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IL28B inhibits Hepatitis C virus replication through the JAK-STAT pathway

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Abstract

Background and Aims—The combination of pegylated interferon (IFN) α and ribavirin (RBV) is standard therapy for patients with chronic HCV infection. However, it produces a sustained virologic response (SVR) in only half of treated individuals and is associated with significant side effects. Recently several single-nucleotide polymorphisms (SNPs) near the *IL28B* locus, also known as *IFN λ 3*, were identified to be strong predictors of SVR in patients receiving PEG-IFN and RBV. We sought to determine whether IL28B was capable of inhibiting HCV replication and to determine the pathway by which IL28B exhibits anti-HCV activity.

Methods—Using the full-length HCV replicon OR6 and the infectious HCV clones JFH1 and Jc1, we assessed the anti-HCV effect of IL28B on HCV and characterized the key steps of the JAK-STAT pathway by real time PCR, luciferase assay, and Western blot. Finally, we evaluated the anti-HCV effect of IL28B in the presence of JAK-STAT pathway inhibitors such as blocking antibodies, a pharmacological inhibitor and siRNAs.

Results—We found that IL28B inhibits HCV replication in a dose- and time- dependent manner. Like IFN α , IL28B induces the phosphorylation of STAT1 and STAT2, ISRE-driven transcription, and expression of known ISGs. The anti-HCV effects of IL28A, IL28B and IL29 were abrogated by an IL10R2 blocking antibody, a pharmacological inhibitor of JAK1/TYK2, and by siRNA against IL28R1, STAT1, STAT2 and IRF9.

Conclusions—Our data demonstrate that IL28A, IL28B and IL29 signal through the JAK-STAT pathway to inhibit HCV. These data suggest possible applications of new approaches in HCV treatment.

Keywords

HCV; JAK; STAT; IL28B

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Introduction

Hepatitis C virus (HCV) is an enveloped, single-stranded, positive sense RNA virus that belongs to the hepacivirus genus in the family Flaviviridae [1,2]. Currently, 4% of the world's population is chronically infected with HCV, of whom as many as 30% will develop cirrhosis within 20 years of infection and a large subset will subsequently develop liver failure and/or hepatocellular carcinoma [1,3]. Infection with HCV has grown into a global epidemic with a death rate surpassing that of HIV/AIDS, and its complications will continue to accumulate for several decades [3].

Combination therapy with pegylated interferon (IFN) α and oral ribavirin is standard treatment for patients with chronic HCV infection. However, it eradicates HCV in only about half of the patients infected with HCV genotype 1, the most frequent genotype in the world. Moreover, severe adverse events are associated with type I IFN therapy, such as myelosuppression, influenza-like symptoms, and neuropsychiatric effects. Because these effects are dose-limiting, many patients are unable to receive higher doses of IFN that might inhibit HCV replication more effectively. These treatment-limiting adverse effects result from the very broad activity of IFN on the immune system, particularly on lymphocytes and neutrophils. It is therefore essential to identify more selective therapeutic agents for the treatment of hepatitis C.

Recently, several groups reported that several single-nucleotide polymorphisms (SNPs) (rs12979860, rs12980275, and rs8099917) near the *IL28B* (*IFN λ 3*) gene locus are strongly associated with SVR to IFN α and ribavirin treatment for hepatitis C [4–8]. *IL28B* is a member of the type III IFN family [9], which also includes IFN λ 1 (*IL29*) and IFN λ 2 (*IL28A*). IFN λ s bind to their cognate receptor, composed of *IL28R1* and *IL10R2*, and then activate the receptor-associated protein kinases *Jak1* and *Tyk2*, leading to activation of downstream STATs by phosphorylating critical serine and tyrosine residues. Activated STAT1 and STAT2 heterotrimerize with *IRF9* to form the ISGF3 complex. ISGF3 then translocates to the nucleus where it binds to the IFN-stimulated response element (ISRE) within the promoter region of IFN-stimulated genes (ISGs). The human genome encodes hundreds of ISGs that are effectors of host responses to viral infection, including *ISG15*, *MxA*, and *PKR*. However, the specific ISGs required for inhibiting HCV replication remain unknown. In this manner, type III IFNs are thought to have considerable functional overlap with type I IFNs [10,11], including IFN α . However, the magnitude of overlap between type I IFN α and IFN λ 3 in their antiviral activity is unknown.

We sought to analyze the role of *IL28B* in limiting hepatitis C virus replication and its regulation of ISG-mediated antiviral pathways. Previous studies in other laboratories have shown antiviral properties for two other closely related IFN λ s, IFN λ 1 and IFN λ 2 against HCV [12–14]. Using both an HCV full length replicon and JFH1-infected Huh7.5.1 cells, we show here that *IL28B* is capable of inhibiting HCV replication in a dose and time dependent manner. *IL28B* treatment stimulates the phosphorylation of STAT1 and STAT2. ISRE activity and several known ISGs are upregulated by *IL28B*. We also show that the anti-HCV effect of *IL28B* is impaired when key components of the JAK-STAT signaling pathway are inhibited.

Materials and methods

Cells, virus and reagents

Huh7.5.1 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The infectious JFH1 plasmid was obtained from Dr. Takaji Wakita and inoculated as previously described [15]. The OR6 cell

line [16], which harbors full-length genotype 1b HCV RNA and co-expresses *Renilla* luciferase, was grown in DMEM supplemented with 10% FBS and 500 µg/ml of G418 (Promega, Madison, WI). The infectious Jc1 plasmid Jc1FLAG2(p7-nsGluc2A) expressing *Gaussia* luciferase was obtained from Dr. Charles Rice [17]. IL28A, IL28B and IL29 were obtained from R&D systems. IL28A and IL29 are recombinant proteins generated from an NSO-derived murine myeloma cell line. IL28B is a recombinant protein generated from the CHO cell line. PEG-IFN α was obtained from Schering Corporation (Kenilworth, NJ). JAK inhibitor I was purchased from EMD Chemicals, Inc., Gibbstown, NJ, dissolved in 1% dimethyl sulfoxide (DMSO). IL10R2 blocking antibody was purchased from R&D Systems (Minneapolis, MN).

Western blotting

Cells were lysed using radioimmune precipitation assay (RIPA) buffer containing 1% NP-40, 0.1% SDS, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 150 mM NaCl and protease inhibitor cocktail, and subsequently sonicated. Proteins were separated by SDS-PAGE with NuPAGE Novex pre-cast 4–12% Bis-Tris gradient gels (Invitrogen, Carlsbad, CA) and transferred to PVDF membranes. The primary antibodies used in this paper were mouse anti-STAT1, rabbit anti-Phospho-STAT1 (Tyr701), rabbit anti-Jak1, anti-Tyk2, anti-STAT2, anti-phospho-STAT2, (Cell Signaling Technology, Inc., Beverly, MA), mouse anti-HCV core, (Affinity BioReagents Inc., Golden, CO), anti-E2, anti-NS4A, anti-NS4B, anti-NS5A, anti-NS5B (Virogen, Watertown, MA), ISG15, MXA (Abcam), mouse anti-actin (Sigma Life Science and Biochemicals, St. Louis, MO), and IL28R1 (Novus Biologicals, Littleton, CO). Secondary antibodies were HRP-conjugated ECL donkey anti-rabbit IgG and HRP-conjugated ECL sheep anti-mouse IgG (Amersham Biosciences, Piscataway, NJ). The ECL Western Blotting Detection Kit (Amersham Biosciences, Piscataway, NJ) was used to detect chemiluminescent signals.

Luciferase Assay

HCV replication in OR6 cells or Jc1FLAG2(p7-nsGluc2A)-infected Huh 7.5.1 cells was determined by monitoring *Renilla* or *Gaussia* luciferase activity (Promega, Madison, WI). To monitor IFN signaling directed by ISRE, the plasmids pISRE-luc (500 ng/well) expressing firefly luciferase and pRL-TK (50 ng/well) expressing *Renilla* luciferase as an internal control were cotransfected using Fugene HD following the manufacturer's protocol. Relative luciferase activity was assessed by the Promega dual-luciferase reporter assay system (Promega, Madison, WI).

siRNA and transfection

Indicated siRNAs were transfected into cells using LipofectamineTM RNAiMAX Transfection Reagent (Invitrogen, Carlsbad, CA). The negative control siRNA was obtained from QIAGEN. All siRNAs used for gene knock-down were SMART pools from Dharmacon and indicated: IL28R1, M-007981-00-0005; Jak1, M-003145-02-0005; Tyk2, M-003182-02-0002; STAT1, M-003543-01-0005; STAT2, M-012064-00-0005; IRF9, M-020858-02-0005. Protein expression of each gene knock down was confirmed by Western blotting as previously described [18].

Cell Viability Assay

Cell viability was assessed using the Cell Titer-Glo Luminescent Cell Viability Assay Kit (Promega, Madison, WI) according to the manufacturer's protocol.

Quantitative PCR

Total cellular and viral RNA was isolated post infection using RNeasy Mini columns (QIAGEN) and reverse transcribed by random priming with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems; Foster City, CA), then quantitated by real-time PCR using the DyNAmo HS SYBR Green qPCR kit (Finnzyme; Espoo, Finland). The primers are listed in Table 1.

Statistics

Data analysis was performed using a 2-tailed Student's t-test. Data are expressed as mean \pm SEM of at least three sample replicates, unless stated otherwise.

