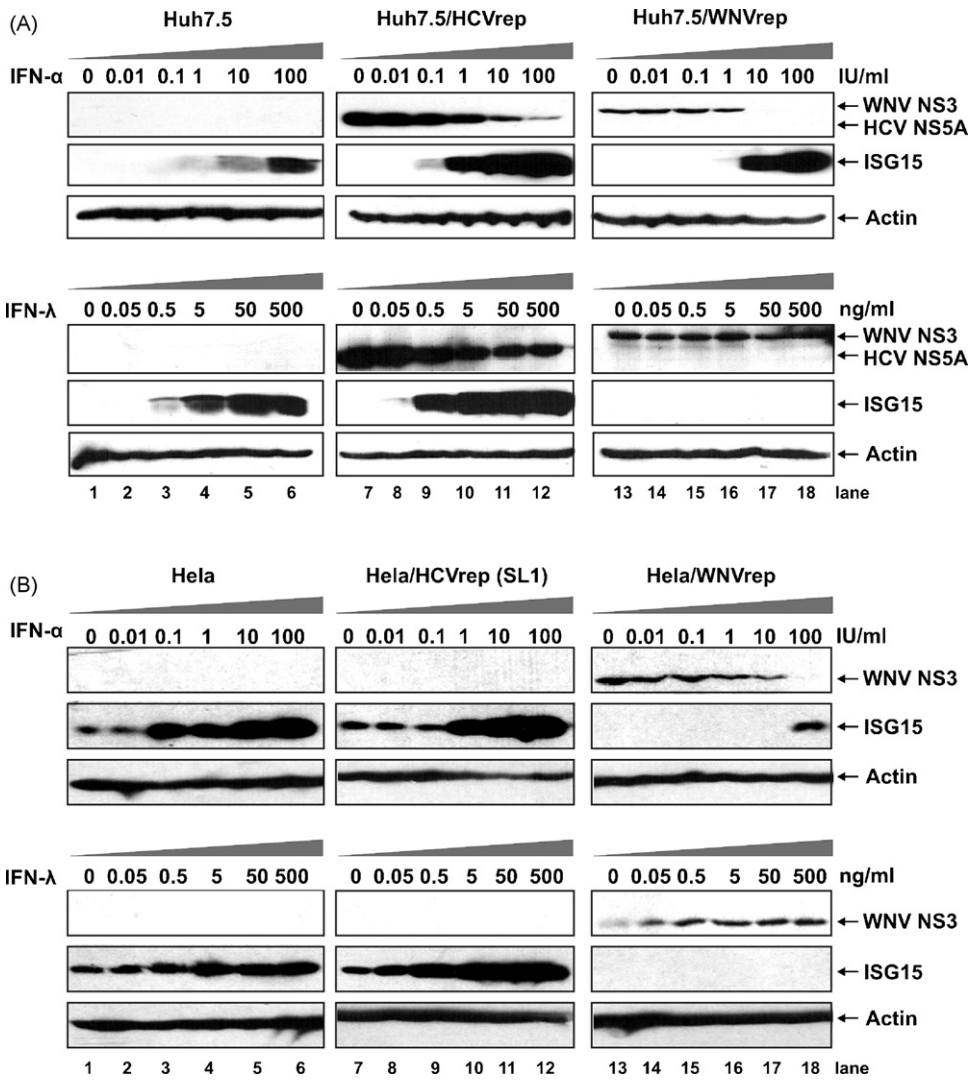

Fig. 3. Effects of IFNs on ISG15 induction and the steady-state levels of viral proteins in Huh7.5 (A) and Hela (B) cells. Parental cells and cells containing HCV and WNV subgenomic replicons were mock-treated or treated with the indicated concentrations of IFN-α and IFN-λ1, respectively, for 48 h. HCV NS5A, WNV NS3, ISG15 in cell lysates were determined by Western blot assay with specific antibodies. β-actin served as a control for the amount of proteins loaded per lane.

obtain G-418-resistant cell colonies from the cured culture after re-application of the antibiotics (data not shown). To examine their ability in response to IFN-λ, the parental Huh7.5, Huh7.5/WNVrep and the cured cells were left untreated or treated with 500 ng/ml of IFN-λ1 for 24 h, levels of ISG15 in the cell lysates were determined by Western blot assay. As shown in Fig. 5A, similar levels of ISG15 protein were induced by IFN-λ in parental and cured WNVrep cells, but not in Huh7.5/WNVrep cells. Consistently, the ISRE-driven luciferase reporter assay further demonstrated that elimination of WNV replicon from Huh7.5/WNVrep cells restored the ability of the cells responding to IFN-λ (Fig. 5B). These results thus imply that the failed response of Huh7.5/WNVrep cells to IFN-λ is due to the presence of WNV replicon, but not selection of IFN-λ non-responsive cells.

