Available online at www.sciencedirect.com
ScienceDirect
Antiviral Research 76 (2007) 194–197
Antiviral Research
www.elsevier.com/locate/antiviral

Short communication

HCV structural proteins interfere with interferon-alpha Jak/STAT signalling pathway

Esther Luquin [a], Esther Larrea [a], Maria P. Civeira [b], Jesús Prieto [a,b], Rafael Aldabe [a,*]

[a] *Division of Hepatology and Gene Therapy (CIMA), University of Navarra, Pamplona, Spain*
[b] *Liver Unit, University Clinic, University of Navarra, Pamplona, Spain*

Received 25 January 2007; accepted 18 June 2007

Abstract

Hepatitis C virus (HCV) is remarkably efficient at establishing persistent infection. The current treatment with IFN-α given alone or in combination with ribavirin is ineffective in eliminating the virus in a large proportion of individuals with chronic hepatitis C. Recent data suggest that HCV blocks IFN-α signalling, an effect that facilitates viral persistence. We have used the HCV genomic and subgenomic replicon system to analyze the effect of structural and non-structural viral proteins on the activation of the Jak/STAT pathway and induction of antiviral activity by IFN-α. Our results show that IFN-α-mediated STAT activation (but not IFN-γ-stimulated STAT phosphorylation) is blocked in Huh7 cell line containing the genomic replicon, while this is not observed in cells with the subgenomic replicon. In agreement with these findings, the transcriptional activity and the antiviral effect of IFN-α were significantly lower in cells harboring the genomic replicon than in cells with the subgenomic replicon. These results indicate that HCV structural proteins play an important role in the escape of HCV from the interferon system.

Keywords: HCV; Replicon; Interferon; STAT phosphorylation; Interferon-stimulated genes

HCV infection affects about 130 million people (Shepard et al., 2005), being one of the most important causes of liver disease worldwide. IFN-α and combination of IFN-α plus ribavirin are the only approved therapies for chronic hepatitis C. However, 40–50% of patients fail to respond to the treatment (Manns et al., 2006). Several mechanisms, including both host and viral factors, have been proposed to explain the lack of response of HCV infection to IFN-α therapy (Gao et al., 2004; Thimme et al., 2006). In patients with chronic hepatitis C the resistance to IFN-α is manifested by a reduced induction of interferon-stimulated genes (ISGs) (He et al., 2006) and by an upregulated baseline expression of a set of ISGs (Chen et al., 2005).

HCV efficiently disturbs the response to IFN-α at several levels. First, the NS3/4 viral protease cleaves TRIF and Cardif (Breiman et al., 2005; Li et al., 2005; Meylan et al., 2005) and since both proteins are important for IRF-3 activation and upregulation of IFN-α genes, cells infected with HCV show an impairment in IFN-α production. Also the expression of the full length HCV polyprotein or of different individual HCV proteins hampers IFN-α response by interfering with IFN-α signal transduction pathway and consequently with the antiviral effects of interferon. It has been reported that the HCV polyprotein or core protein inhibits STAT1 DNA binding probably as result of phosphatase 2Ac upregulation (Heim et al., 1999; Blindenbacher et al., 2003; Duong et al., 2004, 2005). Moreover, it has been reported that the expression of the HCV core protein induces STAT1 degradation (Lin et al., 2005, 2006), downregulates IFN-α induced ISG transcription (de Lucas et al., 2005) and promotes SOCS-3 expression which blocks IFN-α signal transduction (Bode et al., 2003). The antiviral activity promoted by IFN-α can also be disturbed by other HCV proteins such as the E2 and NS5A which interact directly with and inhibit the double-stranded RNA-dependent protein kinase PKR (Gale et al., 1997; Taylor et al., 1999). Moreover, in a previous report we have observed that in the livers of HCV-infected patients STAT2 phosphorylation is absent and there is a reduction of STAT3 levels (Larrea et al., 2006).

In this study we have analyzed the effect of HCV replication and expression of HCV proteins on IFN-α induced Jak/STAT activation. For this purpose we have used the HCV replicon

* Corresponding author at: Division of Gene Therapy and Hepatology, Center for Applied Medical Research, CIMA, Avenida Pío XII, 55, 31008 Pamplona, Spain. Tel.: +34 948 194700; fax: +34 948 194717.
E-mail address: raldabe@unav.es (R. Aldabe).