Results

IL28B demonstrates antiviral activity against HCV in a full-length replicon

As a successful model for HCV infection, the OR6 replicon cell line harbors a full-length genotype 1b HCV RNA with *Renilla* luciferase as a reporter. To determine the antiviral effect of IL28B against HCV, OR6 cells were seeded in 96-well plates for 24 hours and then treated with IL28B at different doses for another 24 hours. *Renilla* luciferase activity reflected the amount of HCV RNA and cell viability was evaluated by assessing cellular ATP levels. As shown in Fig. 1A, IL28B suppressed HCV replication in a dose dependent manner. IL28B at 100 ng/ml inhibited HCV replication to the same extent as 30 IU/ml IFN α (72% reduction).

We next determined the time course of IL28B's anti-HCV effect. As Fig. 1B shows, IL28B (100 ng/ml) inhibited HCV replication in a time dependent manner, achieving 42% suppression within the first 12 hours, and 91% suppression by day 3.

To further confirm IL28B's antiviral effect, expression levels of HCV proteins in IL28B treated OR6 cells were measured by Western blot using antibodies against HCV core, E2, NS4A, NS4B, NS5A, and NS5B. As shown in Fig. 1C, the levels of each of these HCV proteins were reduced by IL28B in the full-length OR6 replicon, confirming that IL28B antiviral for HCV.

To compare the anti-HCV effects of all three types of IFN λ , we treated OR6 cells with IFN α , IL28A, IL28B or IL29 at different doses for 48 hours. As shown in Fig. 1D and 1E, IFN α , IL28A, IL28B and IL29 all suppressed HCV replication in a dose-dependent (Fig. 1D) and time-dependent (Fig. 1E) manner. IL28B appeared to be somewhat more potent than IL28A and IL29.

IL28B inhibits infectious JFH1 replication

We then assessed IL28B's effect on HCV replication in JFH1, an established infectious cell culture model for HCV (genotype 2a). We infected Huh7.5.1 cells with JFH1 for 72 hours and then treated the cells with various doses of IL28B or IFN α for 24 hours. As shown in Fig. 1F, normalized JFH1 RNA levels were suppressed in an IL28B dose-dependent manner, achieving 64% suppression at 10 ng/ml and 92% suppression at 100 ng/ml IL28B (Fig. 1F). IL28B at 10 ng/ml inhibited JFH1 replication in a manner comparable to 15 IU/ml IFN α , while 100 ng/ml IL28B inhibited JFH1 replication to the same extent as 150 IU/ml IFN α .

We next determined the time course of IL28B's anti-HCV effect. As shown in Fig. 1G, IL28B (100 ng/ml) inhibited HCV replication in a time dependent manner, achieving 50% suppression at 6 hours, and 92% suppression by 24 hours.

To confirm the suppression of HCV proteins, the level of HCV core, E2, NS3, and NS5B proteins were measured by immunoblot. We found that IL28 B reduced levels of HCV proteins in a time-dependent manner (Fig. 1H).

IL28B induces phosphorylation of STAT1 and STAT2

IL28R1 and IL10R2 form the cognate receptor complex for IFN λ s. After IFN λ s bind to their receptor, the JAK-STAT pathway is activated. We next measured phosphorylation of STAT1 and STAT2 induced by IL28B. OR6 and JFH1-infected Huh7.5.1 cells were treated with 100 ng/mL IL28B, 30 IU/ml IFN α or mock treated for 30 min, and STAT1 and STAT2 phosphorylation was assessed. As shown in Fig. 2A and B, IL28B treatment induced STAT1 and STAT2 phosphorylation comparable to IFN α , confirming that the JAK-STAT signaling pathway is activated by IL28B in these cells.

IL28B induces ISRE activity and expression of classical ISGs

Like type I IFNs, type III IFNs are thought to mediate signaling through the STAT1 and STAT2 components of the JAK-STAT signal transduction pathways. We used the interferon stimulated response element (ISRE) luciferase reporter assay to assess activity downstream of the STAT1/STAT2 axis. We transfected pISRE-luc and pRL-TK into uninfected Huh7.5.1 cells or JFH1-infected Huh7.5.1 cells for 48 hours and IL28B was then added to the cells for 6 hours. Firefly and *Renilla* luciferase activity were then measured. IL28B significantly stimulated ISRE activity in both uninfected and JFH1-infected Huh7.5.1 cells (Fig. 2C and D). In uninfected Huh7.5.1 cells, ISRE luciferase activity was about 3-fold higher with IL28B treatment than with mock (Fig. 2C). In JFH1-infected Huh7.5.1 cells, ISRE luciferase activity was about double with IL28B treatment compared to mock (Fig. 2D). The increased ISRE luciferase activity by IL28B was similar to the extent of induction by IFN α . The lesser induction of the ISRE reporter activity by IFN in the presence of HCV likely reflected HCV's suppression of the JAK-STAT signaling pathway [19, 20].

Interferons are a family of multifunctional cytokines with the ability to interfere with viral infection through induction of the expression of IFN-stimulated genes. To determine IL28B's effect on ISGs, we analyzed expression of several classic antiviral ISGs. OR6 cells were treated with 10 ng/mL IL28B or 15 IU/ml IFN α or mock for varying lengths of time, and gene expression of several ISGs was assessed. Like IFN α , IL28B significantly increased the expression of IRF9, ISG15, MxA, OAS1, PKR, and STAT1 in a time dependent manner, while mock treatment failed to induce the expression of ISGs (Fig. 3A).

We also assessed ISG protein expression levels with IL28B stimulation. As shown in Fig. 3B and C, protein levels of STAT1, MxA, and ISG15 were significantly increased by IL28B treatment in both OR6 cells and JFH1-infected Huh7.5.1 cells.

To compare the induction of ISGs by the three types of IFN λ , we treated Huh 7.5.1 cells with 100 ng/ml IL28A, IL28B, IL29 or mock treatment for varying lengths of time, and gene expression of several ISGs was assessed. As shown in Fig. 3D, the expression pattern of IRF9, ISG15, MxA, OAS1, PKR, and STAT1 stimulated by IL28A, IL28B or IL29 are similar. These data suggested that the three types of IFN λ likely induce the same set of ISGs.

Taken together, these results imply that IL28B stimulates phosphorylation of STAT1/STAT2 and ISRE activity, thereby leading to the expression of known ISGs.

The antiviral activity of IL28B is dependent on the IFN λ receptor

Type III IFNs bind to the cellular IFN λ receptor (IL28R1 and IL10R2), which in turn engages the tyrosine kinases Jak1 and Tyk2. We tested whether the antiviral activity of

IL28B against HCV is mediated by the IFN λ receptor. We used an IL10R2 blocking antibody to inhibit IL28B signaling in OR6 and JFH1-infected cells (Fig. 4A and C). The induction of known ISGs (STAT1, MxA and ISG15) by IL28B was reduced by IL10R2 antibody. Correspondingly, the reduction of HCV core protein levels by IL28B, as assessed by Western blotting, was rescued by IL10R2 antibody (Fig. 4A and C). To inhibit IL28R1, we used an siRNA approach. IL28R1 knockdown in OR6 was validated by Western blotting as in Fig. 4B. IL28R1 knockdown in JFH1-infected Huh7.5.1 cells was validated by QPCR as in Fig. 4G. The induction of known ISGs (STAT1, MxA and ISG15) by IL28B was also reduced by silencing of IL28R1, indicating that the downstream JAK-STAT pathway was inhibited. As shown in Fig. 4B and D, protein levels of HCV core inhibited by IL28B were rescued by knocking down IL28R1. As shown in Fig. 4B, silencing IL28R1 unexpectedly caused the reduction of HCV core levels in the absence of IL28B, suggesting the possibility of siRNA-mediated off-target effects. Alternatively, IL28R1 may facilitate HCV replication, since the favorable *IL28B* genotype is unexpectedly associated with higher HCV viral loads [4].

To compare the dependence of the anti-HCV effects of the three types of IFN λ on IL10R2 receptor, OR6 cells or Jc1FLAG2(p7-nsGluc2A)-infected Huh 7.5.1 cells were pre-incubated with either IL10R2 or control antibody and then treated with 100 ng/ml of IL28A, IL28B, IL29 or mock treatment for three days. As shown in Fig. 4F and G, levels of normalized luciferase activity inhibited by IL28A, IL28B, IL29 were rescued by IL10R2 antibody.

Similarly, to compare the dependence of the anti-HCV effects of the three types of IFN λ on IL28R1 receptor, OR6 cells or Jc1FLAG2(p7-nsGluc2A)-infected Huh 7.5.1 cells were treated with siRNA against IL28R1 or control siRNA for three days and then incubated with 100 ng/ml of IL28A, IL28B, IL29 or mock treatment for three days. As shown in Fig. 4H and I, levels of normalized luciferase activity inhibited by IL28A, IL28B, IL29 were rescued by siRNA against IL28R1.

Taken together, the anti-HCV effect of IL28B as well as IL28A and IL29 is dependent on its intact IFN λ receptor.