3.4. WNV prevented the accumulation of phosphorylated Tyk2 and STAT proteins induced by IFN-λ in Huh7.5 cells

To determine the initial target of WNV on IFN-λ signal transduction pathway, the signaling events induced by IFN-α and IFN-λ were analyzed in a panel of Huh7.5-derived cell lines. Specifically, parental Huh7.5, Huh7.5/WNVrep and its cured derivative (cured WNVrep) were left untreated or treated with 1000 IU/ml IFN-α and 500 ng/ml IFN-λ1, respectively. Cells were harvested before treatment (0) or at 30 and 120 min after addition of the IFNs. The levels of total and tyrosine phosphorylated Tyk2, STAT1 and STAT2 in cell lysates were measured by Western blot analyses. As shown in Fig. 6, expression of WNV replicons in Huh7.5 cells did not significantly alter the steady-state levels of Tyk2 and STAT1/2 proteins. Moreover, in agreement with the previous result demonstrating that replication of WNV replicons in Huh7.5 cells did not affect the signal transduction of IFN-α (Fig. 4A), the levels and kinetics of Tyk2, STAT1 and STAT2 phosphorylation in response to IFN-α treatment did not differ among the three cell lines. However, IFN-λ-induced tyrosine phosphorylation of Tyk2, STAT1 and STAT2 was efficiently blocked in Huh7.5/WNVrep cells and could be completely restored upon the elimination of the replicons.

Due to the observed differential effects of WNV on the two types of IFNs in Huh7.5 cells and the extensive overlaps of post receptor signaling pathways of IFN-α and IFN-λ, it was inferred that the observed inhibition on IFN-λ signal transduction by WNV could most possibly occur at the level of its receptor. Quantitative RT-PCR analysis suggested that expression of WNV replicons in Huh7.5 and Hela cells did not alter the levels of IL-28Rα and IL-10Rβ mRNAs (data not shown). However, due to the lack of good antibody