0166-3542/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.antiviral.2007.06.004

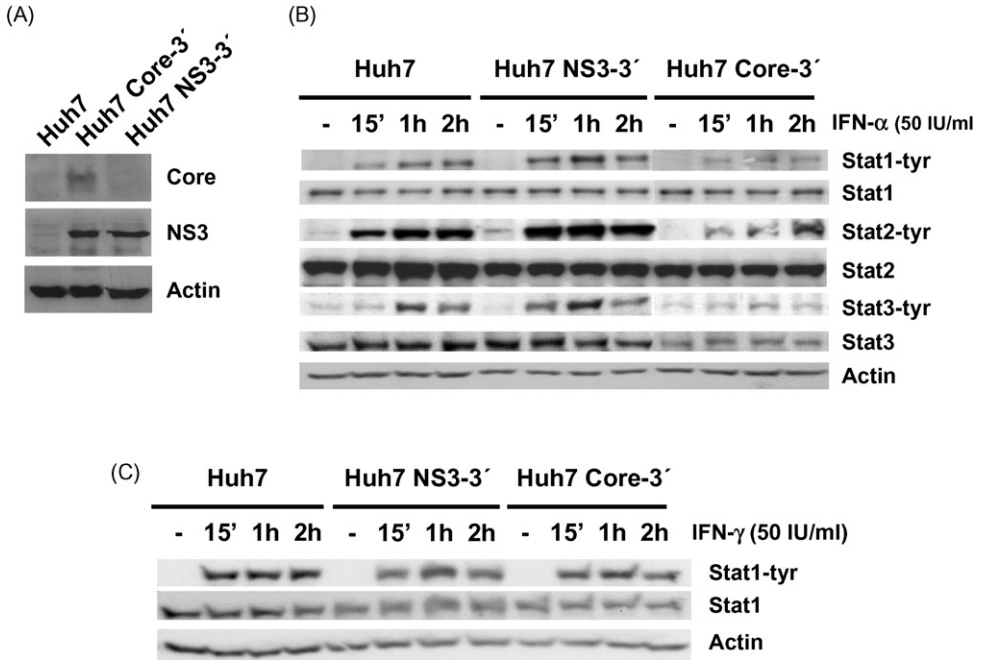

Fig. 1. Phosphorylation of STAT proteins upon stimulation with IFN-α or IFN-γ in HCV replicon cell lines. (A) Detection of HCV Core and NS3 proteins in Huh7 harboring HCV subgenomic replicon (NS3-3′) and Huh7 containing genomic replicon (Core-3′) cell lines. (B) Huh7, Huh7 NS3-3′ and Huh7 Core-3′ cell lines were treated for 15 min, 1 h and 2 h with 50 IU/ml of IFN-α2b (Schering-Plough). Immunoblot analysis of total cell lysates was assessed with the anti-Stat1-tyr, anti-Stat2-tyr and anti-Stat3-tyr antibodies (cell signaling). The membrane was stripped and the presence of the total Stat1, Stat2 and Stat3 proteins was determined using the respective anti-Stat antibodies. Corresponding samples were examined for actin concentration using anti-actin antibody as the protein loading control. (C) Huh7 and Huh7 HCV genomic and subgenomic replicon cell lines were stimulated for 15, 60 and 120 min with 50 IU/ml of IFN-γ (R&D) and STAT1 tyrosine phosphorylation was examined as described previously. Results are representative of three independent experiments.

system (Lohmann et al., 1999), consisting in Huh7 cells containing selectable, self-replicating HCV RNAs. We have analyzed the effects of the replication of the entire HCV viral genome (genomic replicon) and those of a replicon with the proteins present between NS3 and NS5B (subgenomic replicon). The genomic and subgenomic replicon cell lines were generated as previously described (Lohmann et al., 1999; Larrea et al., 2006), and they were used as a pool of clones in all the experiments. As shown in Fig. 1A, HCV core protein can be detected by Western blotting in cells with the genomic replicon but not in control cells nor cells with the subgenomic replicon, while NS3 can be detected in cells with both genomic and subgenomic replicons.

IFN-α induced Jak/STAT activation was analyzed after adding 50 units/ml of IFN-α2b (Shering-Plough) for 15, 60 and 120 min to Huh7 cells harboring genomic replicon (Huh7 Core-3′), subgenomic replicon (Huh7 NS3-3′) and without replicon. We observed a clear impairment of IFN-α-induced phosphorylation of STAT1, STAT2 and STAT3 only when the HCV genomic replicon was present and not when the cells contained the subgenomic replicon (Fig. 1B). This effect was accompanied by a decrease of the amount of STAT3 present in the cell line harboring the full length HCV genome. These findings suggest that the blockade of STAT activation and the reduction of STAT3 levels are provoked by HCV structural proteins.