The antiviral activity of IL28B is dependent on Jak1 and Tyk2

Because Jak1 and Tyk2 are required for STAT1 and STAT2 activation, we conjectured that Jak1 and Tyk2 are critical for the suppression of HCV replication by IL28B. To investigate this, OR6 cells or JFH1-infected Huh7.5.1 cells were incubated with JAK inhibitor I (inhibitor of both Jak1 and Tyk2) for 1 hour prior to treatment with IL28B and cell lysates were collected and analyzed by Western blot (Fig. 5A and B). In the presence of JAK inhibitor I, the induction of known ISGs (STAT1, MxA and ISG15) by IL28B was reduced and HCV core protein levels inhibited by IL28B were rescued by JAK inhibitor I. These data indicate that Jak1 and Tyk2 are required for IL28B's antiviral effect.

To compare the dependence of the anti-HCV effects of the three types of IFN λ on Jak1 and Tyk2, OR6 cells or Jc1FLAG2(p7-nsGluc2A)-infected Huh 7.5.1 cells were pre-treated with either JAK inhibitor I or mock treatment for 1 hour and then incubated with 100 ng/ml of IL28A, IL28B, IL29 or mock treatment for three days. As shown in Fig. 5C and D, levels of normalized luciferase activity inhibited by IL28A, IL28B, IL29 were rescued by JAK inhibitor I. These data indicate that Jak1 and Tyk2 are required for the antiviral effects of all three types of IFN λ .

The antiviral activity of IL28B is dependent on STAT1, STAT2 and IRF9

In the type I IFN signaling cascade, STAT1, STAT2 and IRF9 form the trimetric ISGF3 complex and subsequently undergo nuclear translocation. We therefore tested whether STAT1, STAT2 and IRF9 are required for the antiviral activity of IL28B. We used siRNAs to knock down STAT1, STAT2 and IRF9. In both OR6 cells and JFH1-infected Huh7.5.1 cells, the silencing of STAT1 and STAT2 was validated by Western blotting (Fig. 6A, B, D, and E). Partial knockdown of IRF9 protein was validated by Western blotting in OR6 cells (Fig. 6C). However, knockdown of IRF9 protein in JFH1-infected Huh7.5.1 cells was observed only in the presence of IL28B (Fig. 6F), despite the fact that siRNA against IRF9 was capable of silencing IRF9 mRNA in JFH1-infected Huh7.5.1 cells (Fig. 6G). This relatively weak observed silencing of IRF9 protein may be related to the abundant expression of IRF9 protein. By knocking down STAT1, the induction of STAT1 and MxA by IL28B was reduced; however, ISG15 protein levels remained similar to that of control siRNA (Fig. 6A and D). By knocking down STAT2 or IRF9, the induction of STAT1, MxA, and ISG15 by IL28B was reduced (Fig. 6B, C, E and F). HCV protein levels inhibited in the presence of IL28B were rescued by knocking down STAT1, STAT2, or IRF9. These data indicate that STAT1, STAT2 and IRF9 are required for IL28B antiviral signaling.

To compare the dependence of the anti-HCV effects of the three types of IFN λ on STAT1, STAT2 and IRF9, OR6 cells or Jc1FLAG2(p7-nsGluc2A)-infected Huh 7.5.1 cells either treated with siRNAs against STAT1, STAT2, IRF9 or control siRNA for three days and then incubated with 100 ng/ml of IL28A, IL28B, IL29 or mock treatment for three days. As shown in Fig. 6H and I, levels of normalized luciferase activity inhibited by IL28A, IL28B, IL29 were rescued by siRNAs against STAT1, STAT2 or IRF9. These data indicate that STAT1, STAT2 and IRF9 are required for the antiviral effects of all three types of IFN λ .

Discussion

As the first line of defense against viral pathogens, interferons act on viral RNA translation and sense RNA synthesis directly or indirectly through activation of host interferon stimulated genes (ISGs) [21]. IFN α is the major component of current standard treatment for hepatitis C [22]. The recent discovery of the type III lambda interferon family has opened new avenues of research into novel mechanisms of antiviral activity [3,23,24]. Previously, IFN λ 1 and λ 2 have been shown to inhibit HCV replication in HCV replicon cells [14]. In another study, IFN induced genes were compared by microarrays and different clusters of genes activated by IFN λ 1 were identified [13]. In this report, we have found that IL28B (IFN λ 3) inhibits HCV replication for two different genotypes (1b and 2a) in a time and dose dependent manner, confirming that all three IFN λ s are anti-HCV cytokines.

The mechanisms by which type III IFNs establish an antiviral state are not as well characterized as those for the type I IFNs, but are believed to be similar. We found that IL28B stimulated the phosphorylation of STAT1/STAT2 and ISRE luciferase reporter activities and subsequently induced the expression of known ISGs. Because of a more restricted distribution of the IFN λ receptor, IFN λ may be better tolerated than IFN α , which may justify the use of IFN λ as an alternative or complementary agent for hepatitis C. A recent clinical study found that weekly PEG-IFN λ 1 for 4 weeks is well tolerated with minimal adverse events and hematologic effects and is associated with clear antiviral activity in patients with chronic hepatitis C [25]. Our data provide evidence that IFN λ 3 could also have a role in hepatitis C treatment.

Several GWAS studies identified an association of IL28B SNPs with response to clearance of chronic HCV infection by IFN α and ribavirin [4–8,26]. Whether these SNPs are associated with altered IL28B gene expression or receptor activation remains to be further

established. Furthermore, it is not clear whether IL28B acts solely through its overlap with type I IFN or whether other signaling transduction pathways are also activated.

To elucidate mechanisms contributing to the anti-HCV effect of IL28A, IL28B, and IL29, we examined core components of the JAK-STAT pathway related to IFN λ . We systematically inhibited IL10R2, IL28R1, Jak1, Tyk2, STAT1, STAT2, and IRF9 using chemical, antibody, or siRNA inhibition. The expression of known ISGs, such as STAT1, MxA and ISG15 was measured to reflect the activation of the JAK-STAT pathway. In OR6 cells, JFH1-infected or Jc1-infected Huh7.5.1 cells, HCV suppression mediated by IL28A, IL28B, and IL29 was largely rescued when we inhibited each of these components of the JAK-STAT pathway, indicating that the JAK-STAT pathway is required for the anti-HCV effect of IL28B as well as IL28A and IL29.

In conclusion, our results demonstrate that IL28B inhibits HCV replication in three independent HCV models. Loss-of-function studies by inhibition of the JAK-STAT pathway suggest that the suppression of HCV by IL28B is predominantly mediated by this pathway. Further studies directed at understanding the specific genes induced by IFN λ and the mechanisms of their antiviral effect against HCV will provide valuable insight into HCV pathogenesis. Given that rescue of HCV by blocking JAK-STAT pathway was incomplete, these findings leave open the possibility of independent pathways induced by IL28B. However it is likely that these pathways play a less dominant role than the canonical type I IFN pathway.

Abbreviations

IFN	interferon
RBV	ribavirin
SVR	sustained virologic response
HCV	Hepatitis C virus
SNP	single-nucleotide polymorphism
ISRE	interferon-stimulated response element
ISG	IFN-stimulated genes
DMEM	Dulbecco's Modified Eagle's Medium
FBS	fetal bovine serum
RIPA	radioimmune precipitation assay