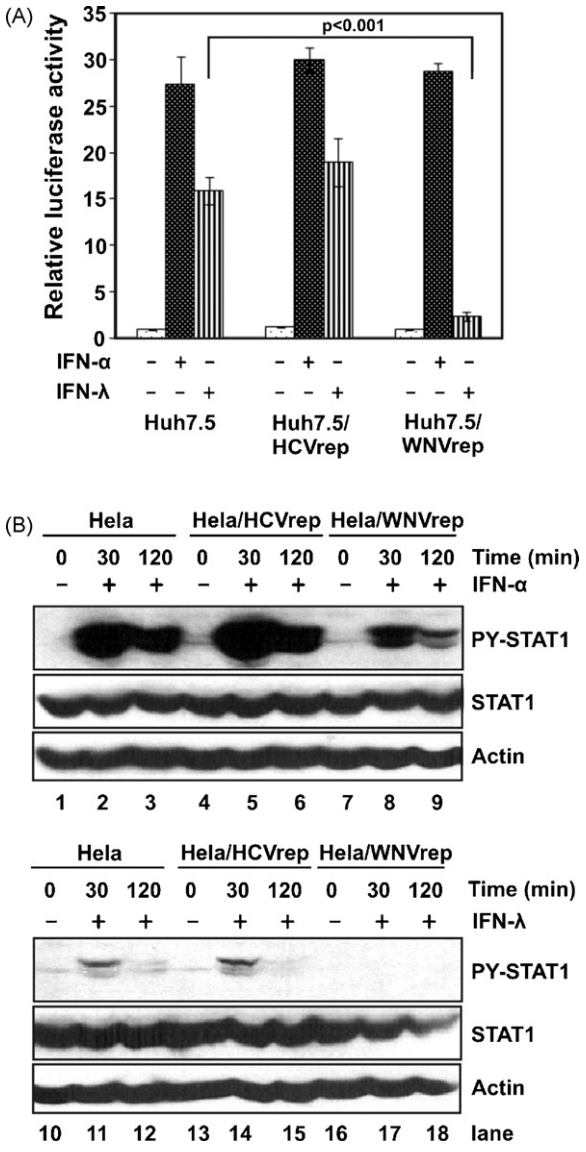

Fig. 4. IFN-λ signal transduction was impaired in cells expressing WNV replicons. (A) Activation of ISRE promoter by IFN-λ1, but not IFN-α, was inhibited in Huh7.5 cells containing WNV replicons. Parental Huh7.5, Huh7.5/HCVrep and Huh7.5/WNVrep were transfected with a plasmid pIRSE-Luc in that the firefly luciferase gene expression is under the control of ISG15 promoter. Twenty-four hours after transfection, cells were mock-treated or treated with IFN-α (1000 IU/ml) and IFN-λ1 (500 ng/ml), respectively, for 16 h. Luciferase activities in cell lysates were determined and expressed as fold of induction (mean ± standard derivations, $n = 6$). (B) IFN-λ-induced STAT1 phosphorylation was abolished in Hela cells containing WNV replicons. Parental Hela, SL1 and Hela/WNVrep cells were treated with 1000 IU/ml of IFN-α and 500 ng/ml of IFN-λ1 for 0, 30, and 120 min. Levels of tyrosine phosphorylated STAT1 (PY-STAT1) and total STAT1 in cell lysates were determined by Western blot assay. β-actin served as a control for the amount of proteins loaded per lane.

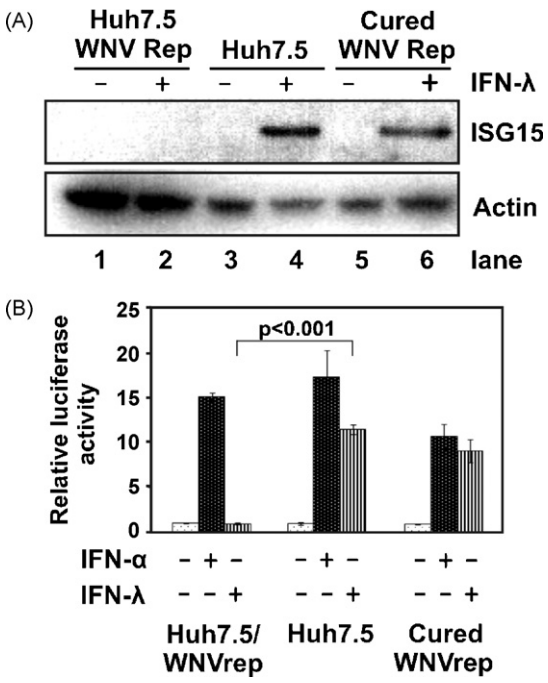

Fig. 5. Expression of WNV replicons in Huh7.5 cells inhibited IFN-λ signal transduction. (A) Huh7.5/WNVrep, parental Huh7.5 and cured Huh7.5/WNVrep cells were mock-treated or treated with 500 ng/ml of IFN-λ1 for 24 h and levels of ISG15 protein in cell lysates were assayed by Western blot. β-actin served as a control for the amount of proteins loaded per lane. (B) Huh7.5/WNVrep, parental Huh7.5 and cured Huh7.5/WNVrep cells were transfected with a plasmid pIRSE-Luc in that the firefly luciferase gene expression is under the control of ISRE promoter. Twenty-four hours after transfection, cells were mock-treated or treated with IFN-α (1000 IU/ml) and IFN-λ1 (500 ng/ml), respectively, for 16 h. Luciferase activities in cell lysates were determined and expressed as fold of induction (mean ± standard derivations, $n = 6$).

reagents, effects of WNV on the total levels of IFN-λ receptors as well as their surface expression remain to be determined.