In order to evaluate if the inhibitory effect of HCV on interferon signalling is IFN-α specific we treated the cells with 50 units/ml of IFN-γ (R&D) for 15, 60 and 120 min and we examined the amount of tyrosine-phosphorylated STAT1. We found similar levels of STAT1 activation in the three cell lines (Fig. 1C) indicating that IFN-γ signaling is not impaired, at variance with what was observed with IFN-α. Of interest, we did not observe STAT1 degradation in the cells with the genomic replicon in contrast with data from previous reports (Lin et al., 2005). The reason for this discrepancy is not obvious, but it could be due to differences in the level of expression of viral proteins or the protein expression systems used in the two studies. To confirm that IFN-γ signalling was not blocked in cells with the genomic replicon, we analyzed the induction of IFN-γ-sensitive genes such as IRF1 after incubation of the cells with 50 units/ml of IFN-γ for 15, 60 and 120 min. As shown in Fig. 2D, we found that the upregulation of this gene was not impaired in Huh-7 cells harboring the genomic replicon. It seems therefore that replication of the entire HCV RNA interferes selectively with IFN-α signalling leaving intact the IFN-γ signal transduction, a finding that argues against degradation of STAT1 by HCV structural proteins in our system.

Next, we compared the transcriptional response to IFN-α in cells harboring the genomic replicon, cells with the subgenomic replicon and cells without replicon. At this end we used a luciferase gene expression system in which the reporter gene is driven by the interferon-stimulated response element (ISRE) that is activated upon binding of IFN-α to its receptor (Platanias, 2005). Cells transfected for 24 h with the plasmid pISREluc (Stratagene) were incubated with IFN-α (50 units/ml) and were sampled at 8 h of culture. As shown in Fig. 2A, luciferase expression was lower in cells supporting replication of the whole