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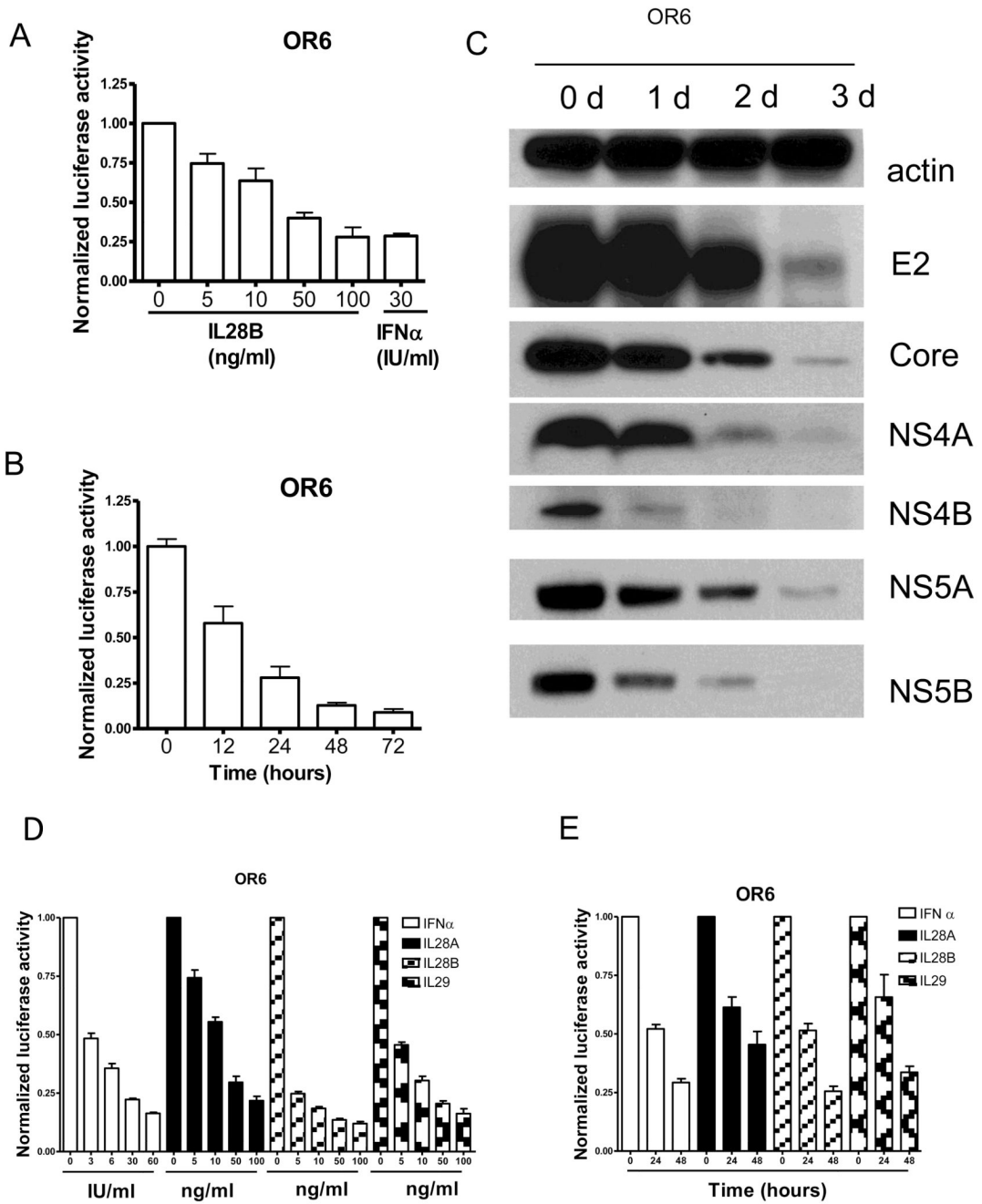
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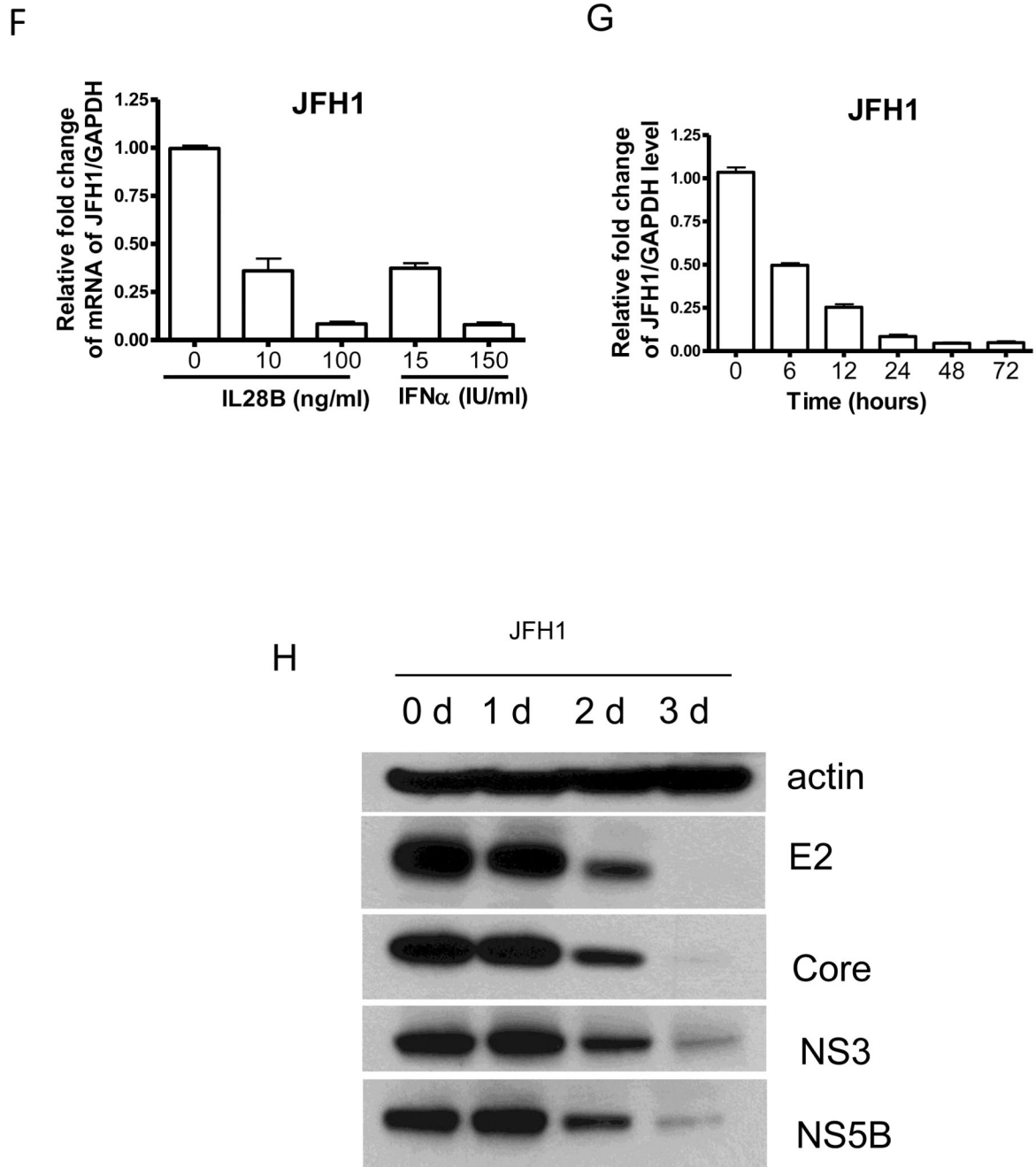


Figure 1. IL28B inhibits HCV replication in OR6 cells and JFH1 infection model

(A) OR6 cells were incubated with different doses of IL28B or 30 IU/ml IFN α as indicated for 24 hours. *Renilla* luciferase activity and cellular ATP levels were measured. (B) OR6 cells were incubated with 100 ng/ml IL28B for different times as indicated. *Renilla* luciferase activity and cellular ATP levels were measured. (C) OR6 cells were incubated with 100 ng/ml IL28B for different times as indicated. Cell lysates were analyzed by immunoblotting with the indicated antibodies (D) OR6 cells were incubated with different doses of IFN α , IL28A, IL28B or IL29 as indicated for 48 hours. *Renilla* luciferase activity and cellular ATP levels were measured. (E) OR6 cells were incubated with 100 ng/ml IL28A, IL28B, IL29 or 30IU/ml IFN α for different times as indicated. *Renilla* luciferase

activity and cellular ATP levels were measured. (F) JFH1-infected Huh7.5.1 cells were incubated with different doses of IL28B or IFN α as indicated for 24 hours. Total RNA was isolated and reversely transcribed and then quantitative PCR was performed. (G) JFH1-infected Huh7.5.1 cells were incubated with 100 ng/ml IL28B for different times as indicated. Total RNA was isolated and reversely transcribed and then quantitative PCR was performed. (H) JFH1-infected Huh7.5.1 cells were incubated with 100 ng/ml IL28B for different times as indicated. Cell lysates were analyzed by immunoblotting with the indicated antibodies.