4. Discussion

The cell culture studies presented in this report appear to suggest that compared to IFN-α, IFN-λ is a weaker antiviral cytokine against WNV. Moreover, consistent with previous reports (Guo et al., 2005; Rossi et al., 2005), IFN-α signal transduction can be partially disrupted by WNV replication in Hela cells. However, in marked contrast, WNV infection completely abrogated the signal transduction and ISG induction by IFN-λ in both Huh7.5 and Hela cells. Our results thus suggest that IFN-λ might not be a good candidate for treatment of WNV infection

Our efforts toward determining the target(s) of WNV inhibition on IFN-λ signal transduction revealed that as observed with IFN-α in Hela and Vero cells (Guo et al., 2005), WNV efficiently abolished IFN-λ-induced accumulation of phosphorylated Tyk2 (Fig. 6). Our results showed that compared to IFN-α, IFN-λ demonstrated a weaker activation of JAK-STAT signaling pathway (Figs. 4 and 6). It is, therefore, possible that the signaling pathway of IFN-λ ιs more prone to be disrupted by WNV than that of IFN-α. However, the possibility that distinct mechanisms are involved in disruption of the signaling pathways of the two cytokines by WNV cannot be ruled out. Considering their share of post receptor signaling components by the two cytokines, the most possible scenario is that expression of IFN-λ receptor proteins (IL-10Rβ and IL-28Rα) and/or their function may be directly disrupted by one or several viral NS proteins or indirectly targeted by cellular factor(s) that are induced or activated by WNV infection.

Concerning effects of WNV on the expression of IFN receptors, it is possible that WNV infection could reduce the levels of receptor proteins *via* either inhibition of their transcription and/or translation or acceleration of their degradation. Alternatively, the cell surface expression of the receptor proteins could be disrupted by direct or indirect interaction with viral proteins. However, our previous studies revealed that despite the significant inhibition of Tyk2 and JAK1 phosphorylation, replication of Kunjin (a sub-type WNV) replicons in Hela cells and infection of Vero cells by WNV did not apparently alter the total level of IFNAR1 as well as cell surface expression of IFNAR2, suggesting a functional suppression of type I IFN receptor by WNV (Guo et al., 2005). Similarly, it was reported

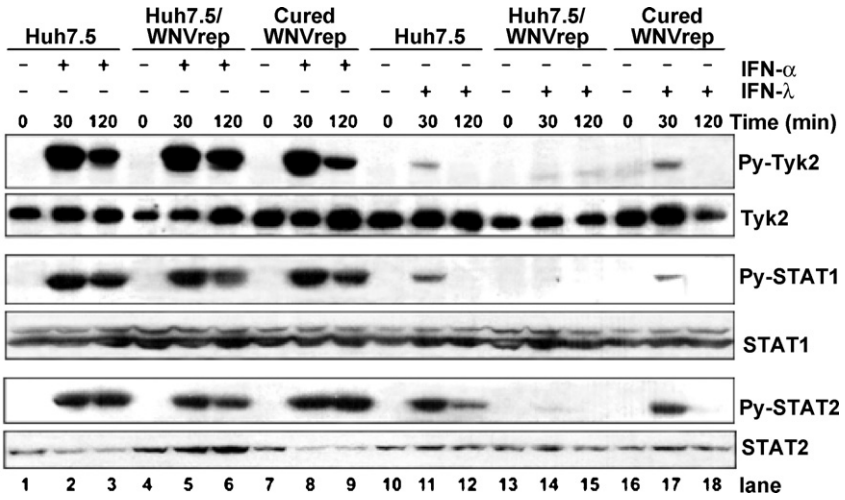

Fig. 6. Effects of WNV on the kinetics of Tyk2, STAT1 and STAT2 tyrosine phosphorylation induced by IFN-α and IFN-λ1 treatment in Huh7.5 cells. Parental Huh7.5 cells, Huh7.5/WNVrep and cured Huh7.5/WNVrep cells were treated with 1000 IU/ml of IFN-α or 500 ng/ml IFN-λ1 for 0, 30, and 120 min. Levels of tyrosine – phosphorylated Tyk2 (PY-Tyk2), total Tyk2, tyrosine phosphorylated (PY-STAT1), total STAT1, tyrosine phosphorylated (PY-STAT2) and total STAT2 in cell lysates were determined by Western blot assay.

that although dengue virus reduced the level of IFN-α-induced Tyk2 phosphorylation in dendritic cells, the cell surface expression of IFNAR1 and 2 was not affected by the virus infection (Ho et al., 2005). In the case of WNV inhibition of IFN-λ signaling, we demonstrated that the steady-state levels of IL-10Rβ and IL-28Rα mRNAs are not altered by WNV replicon replication in both Huh7.5 and Hela cells, but our attempts to determine the level and cell surface expression of the receptor proteins failed, due to the poor quality of antibodies.