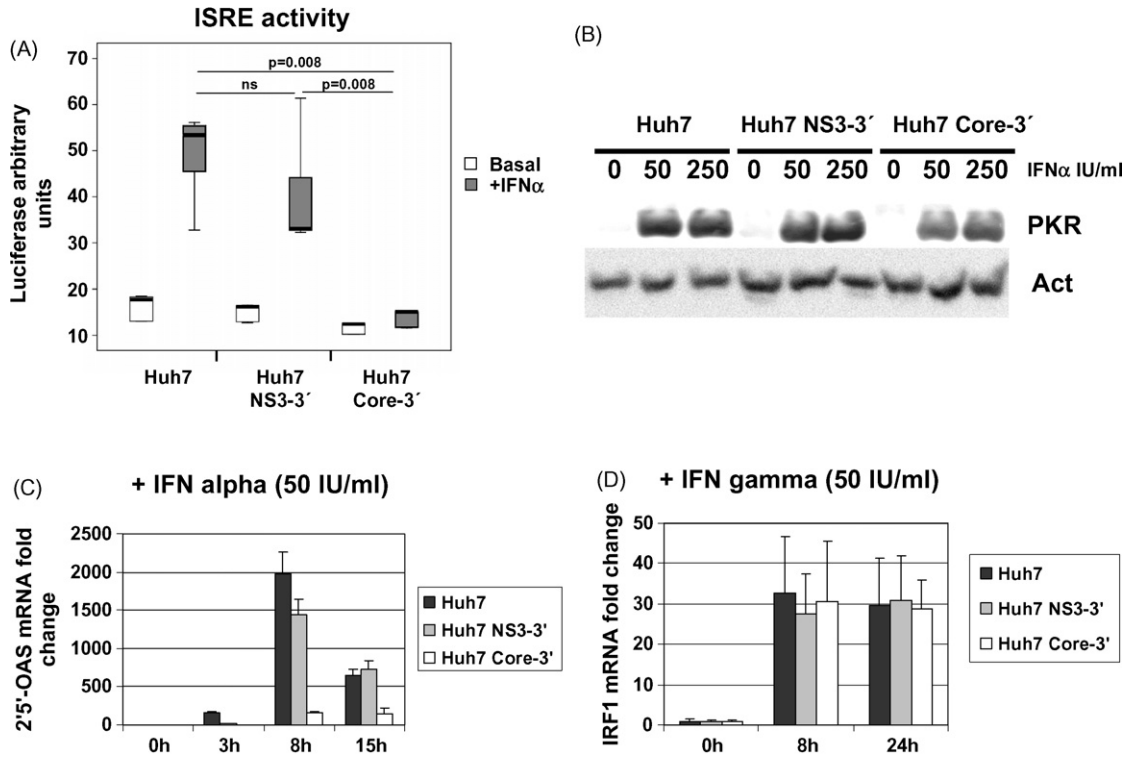

Fig. 2. Differential effects of interferon in Huh7, Huh7 cells with HCV subgenomic replicon (Huh7 NS3-3′) and Huh7 with HCV genomic replicon (Huh7 Core-3′) cell lines. (A) Measurement of luciferase activity in cells transfected with the plasmid pISREluc (Stratagene) for 24 h in basal conditions and after treatment with IFN-α for 8 h. The plasmid pISREluc was cotransfected with the plasmid ptkRluc that expresses *Renilla* luciferase under the control of the thymidine kinase promoter and served to normalize the luciferase activity obtained. Statistical analyses were performed using non-parametric, Kruskal–Wallis and Mann–Whitney *U* tests. Data are reported as medians and interquartile range from three independent experiments. ns, not significant. (B) PKR induction in Huh7 and Huh7 HCV genomic and subgenomic replicon cell lines stimulated with 50 or 250 IU/ml of IFN-α for 18 h. Cell lysates were immunoblotted with PKR antibody (Santa Cruz) as described in Fig. 1. (C) Differential 2′5′-OAS mRNA induction after incubating Huh7 and Huh7 HCV genomic and subgenomic replicon cell lines with IFN-α (50 IU/ml) for the indicated periods of time. Total RNA was extracted with Trizol (Invitrogen) and reverse transcribed prior to measure 2′5′-OAS expression by quantitative real-time PCR using an iQ and the SYBR GRN supermix (BioRad). As an internal control for each sample, PCR amplification of a fragment of actin cDNA was performed. The amount of each transcript was expressed by the formula $2^{cpactin-cpgene}$, where cp is the point at which the fluorescence rises appreciably above the background fluorescence. (D) IRF1 mRNA induction after treating Huh7 and Huh7 containing HCV subgenomic and genomic replicons with IFN-γ (50 IU/ml) for 8, 15 and 24 h. The samples were treated as described before. Results are expressed as the mean ± standard deviations of one representative experiment out of three independent experiments performed in triplicate (C and D).

HCV genome as compared with those with subgenomic replicon or with control cells. To confirm these observations we measured in the three types of cells PKR expression (Fig. 2B) and mRNA expression of 2′,5′ oligoadenylate synthetase (2′5′-OAS) (Fig. 2C), classical IFN-α sensitive genes (Rebouillat and Hovanessian, 1999), in the presence and absence of IFN-α. As represented in Fig. 2B and C, after addition of IFN-α the increase in PKR protein and 2′5′-OAS mRNA is lower in cells with the genomic replicon, confirming the critical role of HCV structural proteins in the blockade of IFN-α effects. These findings are in agreement with recent publications showing that HCV core protein downregulates the expression of genes stimulated by IFN-α (de Lucas et al., 2005).

We then analyzed if the attenuation of IFN-α signalling and the reduction in IFN-α transcriptional activity observed in cells with genomic replicon were accompanied by a reduced antiviral effect of IFN-α in these cells. We incubated the cells with 50 units/ml of IFN-α for 72 h and we determined the reduction of the amount of viral RNA in cells with genomic and subgenomic replicon. As shown in Fig. 3A, IFN-α induced a more pronounced decrease in the amount of HCV RNA in these cells with the subgenomic replicon than in those with the genomic replicon. But when the genomic and subgenomic replicon cell lines were treated with IFN-γ (50 units/ml) for 72 h, HCV RNA was reduced to the same extent in both cell lines (Fig. 3B) indicating again the selective impairment of IFN-α signalling. This finding emphasizes the role of HCV structural proteins in the decrease of the antiviral effect of IFN-α in HCV-infected cells and is in agreement with previous reports implicating HCV core protein in the escape of HCV from the interferon system (Blindenbacher et al., 2003; Bode et al., 2003; de Lucas et al., 2005; Lin et al., 2005).