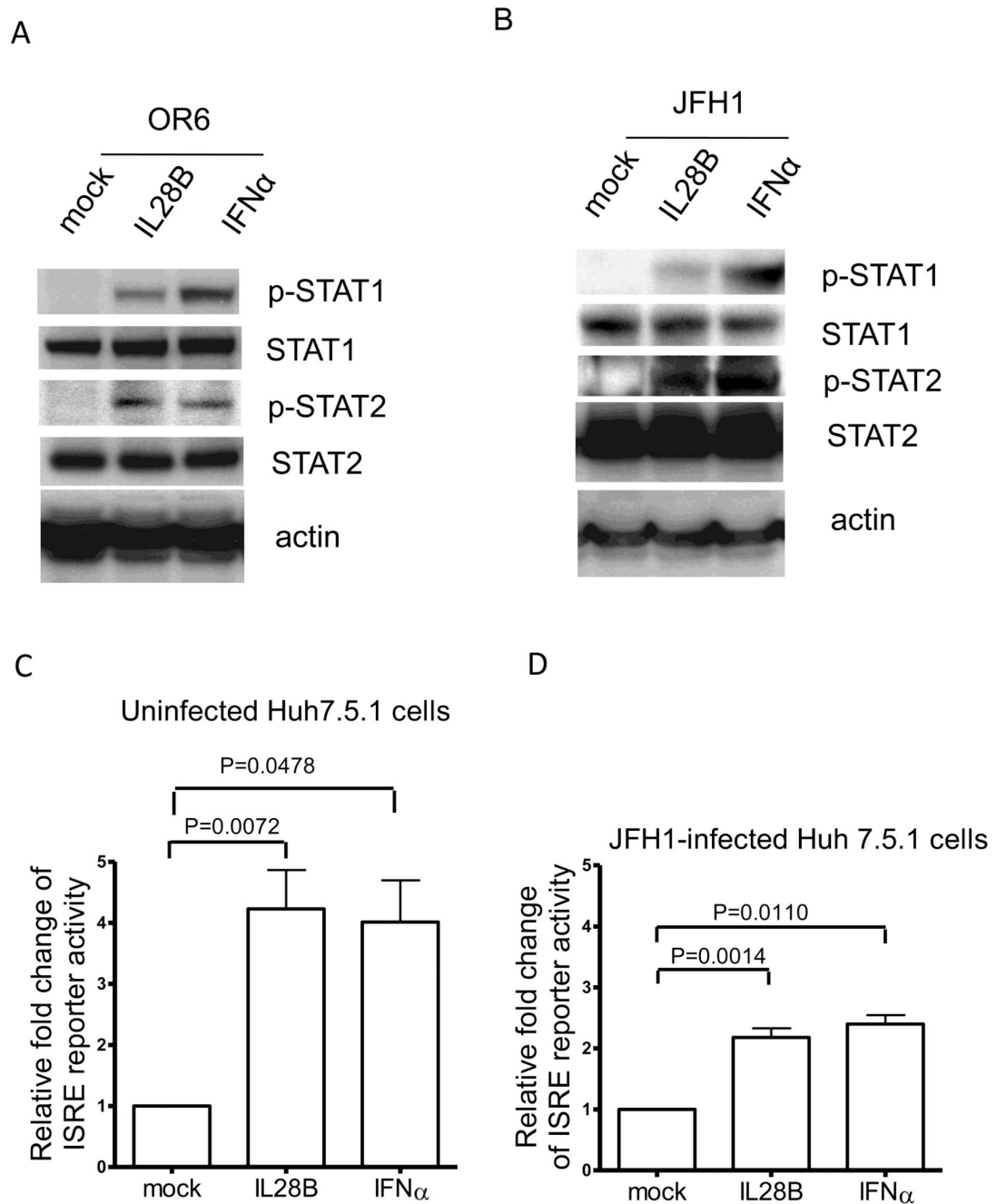
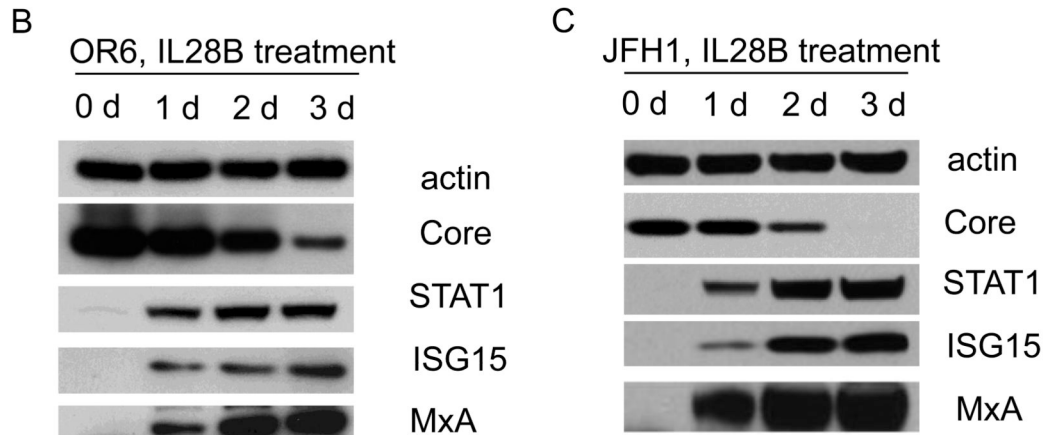
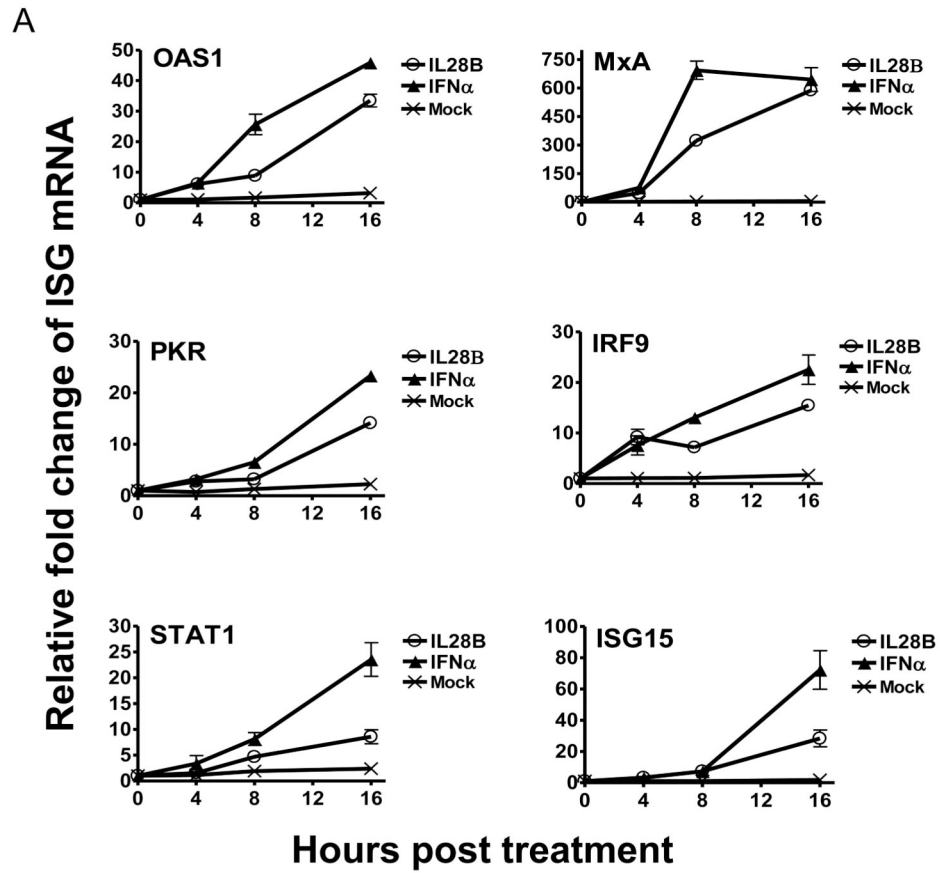


Figure 2. IL28B induces STAT1 and STAT2 phosphorylation and ISRE reporter activity
OR6 cells (A) or JFH1-infected Huh7.5.1 cells (B) were treated with 100 ng/ml IL28B, 30 IU/ml IFN α or mock treatment for 30 min and the cell lysates were analyzed by Western blotting with the indicated antibodies. pISRE-luc encoding firefly luciferase under the control of the ISRE and pRL-TK expressing *Renilla* luciferase were transfected to Huh7.5.1 cells (C) or JFH1-infected Huh7.5.1 cells (D) for 48 hours and 100 ng/ml IL28B or 30 IU/ml IFN α were added to the cells for 6 hours. The firefly and *Renilla* luciferase activity was measured.