Alternatively, the signaling function of IFN receptors could be impaired by interaction with either viral NS proteins or cellular factors that are induced or activated by WNV infection. In support of the role of viral proteins, it was reported that several WNV nonstructural proteins, including NS2A, NS2B, NS3, NS4A and NS4B, when expressed individually, were able to inhibit IFN-α signaling (Liu et al., 2005). Most consistently, it was shown that NS4B protein derived from several flaviviruses, including DENV, WNV and YFV, potently inhibit IFN-α-induced STAT1 phosphorylation (Munoz-Jordan et al., 2005). Employing a similar approach, our preliminary study showed that over-expression of WNV NS4B and NS5 proteins inhibited the activation of IRSE-directed luciferase gene expression by IFN-λ (data not shown), suggesting that the inhibitory effects of WNV on IFN-λ signal transduction could be potentially mediated by these two viral proteins.

Considering the WNV-induced cellular factors that might potentially disrupt IFN signaling, a recent report suggested that WNV infection disrupted cellular cholesterol synthesis and caused intracellular cholesterol redistribution, which resulted in changes of IFN-α receptor-containing membrane lipid rafts and thus compromised the signal transduction of the cytokine (Mackenzie et al., 2007). To determine if this is the case for IFN-λ signal transduction, we examined the effects of cholesterol synthesis inhibition with fluvastain and/or depletion of plasma membrane cholesterol with 2-hydroxyprophyl-beta-cyclodextran (HP-β-CD) on WNV replication and IFN signal transduction in Huh7.5 cells. However, consistent with Wang and colleagues (Wang et al., 2005), but contradictory to Mackenzie (Mackenzie et al., 2007), treatment of Huh7.5/WNVrep as well as parental Huh7.5 cells with fluvastain and HP-β-CD, alone or in combination, did neither affect the replication of WNV replicon nor inhibit the induction of ISG56 expression by IFN-α or IFN-λ (data not shown).

In summary, we demonstrated in this report that IFN-λ was able to prevent WNV infection of naïve cells, but failed to inhibit the virus replication in cells that viral infection had been established, which is most possibly due to the inhibition of the cytokine-induced phosphorylation of receptor-associated tyrosine kinase Tyk2 *via* an unknown mechanism by WNV.

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References

Ank, N., West, H., Bartholdy, C., Eriksson, K., Thomsen, A.R., Paludan, S.R., 2006. Lambda interferon (IFN-lambda), a type III IFN, is induced by viruses and IFNs and displays potent antiviral activity against select virus infections in vivo. J. Virol. 80 (9), 4501–4509.

Bartlett, N.W., Buttigieg, K., Kotenko, S.V., Smith, G.L., 2005. Murine interferon lambdas (type III interferons) exhibit potent antiviral activity in vivo in a poxvirus infection model. J. Gen. Virol. 86 (Pt 6), 1589–1596.

Blight, K.J., McKeating, J.A., Rice, C.M., 2002. Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. J. Virol. 76 (24), 13001–13014.

Borden, E.C., Sen, G.C., Uze, G., Silverman, R.H., Ransohoff, R.M., Foster, G.R., Stark, G.R., 2007. Interferons at age 50: past, current and future impact on biomedicine. Nat. Rev. Drug Discov. 6 (12), 975–990.

Brinton, M.A., 2002. The molecular biology of West Nile virus: a new invader of the western hemisphere. Annu. Rev. Microbiol. 56, 371–402.

Chan-Tack, K.M., Forrest, G., 2005. Failure of interferon alpha-2b in a patient with West Nile virus meningoencephalitis and acute flaccid paralysis. Scand. J. Infect. Dis. 37 (11–12), 944–946.

Guo, J.T., Bichko, V.V., Seeger, C., 2001. Effect of alpha interferon on the hepatitis C virus replicon. J. Virol. 75 (18), 8516–8523.

Guo, J.T., Hayashi, J., Seeger, C., 2005. West Nile virus inhibits the signal transduction pathway of alpha interferon. J. Virol. 79 (3), 1343–1350.

Guo, J.T., Zhu, Q., Seeger, C., 2003. Cytopathic and noncytopathic interferon responses in cells expressing hepatitis C virus subgenomic replicons. J. Virol. 77 (20), 10769–10779.

