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Hepatitis C virus replication in mouse cells is restricted by IFN-dependent and -independent mechanisms

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Title: Hepatitis C Virus Replication in Mouse Cells is Restricted by IFN-Dependent and -Independent Mechanisms

Short title: IFN-independent inhibition of HCV replication

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Keywords: Hepatitis C virus, Interferon regulatory factor, IFN-independent, mouse embryonic fibroblasts, primary mouse hepatocytes

Abstract:

BACKGROUND & AIMS: Current treatment strategies for Hepatitis C virus (HCV) infection include pegylated interferon (IFN)-a and ribavirin. Approximately 50% of patients control HCV infection following treatment, but the broad range of patients' outcomes and responses to treatment, among all geneotypes, indicates a role for host factors. Although the IFN system is important in limiting HCV replication, the virus has evolved mechanisms to circumvent the IFN response. However, direct, IFN-independent antiviral processes might also help control HCV replication. We examined the role of IFN-independent responses against HCV replication.

METHODS: We analyzed replication of the subgenomic JFH1 replicon in embryonic fibroblasts and primary hepatocytes from mice with disruptions in genes encoding factors in the IFN-dependent and alternative antiviral pathways (STAT1, PKR, IRF1, IRF3, IRF5, IRF7, MAVS, and IFNAR). We also assessed the effects of expression of these factors by mouse primary hepatocytes on HCV replication.

RESULTS: In addition to IRF3- and IFN-mediated antiviral responses, IFN-independent, but IRF1- and IRF5-dependent mechanisms, restrict HCV replication in MEFs. In primary hepatocytes these IFN-independent require MAVS and IRF-1.

CONCLUSIONS: HCV replication is limited by interferon-mediated pathways as well pathways that are independent of type I IFNs. IRF1 and IRF5 control IFN-independent signaling events that lead to antiviral responses. We observed antiviral roles of IRF1 and

IRF5 that were IFN-independent and cell-type specific. These mechanisms are important in controlling viruses that interfere with the IFN signaling, as cells retain the ability to induce functional but local antiviral states through expression of ISGs.

Keywords: interferon regulatory factor, MEF, therapy, cell culture

Introduction

Hepatitis C virus (HCV) affects an estimated 3% of the world population and is the leading cause of liver-associated morbidity[1, 2]. Evasion or attenuation of host mediated antiviral responses results in chronic infection, liver cirrhosis and hepatocellular carcinoma[3]. The current combination therapy of IFN-α and ribavirin for chronic Hepatitis C has proven only partly successful. Viral clearance depends on the viral genotype, stage of the infection and only 50% of patients achieve the desired sustained antiviral response.

The HCV genome is a 9.6-kilobase uncapped, linear, single-stranded positive-sense RNA with an inherent ability to trigger innate antiviral responses. The molecular patterns of HCV result in the induction of type I IFN through RIG-I signaling[4-6]. IFNs are known to suppress HCV replication *in vitro*, by the induction of antiviral responses through activation of interferon stimulated genes (ISGs). Secreted type I IFNs bind to a heterodimeric IFN receptor (IFNAR). IFNAR activation and the subsequent phosphorylation of STAT1 and STAT2 results in interaction with IRF-9 and to the induction of ISGs that execute the establishment of an antiviral state [7]. One such target, IRF-7, responds to IFN stimulation by phosphorylation and induces IFNαs, leading to an amplification of the IFN response.

Given the critical role of innate immune responses in inhibiting HCV replication, it is not surprising that a number of factors of the IFN circuits (like MAVS, TRIF and STAT1) are actively counteracted[8-11] or manipulated[12] by viral proteins. The existence of these viral mechanisms resulted in the evolution of redundant pathways for detection and inhibition of viral infections[13]. Interferon-independent signaling following peroxisomal activation of MAVS has been shown to induce an immediate albeit transient induction of an innate immune response[14]. In this study, we investigated the impact of IFN-dependent and IFN-

independent actions on restriction of HCV replication. Our data acknowledge the functional role of interferon regulatory factors that interfere with viruses, independent of type I interferons.

Material and Methods

Cell culture

STAT1$^{-/-}$ and PKR$^{-/-}$ mouse embryonic fibroblasts (MEFs) were kindly provided by T. Decker (University of Vienna, Austria) and C. Weissmann (University of Zürich, Switzerland). IRF-1$^{-/-}$, IRF-3$^{-/-}$, IRF-5$^{-/-}$, IRF-7$^{-/-}$, MAVS$^{-/-}$ and IFNAR$^{-/-}$ MEFs were isolated form E13.5 embryos from C57BL/6 knock-out mice. Isolated MEFs were infected with recombinant lentiviruses containing the SV40TAg under the control of the tetracycline promoter to generate conditionally immortalized cells[15].