D

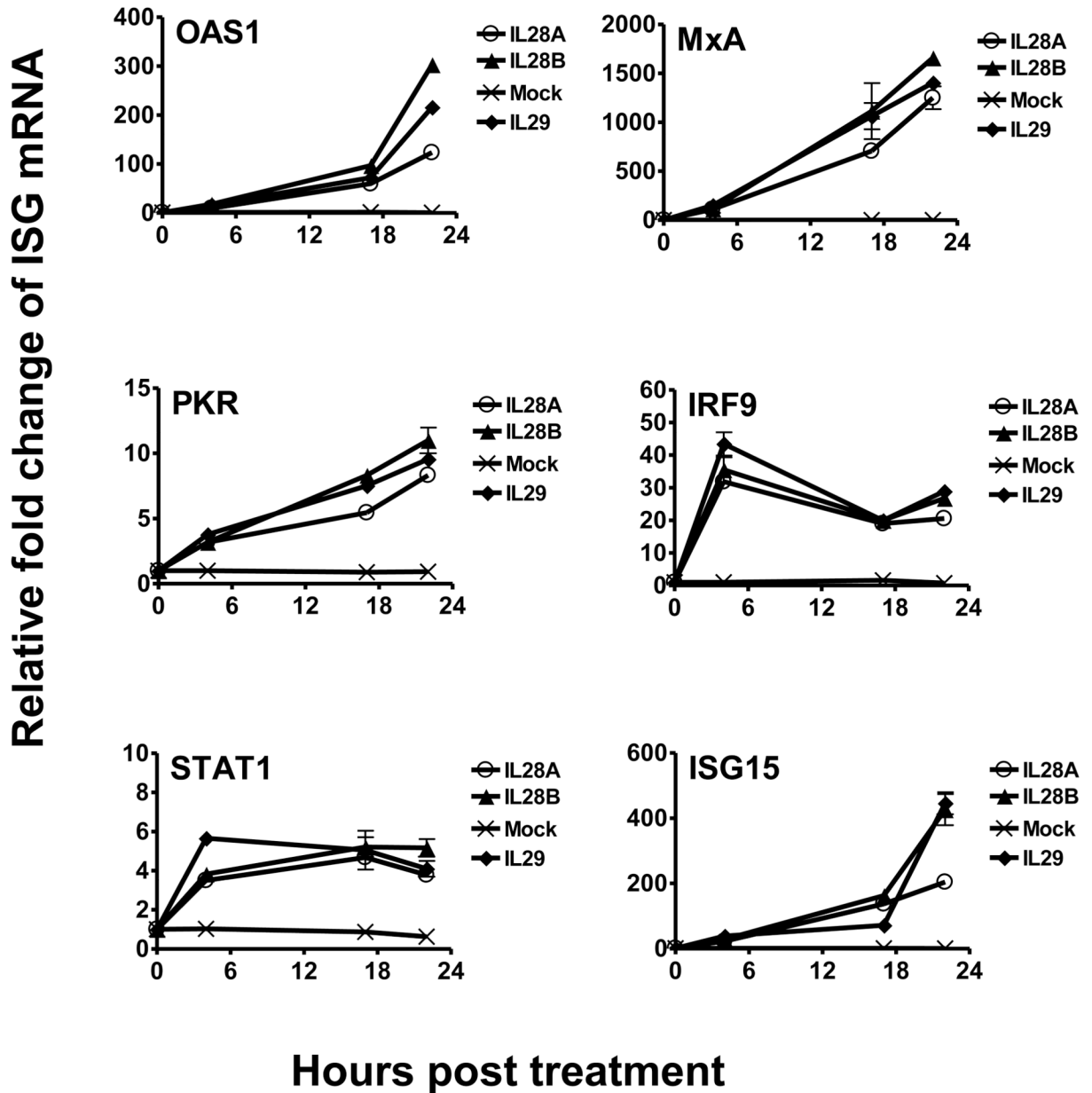
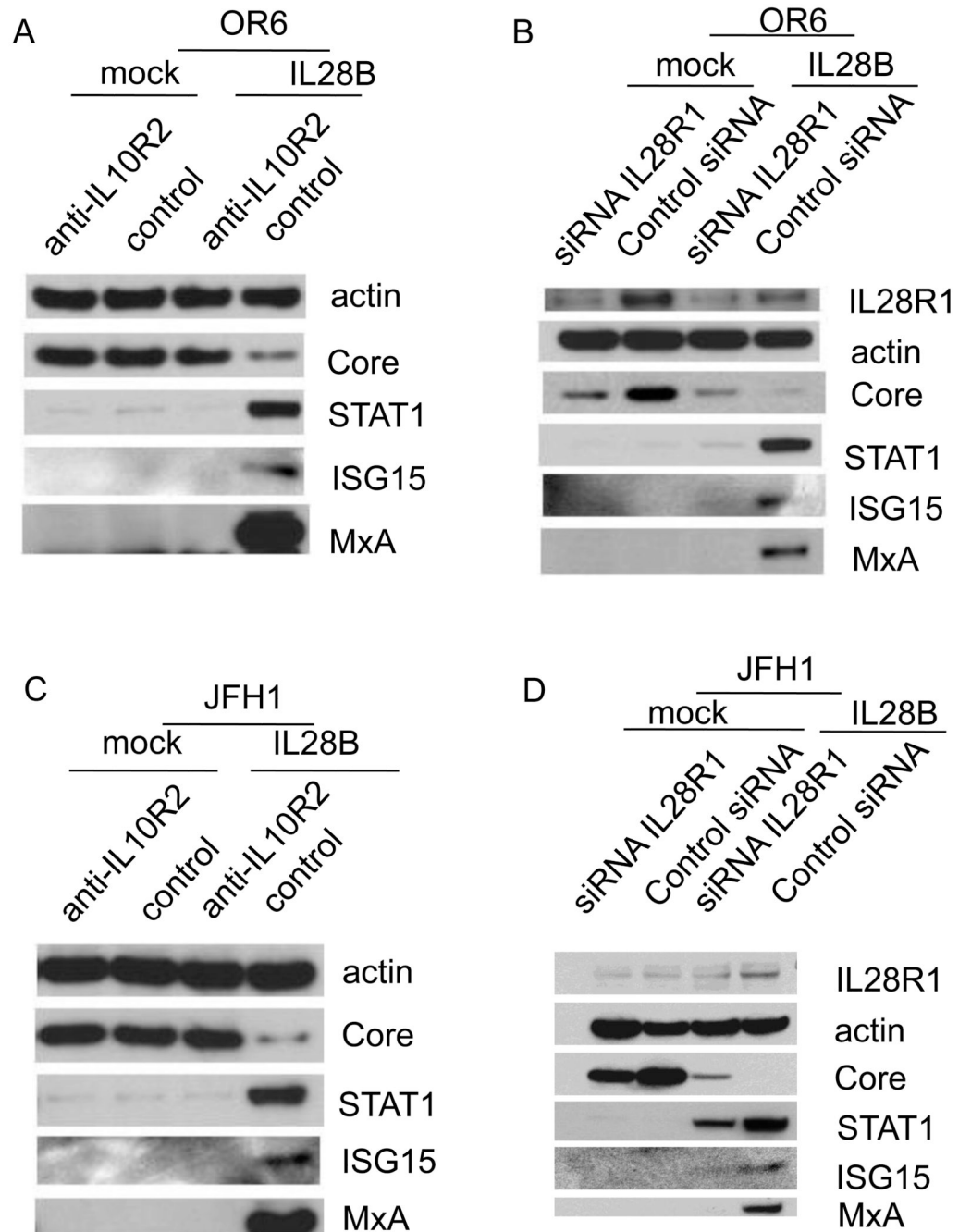


Figure 3. IL28B stimulates known ISGs

(A) OR6 cells were incubated 10 ng/ml IL28B or 15 IU/ml IFN α or mock treatment for different times as indicated. Total RNA was isolated and reverse transcribed followed by real time PCR. OR6 cells (B) and JFH1-infected Huh7.5.1 cells (C) were incubated with 100 ng/ml IL28B for different times as indicated. Cell lysates were analyzed by immunoblotting with the indicated antibodies. (D) Huh 7.5.1 cells were incubated 100 ng/ml IL28A, IL28B, IL29 or mock treatment for different times as indicated. Total RNA was isolated and reverse transcribed followed by real time PCR.



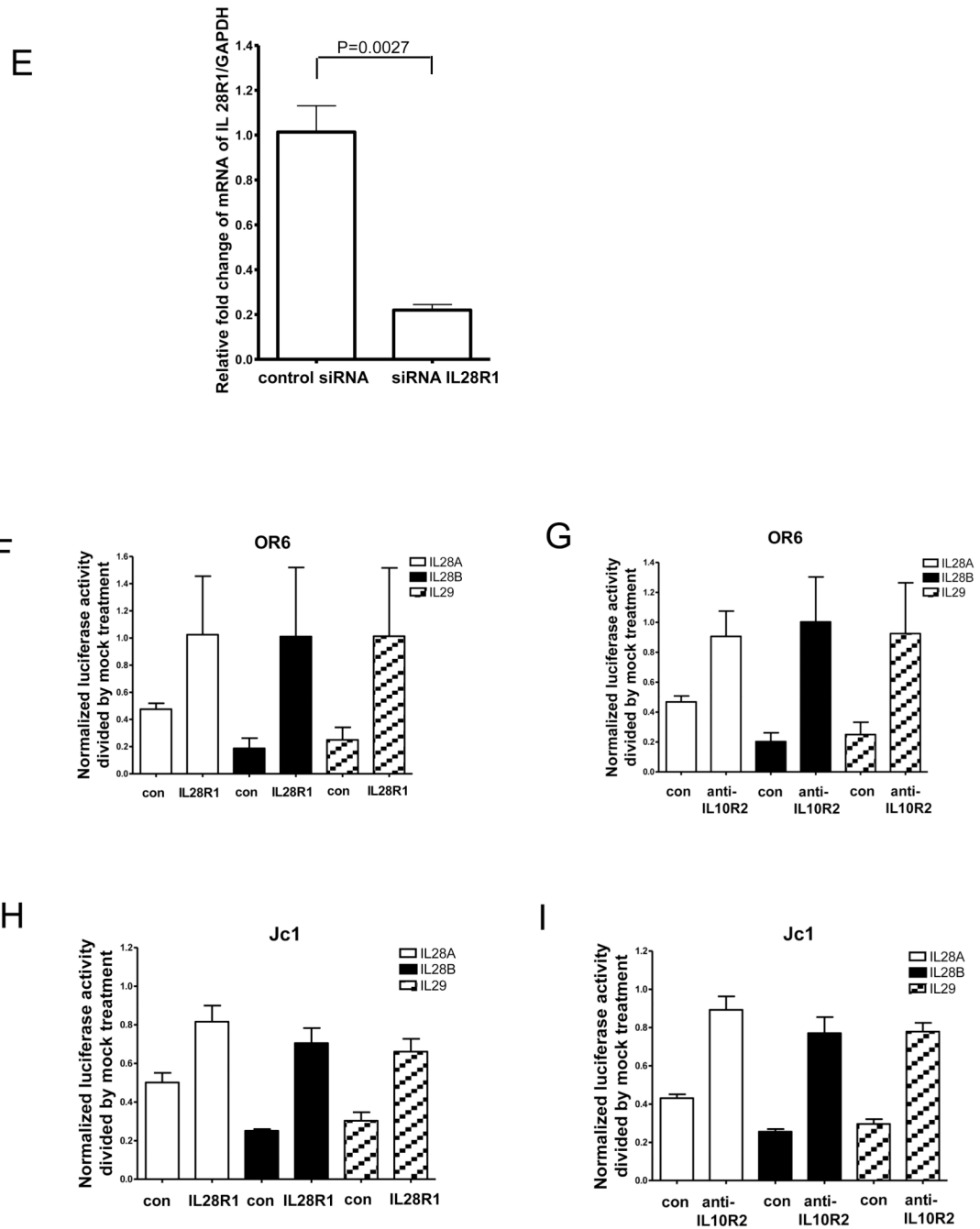


Figure 4. The IFN λ receptor is required for the anti-HCV effect of IL28B

OR6 cells (A) and JFH1-infected Huh7.5.1 cells (C) were incubated with IL10R2 blocking antibody or control goat IgG for 2 hours and were then treated with 100 ng/ml IL28B or control mock treatment for 3 days. Cell lysates were analyzed by immunoblotting with the indicated antibodies. OR6 cells (B) and JFH1-infected Huh7.5.1 cells (D) were treated with siRNA against IL28R1 for 3 days and incubated with 100 ng/ml IL28B for 3 days. Cell lysates were analyzed by immunoblotting with the indicated antibodies. (E) JFH1-infected Huh7.5.1 cells were treated with siRNA against IL28R1 for 3 days. Total RNA was isolated and reversely transcribed and then quantitative PCR was performed. OR6 cells (F) and Jc1-infected Huh7.5.1 cells (H) were incubated with either IL10R2 or control goat antibodies for