Ho, L.J., Hung, L.F., Weng, C.Y., Wu, W.L., Chou, P., Lin, Y.L., Chang, D.M., Tai, T.Y., Lai, J.H., 2005. Dengue virus type 2 antagonizes IFN-alpha but not IFN-gamma antiviral effect via down-regulating Tyk2-STAT signaling in the human dendritic cell. J. Immunol. 174 (12), 8163–8172.

Jiang, D., Guo, H., Xu, C., Chang, J., Gu, B., Wang, L., Block, T.M., Guo, J.T., 2008. Identification of three interferon-inducible cellular enzymes that inhibit the replication of hepatitis C virus. J. Virol. 82 (4), 1665–1678.

Keller, B.C., Fredericksen, B.L., Samuel, M.A., Mock, R.E., Mason, P.W., Diamond, M.S., Gale Jr., M., 2006. Resistance to alpha/beta interferon is a determinant of West Nile virus replication fitness and virulence. J. Virol. 80 (19), 9424–9434.

Kotenko, S.V., Gallagher, G., Baurin, V.V., Lewis-Antes, A., Shen, M., Shah, N.K., Langer, J.A., Sheikh, F., Dickensheets, H., Donnelly, R.P., 2003. IFN-lambdas mediate antiviral protection through a distinct class II cytokine receptor complex. Nat. Immunol. 4 (1), 69–77.

Kramer, L.D., Li, J., Shi, P.Y., 2007. West Nile virus. Lancet Neurol. 6 (2), 171–181.

Lanciotti, R.S., Kerst, A.J., Nasci, R.S., Godsey, M.S., Mitchell, C.J., Savage, H.M., Komar, N., Panella, N.A., Allen, B.C., Volpe, K.E., Davis, B.S., Roehrig, J.T., 2000. Rapid detection of West Nile virus from human clinical specimens, field-collected mosquitoes, and avian samples by a TaqMan reverse transcriptase-PCR assay. J. Clin. Microbiol. 38 (11), 4066–4071.

Lanciotti, R.S., Roehrig, J.T., Deubel, V., Smith, J., Parker, M., Steele, K., Crise, B., Volpe, K.E., Crabtree, M.B., Scherret, J.H., Hall, R.A., MacKenzie, J.S., Cropp, C.B., Panigrahy, B., Ostlund, E., Schmitt, B., Malkinson, M., Banet, C., Weissman, J., Komar, N., Savage, H.M., Stone, W., McNamara, T., Gubler, D.J., 1999. Origin of the West Nile virus responsible for an outbreak of encephalitis in the northeastern United States. Science 286 (5448), 2333–2337.

Lin, R.J., Liao, C.L., Lin, E., Lin, Y.L., 2004. Blocking of the alpha interferon-induced Jak-Stat signaling pathway by Japanese encephalitis virus infection. J. Virol. 78 (17), 9285–9294.

Liu, W.J., Wang, X.J., Mokhonov, V.V., Shi, P.Y., Randall, R., Khromykh, A.A., 2005. Inhibition of interferon signaling by the New York 99 strain and Kunjin subtype of West Nile virus involves blockage of STAT1 and STAT2 activation by nonstructural proteins. J. Virol. 79 (3), 1934–1942.

Mackenzie, J.M., Khromykh, A.A., Parton, R.G., 2007. Cholesterol manipulation by West Nile virus perturbs the cellular immune response. Cell Host Microbe 2 (4), 229–239.

Mackenzie, J.S., Gubler, D.J., Petersen, L.R., 2004. Emerging flaviviruses: the spread and resurgence of Japanese encephalitis, West Nile and dengue viruses. Nat. Med. 10 (12 Suppl.), S98–109.

Manns, M.P., Foster, G.R., Rockstroh, J.K., Zeuzem, S., Zoulim, F., Houghton, M., 2007. The way forward in HCV treatment—finding the right path. Nat. Rev. Drug Discov. 6 (12), 991–1000.

Marcello, T., Grakoui, A., Barba-Spaeth, G., Machlin, E.S., Kotenko, S.V., MacDonald, M.R., Rice, C.M., 2006. Interferons alpha and lambda inhibit hepatitis C virus replication with distinct signal transduction and gene regulation kinetics. Gastroenterology 131 (6), 1887–1898.

Morales, M.A., Barrandeguy, M., Fabbri, C., Garcia, J.B., Vissani, A., Trono, K., Gutierrez, G., Pigretti, S., Menchaca, H., Garrido, N., Taylor, N., Fernandez, F., Levis, S., Enria, D., 2006. West Nile virus isolation from equines in Argentina, 2006. Emerg. Infect. Dis. 12 (10), 1559–1561.