To obtain mouse primary hepatocytes, 6-8 week-old mice were anesthetized with 0.1ml/10g body mass of ketamine (10%) xylazine (5%). Liver perfusion medium (17701038, Life technologies) supplemented with heparin (500U/ml) was injected into the vena cava followed by liver digest medium (17703-034, Life technologies) supplemented with collagenase H (55mg/150ml, Roche). The digested liver was excised and hepatocytes were released from the liver. Cells were passed through a 100 µm mesh. The cell suspension was centrifuged at 300 rpm for 5 minutes and the pellet washed. Total hepatocyte numbers were seeded in the desired concentration on collagen R-coated (47254.02, Serva) plastic. Cells were cultured in Dulbecco´s Modified Eagle Medium (Invitrogen) supplemented with 2 mM L-glutamine, nonessential amino acids, 100 U/ml of penicillin, 100 µg/ml of streptomycin, and 10% fetal calf serum.

Oligos encompassing the sequence of miR-122 (5´TGGAGTGTGACAATGGTGTTTG3´) were cloned into S-miR-30-IEW vector[16]. The miR-RNA-lentiviral vector encodes for the miR-RNA followed by an IRES element and the gene that encodes for enhanced green fluorescent protein (eGFP). Infected cells were sorted for GFP expression. Type I IFNs

were neutralized by treating cells with 2 µg/ml of anti-IFNα (4EA1) and anti-IFN-β (7FD3) antibodies neutralizing 250U of IFN.

In vitro transcription and electroporation

The generation of *in vitro* transcripts from WT JFH1 and the replication-deficient NS5B-inactive JFH1ΔGDD has been described elsewhere[17-19]. Following electroporation, cells were immediately transferred to 10 ml DMEM and 1 ml of the cell suspension was seeded per well in 12 well plates.

Luciferase reporter assay

Cell lysates were prepared at the indicated time points after electroporation using Passive Lysis Buffer according to manufacturer's instructions (E1941, Promega). Firefly luciferase was determined using a Luciferase Kit (Luciferase 1000 Assay System, Promega). All experiments were performed at least twice with triplicates before calculating mean values and standard deviations.

Western blot analysis and antibodies

For preparation of whole cell extracts, cells were lysed in buffer containing 250 mM Tris, 0.5% Triton X-100, and Halt protease inhibitor cocktail (Thermo Scientific). Western blot analysis was performed according to standard procedures. The following primary antibodies were used: anti-IRF-1 (M-20; SC-640 Santa Cruz), anti-IRF-3 (FL-425; SC-9082 Santa Cruz), anti-IRF-5 (4950 Cell Signaling), anti-IFNAR (127313 Bio Legend), anti-PKR (M515; SC-1702 Santa Cruz), anti-STAT1 (9172 Cell Signaling), anti-NS5A [20], anti-MAVS

(4983 Cell Signaling) and anti-Actin (MAB 1501R Chemicon). HRP–conjugated anti-rabbit and anti-mouse Abs (Amersham) were used as secondary Abs using ECL detection (Bio-Rad). The chemiluminescence signal was recorded digitally by a ChemiDoc DRS imaging system (Bio-Rad). Digital signal acquisition and analysis were performed using Quantity One Program, Version 4.6 (Bio-Rad).

DNA constructs

Murine genes encoding IRF-1, IRF-5 and IRF-7 were cloned under the control of an MPSV/MT7 hybrid promoter. Constitutively active IRF-5(4D)[21] and IRF-7(M15)[22] were generated by Quick Change site directed mutagenesis kit (Stratagene) and verified by sequencing. Expression constructs of the NS3/4AS139A[10] mutant and MAVS[23] were a kind gifts from Z.J. Chen and M. Langereis

IFN-Assay

To determine the amount of secreted IFN, type I and III IFN sensitive epithelial cells isolated from Mx2-Luc reporter mice[24] were treated for 24 hours with supernatants from MEFs transfected with HCV subgenomic replicon RNA. IFN concentrations were obtained by administering serial dilutions of IFN-β. To determine the IFN species, cells were incubated with 2 µg/ml of each antibody neutralizing 250U of IFNα (4EA1) and IFN-β (7FD3).

Indirect Immunoflourescence

Electroporated cells were seeded on cover slips in 24-well plates and cultured at 37°C, 5% CO_2. Cells were fixed 3 days post electroporation with 3% paraformaldehyde (w/v) in PBS for 10 minutes at room temperature. Cells were permeabilized in PBS constituted with 0.5% Triton X-100. Subsequently, cells were washed three times with PBS and incubated with the primary antibody anti-HCV NS3 (F3A6) for 45 minutes at room temperature. Washed cover slips were subjected to staining with secondary goat antibodies specific to IgG conjugated with Cy5 (Dianova) at a dilution of 1:800. Coverslips were mounted on a glass slide over a drop of Fluoroshield with DAPI (F6057, Sigma) and analyzed for fluorescence.