2 hours and then treated with 100 ng/ml IL28A, IL28B, IL29 or mock treatment for 3 days. *Renilla* (F) or *Gaussia* (H) luciferase activity and cellular ATP levels were measured. The normalized luciferase activity were then divided by the normalized luciferase activity from mock treatment. OR6 cells (G) and Jc1-infected Huh7.5.1 cells (I) were treated with siRNA against IL28R1 for 3 days and then treated with 100 ng/ml IL28A, IL28B, IL29 or mock treatment for 3 days. *Renilla* (G) or *Gaussia* (I) luciferase activity and cellular ATP levels were measured. The normalized luciferase activity was then divided by the normalized luciferase activity from mock treatment.

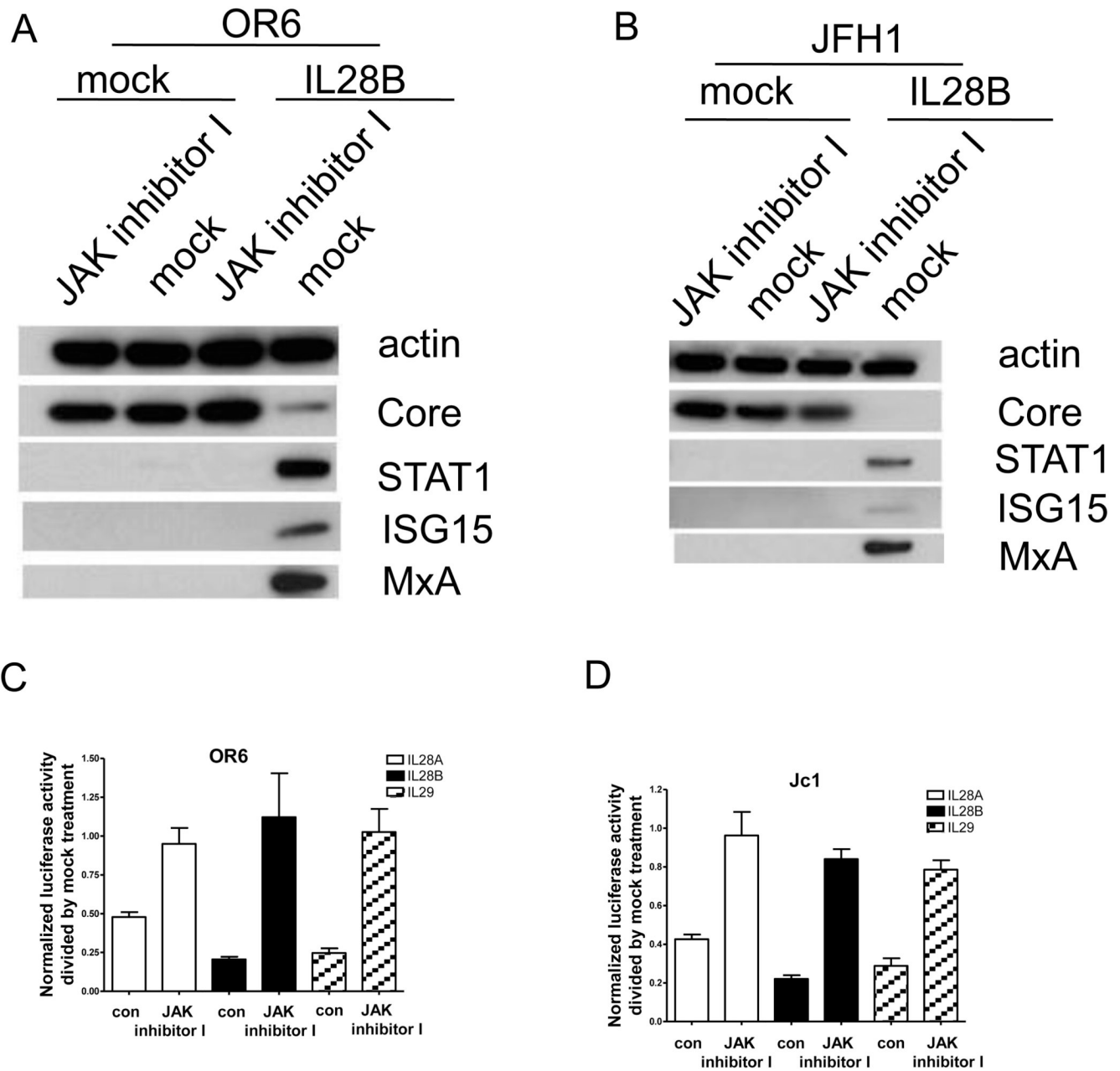
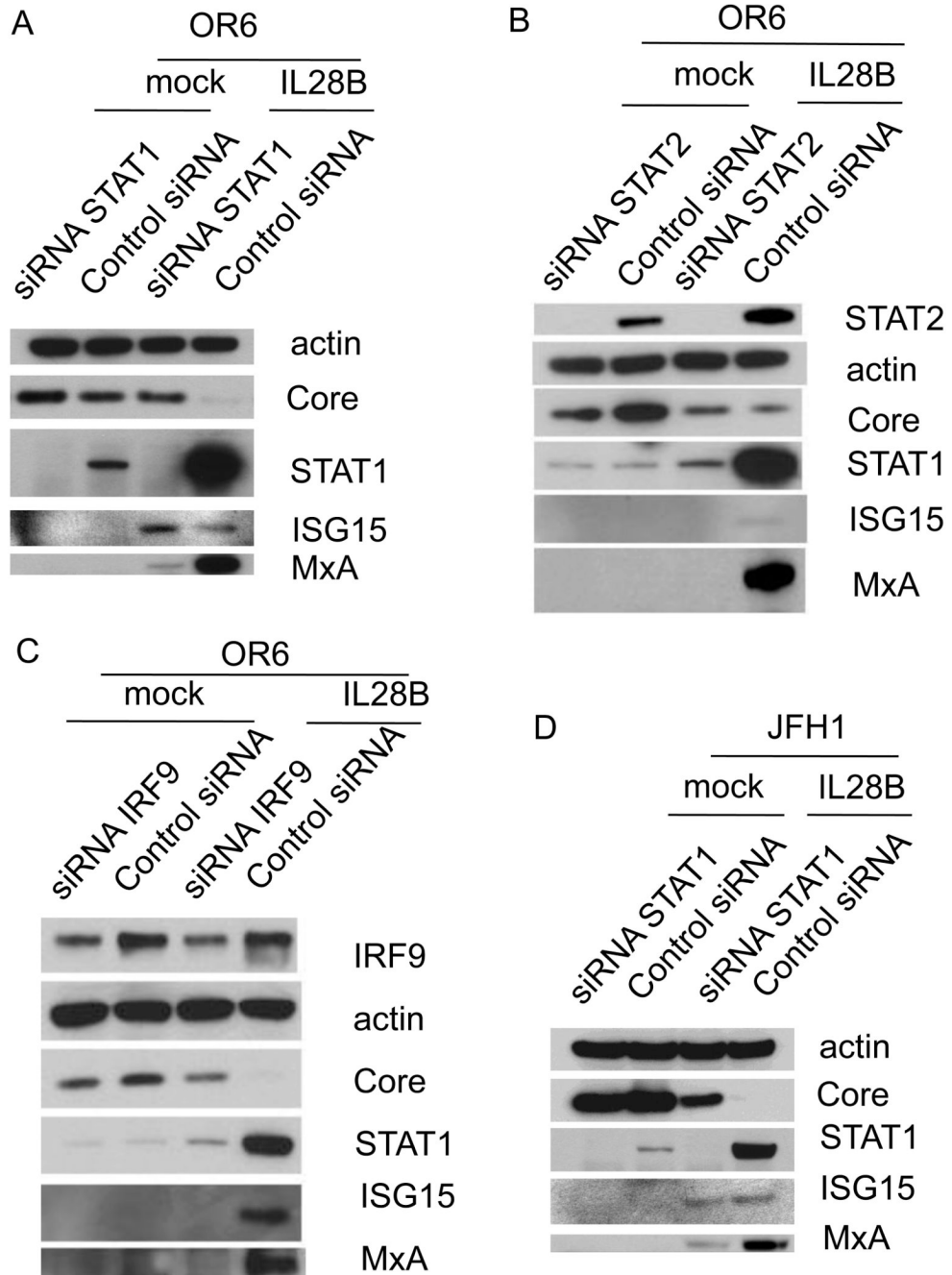
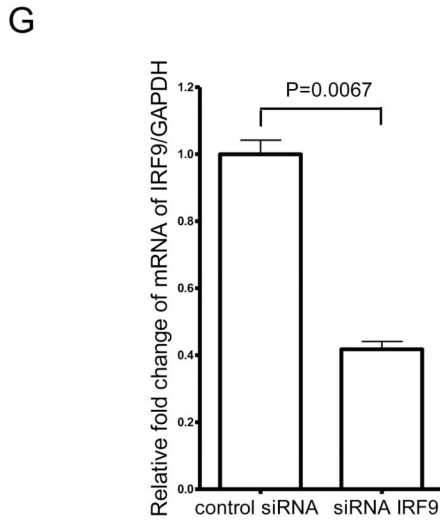
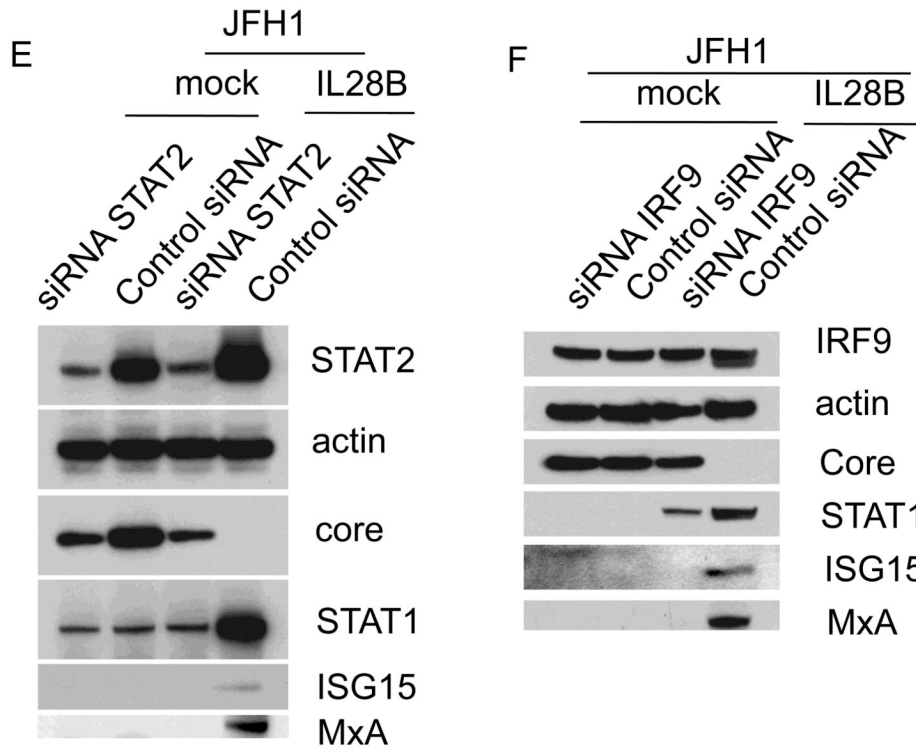