Morrey, J.D., Day, C.W., Julander, J.G., Blatt, L.M., Smee, D.F., Sidwell, R.W., 2004. Effect of interferon-alpha and interferon-inducers on West Nile virus in mouse and hamster animal models. Antivir. Chem. Chemother. 15 (2), 101–109.

Munoz-Jordan, J.L., Laurent-Rolle, M., Ashour, J., Martinez-Sobrido, L., Ashok, M., Lipkin, W.I., Garcia-Sastre, A., 2005. Inhibition of alpha/beta interferon signaling by the NS4B protein of flaviviruses. J. Virol. 79 (13), 8004–8013.

Munoz-Jordan, J.L., Sanchez-Burgos, G.G., Laurent-Rolle, M., Garcia-Sastre, A., 2003. Inhibition of interferon signaling by dengue virus. Proc. Natl. Acad. Sci. USA 100 (24), 14333–14338.

Onoguchi, K., Yoneyama, M., Takemura, A., Akira, S., Taniguchi, T., Namiki, H., Fujita, T., 2007. Viral infections activate types I and III interferon genes through a common mechanism. J. Biol. Chem. 282 (10), 7576–7581.

Osterlund, P.I., Pietila, T.E., Veckman, V., Kotenko, S.V., Julkunen, I., 2007. IFN regulatory factor family members differentially regulate the expression of type III IFN (IFN-lambda) genes. J. Immunol. 179 (6), 3434–3442.

Puig-Basagoiti, F., Deas, T.S., Ren, P., Tilgner, M., Ferguson, D.M., Shi, P.Y., 2005. High-throughput assays using a luciferase-expressing replicon, virus-like particles, and full-length virus for West Nile virus drug discovery. Antimicrob. Agents Chemother. 49 (12), 4980–4988.

Rossi, S.L., Zhao, Q., O'Donnell, V.K., Mason, P.W., 2005. Adaptation of West Nile virus replicons to cells in culture and use of replicon-bearing cells to probe antiviral action. Virology 331 (2), 457–470.

Sadler, A.J., Williams, B.R., 2008. Interferon-inducible antiviral effectors. Nat. Rev. Immunol. 8 (7), 559–568.

Scholle, F., Girard, Y.A., Zhao, Q., Higgs, S., Mason, P.W., 2004. Trans-packaged West Nile virus-like particles: infectious properties in vitro and in infected mosquito vectors. J. Virol. 78 (21), 11605–11614.

Sen, G.C., 2001. Viruses and interferons. Annu. Rev. Microbiol. 55, 255–281.

Sheppard, P., Kindsvogel, W., Xu, W., Henderson, K., Schlutsmeyer, S., Whitmore, T.E., Kuestner, R., Garrigues, U., Birks, C., Roraback, J., Ostrander, C., Dong, D., Shin, J., Presnell, S., Fox, B., Haldeman, B., Cooper, E., Taft, D., Gilbert, T., Grant, F.J., Tackett, M., Krivan, W., McKnight, G., Clegg, C., Foster, D., Klucher, K.M., 2003. IL-28, IL-29 and their class II cytokine receptor IL-28R. Nat. Immunol. 4 (1), 63–68.

Stark, G.R., Kerr, I.M., Williams, B.R., Silverman, R.H., Schreiber, R.D., 1998. How cells respond to interferons. Annu. Rev. Biochem. 67, 227–264.

Wang, C., Gale Jr., M., Keller, B.C., Huang, H., Brown, M.S., Goldstein, J.L., Ye, J., 2005. Identification of FBL2 as a geranylgeranylated cellular protein required for hepatitis C virus RNA replication. Mol. Cell 18 (4), 425–434.

Zhou, Z., Hamming, O.J., Ank, N., Paludan, S.R., Nielsen, A.L., Hartmann, R., 2007. Type III interferon (IFN) induces a type I IFN-like response in a restricted subset of cells through signaling pathways involving both the Jak-STAT pathway and the mitogen-activated protein kinases. J. Virol. 81 (14), 7749–7758.

Zhu, Q., Guo, J.T., Seeger, C., 2003. Replication of hepatitis C virus subgenomes in nonhepatic epithelial and mouse hepatoma cells. J. Virol. 77 (17), 9204–9210.