In conclusion, we have observed that activation of Jak/STAT pathway by IFN-α as well as the transcriptional activity and antiviral effect of IFN-α are attenuated in cells with replication of the entire HCV genome but not in cells with replicating HCV RNA lacking structural proteins. These findings reveal a key role of HCV structural proteins in the escape of HCV to the interferon system and in the progression to chronicity of this viral infection.

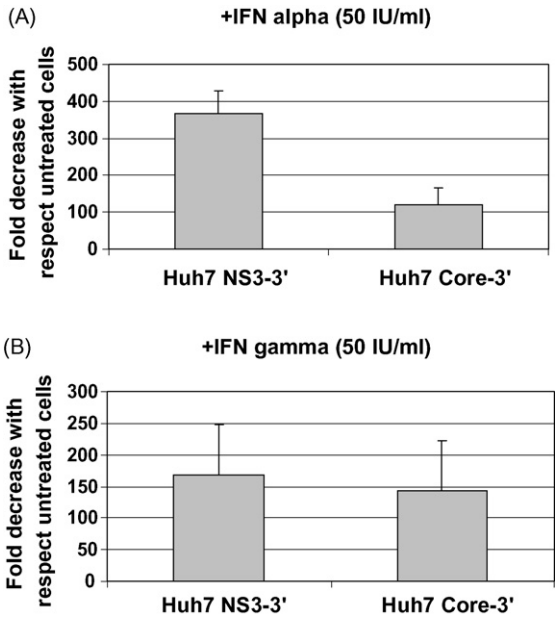

Fig. 3. Antiviral effects of IFN-α and IFN-γ in HCV replicon cell lines. Quantitation of HCV RNA reduction in Huh7 cells with the genomic (Huh7 Core-3′) or subgenomic (Huh7 NS3-3′) replicon after treatment with IFN-α (50 IU/ml) (A) or IFN-γ (50 IU/ml) (B) for 72 h. The medium was replaced every 24 h and when treatment was finished, total RNA was extracted using Trizol. 1 μg of total RNA was used for generating cDNA, and HCV-RNA was measured by quantitative real-time PCR using primers for the HCV 5′ UTR and an iQ and the SYBR GRN supermix (BioRad). As an internal control for each sample, PCR amplification of a fragment of actin cDNA was performed. The amount of each transcript was expressed by the formula $2^{\text{cpactin-cp5'UTR}}$. Results are expressed as the means ± standard deviations of one representative experiment out of three independent experiments performed in triplicate.

Acknowledgements

E. Luquin is supported by the program Torres Quevedo and R.A. by the program Contratos de apoyo a la investigación en el Sistema Nacional de Salud. HCV replicon cDNA was kindly provided by Dr R. Bartenschlager. We thank Sandra Jusue, Maite Corres and Beatriz Carte for excellent technical assistance.

References

Blindenbacher, A., Duong, F.H.T., Hunziker, L., Stutvoet, S.T.D., Wang, X., Terracciano, L., Moradpour, D., Blum, H.E., Alonzi, T., Tripodi, M., La Monica, N., Heim, M.H., 2003. Expression of hepatitis C virus proteins inhibits interferon-α signaling in the liver of transgenic mice. Gastroenterology 124, 1465–1475.

Bode, J.G., Ludwig, S., Ehrhardt, C., Erhardt, A., Albrecht, U., Schaper, F., Heinrich, P.C., Haussinger, D., 2003. IFN-α; antagonistic activity of HCV core protein involves induction of suppressor of cytokine signaling-3. FASEB J. 17, 488–490.

Breiman, A., Grandvaux, N., Lin, R., Ottone, C., Akira, S., Yoneyama, M., Fujita, T., Hiscott, J., Meurs, E.F., 2005. Inhibition of RIG-I-dependent signaling to the interferon pathway during hepatitis C virus expression and restoration of signaling by IKKepsilon. J. Virol. 79, 3969–3978.

Chen, L., Borozan, I., Feld, J., Sun, J., Tannis, L.-L., Coltescu, C., Heathcote, J., Edwards, A.M., McGilvray, I.D., 2005. Hepatic gene expression discriminates responders and nonresponders in treatment of chronic hepatitis C viral infection. Gastroenterology 128, 1437–1444.

de Lucas, S., Bartolomé, J., Carreño, C., 2005. Hepatitis C virus core protein down-regulates transcription of interferon-induced antiviral genes. J. Infect. Dis. 191, 93–99.