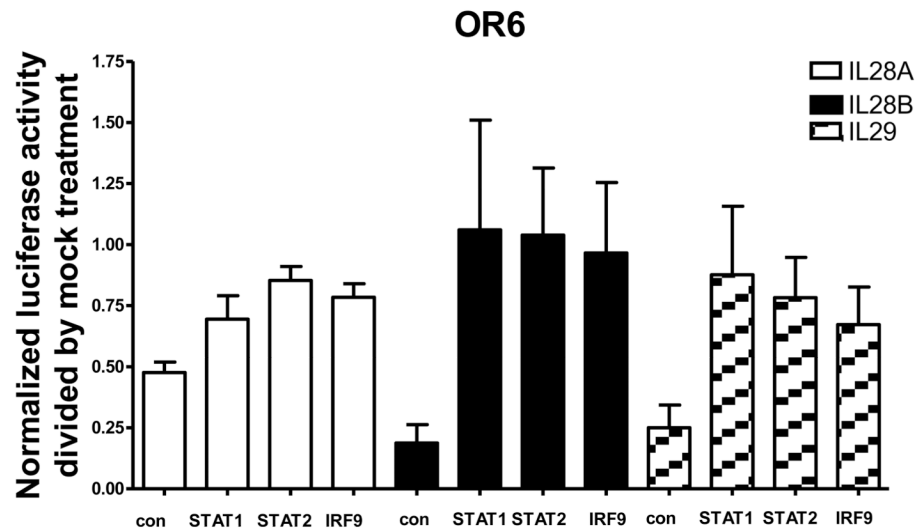
Figure 5. Jak1 and Tyk2 are required for the anti-HCV effect of IL28B

OR6 cells (A) and JFH1-infected Huh7.5.1 cells (B) were incubated with JAK inhibitor I or mock for 1 hour and then treated with 100 ng/ml IL28B or mock for 3 days. Cell lysates were analyzed by immunoblotting with the indicated antibodies. OR6 cells (C) and Jc1-infected Huh7.5.1 cells (D) were incubated with JAK inhibitor I or mock for 1 hour and then treated with 100 ng/ml IL28A, IL28B, IL29 or mock treatment for 3 days. *Renilla* (C) or *Gaussia* (D) luciferase activity and cellular ATP levels were measured. The normalized luciferase activity were then divided by the normalized luciferase activity from mock treatment.





H



I

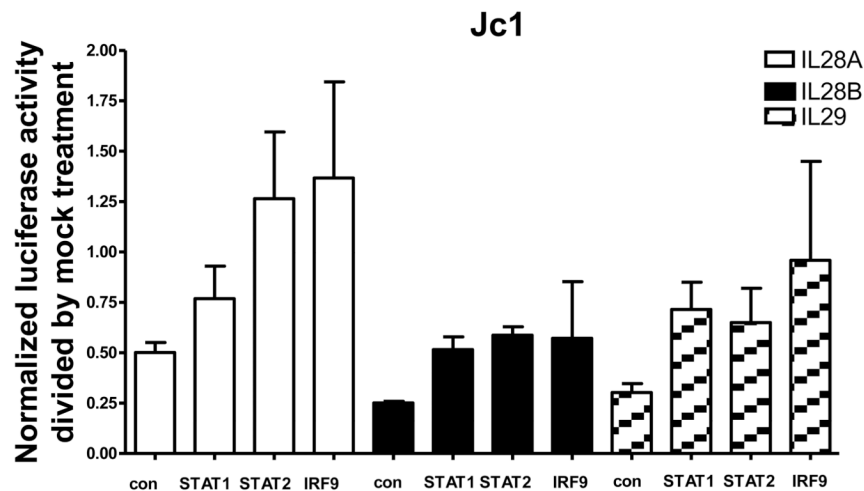


Figure 6. STAT1, STAT2 and IRF9 are required for the anti-HCV effect of IL28B

OR6 cells were treated with siRNAs against STAT1 (A), STAT2 (B) or IRF9 (C) for 3 days and incubated with 100 ng/ml IL28B or mock for 3 days. Cell lysates were analyzed by immunoblotting with the indicated antibodies. JFH1-infected Huh7.5.1 cells were treated with siRNAs against STAT1 (D), STAT2 (E) or IRF9 (F) for 3 days and incubated with 100 ng/ml IL28B or mock for 3 days. Cell lysates were analyzed by immunoblotting with the indicated antibodies. (G) JFH1-infected Huh7.5.1 cells were treated with siRNAs against IRF9 for 3 days. Total RNA was isolated and reversely transcribed and then quantitative PCR was performed. OR6 cells (H) and Jc1-infected Huh7.5.1 cells (I) were treated with siRNAs against STAT1, STAT2 or IRF9 for 3 days and then treated with 100 ng/ml IL28A, IL28B,

IL29 or mock treatment for 3 days. *Renilla* (H) or *Gaussia* (I) luciferase activity and cellular ATP levels were measured. The normalized luciferase activity was then divided by the normalized luciferase activity from mock treatment.

Table 1

Primers used for quantitative RT-PCR.

Target gene	Primer ^a	Nucleotide sequence
GAPDH	F	5'-CAACTGGTCGTGGACAACCAT-3'
	R	5'-GCACGGACACTCACAATGTTTC-3'
IL28R1	F	5'-GACATGACCGGGGACTGCATG-3'
	R	5'-GACACACAGGTCCCCGCTGG-3'
PKR	F	5'-TCTACGCTTTGGGGCTAAT-3'
	R	5'-AGATGATGCCATCCCGTAG-3'
STAT1	F	5'-GTGGAAAGACAGCCCTGCA-3'
	R	5'-ACTGGACCCCTGTCTTCAA-3'
IRF9	F	5'-CCCGAAAACCTCCGGAAC TG -3'
	R	5'-CAGCACACTCCGGGAAACT-3'
ISG15	F	5'-GGTGGACAAATGCGACGAA-3'
	R	5'-ATGCTGGTGGAGGCCCTTA-3'
OAS1	F	5'-AGAAGGCAGCTCACGAAAC-3'
	R	5'-CCACCACCAAGTTTCCTG-3'
MxA	F	5'-GCCGGCTGTGGATATGCTA-3'
	R	5'-TTTATCGAAACATCTGTGA-3'
JFH1	F	5'-TCTGCGGAACCGGTGAGTA-3'
	R	5'-TCAGGCAGTACCACAAGGC-3'

^aF, forward; R, reverse.