Duong, F.H.T., Christen, V., Berke, J.M., Penna, S.H., Moradpour, D., Heim, M.H., 2005. Upregulation of protein phosphatase 2Ac by hepatitis C virus modulates NS3 helicase activity through inhibition of protein arginine methyltransferase 1. J. Virol. 79, 15342–15350.

Duong, F.H.T., Filipowicz, M., Tripodi, M., La Monica, N., Heim, M.H., 2004. Hepatitis C virus inhibits interferon signaling through up-regulation of protein phosphatase 2A. Gastroenterology 126, 263–277.

Gale, J.M.J., Korth, M.J., Tang, N.M., Tan, S.-L., Hopkins, D.A., Dever, T.E., Polyak, S.J., Gretch, D.R., Katze, M.G., 1997. Evidence that hepatitis C virus resistance to interferon is mediated through repression of the PKR protein kinase by the nonstructural 5A protein. Virology 230, 217–227.

Gao, B., Hong, F., Radaeva, S., 2004. Host factors and failure of interferon-α treatment in hepatitis C virus. Hepatology 39, 880–890.

He, X.-S., Ji, X., Hale, M.B., Cheung, R., Ahmed, A., Guo, Y., Nolan, G.P., Pfeffer, L.M., Wright, T.L., Risch, N., Tibshirani, R., Greenberg, H.B., 2006. Global transcriptional response to interferon is a determinant of HCV treatment outcome and is modified by race. Hepatology 44, 352–359.

Heim, M.H., Moradpour, D., Blum, H.E., 1999. Expression of hepatitis C virus proteins inhibits signal transduction through the Jak-STAT pathway. J. Virol. 73, 8469–8475.

Larrea, E., Aldabe, R., Molano, E., Fernandez-Rodriguez, C.M., Ametzazurra, A., Civeira, M.P., Prieto, J., 2006. Altered expression and activation of signal transducers and activators of transcription (STATs) in hepatitis C virus infection: in vivo and in vitro studies. Gut 55, 1188–1196.

Li, K., Foy, E., Ferreon, J.C., Nakamura, M., Ferreon, A.C.M., Ikeda, M., Ray, S.C., Gale Jr., M., Lemon, S.M., 2005. Immune evasion by hepatitis C virus NS3/4A protease-mediated cleavage of the Toll-like receptor 3 adaptor protein TRIF. Proc. Natl. Acad. Sci. U.S.A. 102, 2992–2997.

Lin, W., Choe, W.H., Hiasa, Y., Kamegaya, Y., Blackard, J.T., Schmidt, E.V., Chung, R.T., 2005. Hepatitis C virus expression suppresses interferon signaling by degrading STAT1. Gastroenterology 128, 1034–1341.

Lin, W., Kim, S.S., Yeung, E., Kamegaya, Y., Blackard, J.T., Kim, K.A., Holtzman, M.J., Chung, R.T., 2006. Hepatitis C virus core protein blocks interferon signaling by interaction with the STAT1 SH2 domain. J. Virol. 80, 9226–9235.

Lohmann, V., Körner, F., Koch, J.O., Herian, U., Theilmann, L., Bartenschlager, R., 1999. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science 285, 110–113.

Manns, M.P., Wedemeyer, H., Cornberg, M., 2006. Treating viral hepatitis C: efficacy, side effects, and complications. Gut 55, 1350–1359.

Meylan, E., Curran, J., Hofmann, K., Moradpour, D., Binder, M., Bartenschlager, R., Tschopp, J., 2005. Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. Nature 437, 1167–1172.

Platanias, L.C., 2005. Mechanisms of type-I and type-II-interferon-mediated signalling. Nat. Rev. Immunol. 5, 375–386.

Rebouillat, D., Hovanessian, A.G., 1999. The human 2′,5′-oligoadenylate synthetase family: interferon-induced proteins with unique enzymatic properties. J. Interferon Cytokine Res. 19, 295–308.

Shepard, C.W., Finelli, L., Alter, M.J., 2005. Global epidemiology of hepatitis C virus infection. Lancet Infect. Dis. 5, 558–567.

Taylor, D.R., Shi, S.T., Romano, P.R., Barber, G.N., Lai, M.M., 1999. Inhibition of the interferon-inducible protein kinase PKR by HCV E2 protein. Science 285, 107–110.

Thimme, R., Lohmann, V., Weber, F., 2006. A target on the move: innate and adaptive immune escape strategies of hepatitis C virus. Antiviral Res. 69, 129–141.