Transfection of primary hepatocytes

Transfection of primary hepatocytes was performed one day post isolation with 3 µg/ml RNA transcribed from subgenomic replicon JFH1 using Lipofectamine-2000 (Invitrogen) according to manufacturer's instructions. Cells were lysed and luciferase signals were measure as described above.

PCR

Genotyping for IRF-$7^{-/-}$ and $IFNAR^{-/-}$ cells were performed using primers Uzu(GTGGTACCACTCCCCTCTTTATAATCT), R813long(AGTAGATCCAAGCTCCCG GCTAAGTTCGTAC) and Neo16c(TCGTGCTTTACGGTATCGCCGCTCCCGATTC) and IFNAR1(CCAAGCGAAACATCGCATCG), IFNAR2(GTTCCCTTCCTCTGCTCTG) and IFNAR3(ACACAAAGACGAGGCGAAGTG) respectively.

Results

The IFN system restricts HCV replication

To determine the role of the type I IFN system in the restriction of HCV replication we compared the replication of subgenomic genotype 2a strain (JFH1) luciferase replicon[25] in fibroblasts knocked-out for factors involved in induction, reaction and amplification of type I IFNs as well as antiviral responses induced by alternative pathways. Replication of JFH1 has been described in mouse cells[26, 27] and HCV replication is enhanced by miR-122 in non-hepatic human and mouse cells[26, 28]. Conditionally immortalized[15] wild type (WT) and gene disrupted MEFs expressing miR-122 (Figure 1*B*) were electroporated with JFH1 and polymerase deficient JFH1ΔGDD RNA (Figure 1*A*). Luciferase expression in WT MEFs transfected with JFH1 or JFH1ΔGDD replicon was indistinguishable up to 5 days post electroporation, suggesting that WT MEFs were unable to maintain detectable HCV replication. Since HCV replication is sensitive to type I IFN, we analyzed cells deficient in factors involved in IFN induction and response. MAVS$^{-/-}$, IRF-3$^{-/-}$, IFNAR$^{-/-}$ and STAT1$^{-/-}$ cells showed clearly elevated luciferase expression after transfection of the WT replicon in comparison to JFH1ΔGDD (Figure 1*C*). As shown before for IRF-3 [26], these results indicate an initial production and action of type I IFN to repress HCV replication. PKR$^{-/-}$ cells showed low levels of JFH1 replication in comparison to IRF-3$^{-/-}$ and IFNAR$^{-/-}$ cells, indicating an involvement but not pivotal role of PKR in limiting HCV replication. Similar to WT cells in IRF-7$^{-/-}$ cells replication was undetectable. Thus, the amplification of the IFN response has little or no impact on HCV restriction in these cells. IRF-1[13, 14] and IRF-5[29, 30] are known to stimulate ISG expression independent of IFN signals. Cells deficient in IRF-1

or IRF-5 showed a rapid decline of luciferase expression indicating that deletion of these factors was not sufficient to rescue sufficient HCV replication.

Type I IFN signaling inhibits JFH1 replication in mouse embryonic fibroblasts

To validate JFH1 replication in MEFs, cells transfected with JFH1 RNA were analyzed for HCV NS5A protein expression. As a positive control we used the highly permissive Huh7.5 cell line. Expression of NS5A was detectable by Western blotting upon electroporation of JFH1 in IFNAR$^{-/-}$ cells, whereas no expression was detectable upon JFH1ΔGDD electroporation (Figure 2*A*). However, expression level of NS5A was significant lower in MEFs in comparison to Huh7.5 cells, indicating a lower permissiveness of MEFs for HCV replication. Furthermore, three days post electroporation IFNAR$^{-/-}$ cells transfected with JFH1 exhibited cytoplasmic distribution of NS3 whereas; no NS3 was detectable in the JFH1 transfected WT or JFH1ΔGDD transfected WT or IFNAR MEFs (Figure 2*B*). These results demonstrate that HCV RNA is able to replicate in MEFs and that the replication efficiency is enhanced by deletion of the type I IFN pathway.

Type I IFN induction in MEFs by HCV replication

To determine if HCV replication in MEFs induces IFN, we tested the supernatant of transfected cells on type I and III IFN sensitive reporter cells[24] expressing luciferase under the control of the interferon-regulated mouse Mx2 promoter[31]. Luciferase values from IFN-reporter cells incubated with supernatants from MEFs transfected with HCV RNA were determined and normalized to WT MEFs electroporated without RNA (mock) (Figure 3). Replication incompetent JFH1ΔGDD RNA induces low amounts of type I IFNs in WT MEFs

indicating replication-independent triggering of the RIG-I signaling. A comparison of IFN production from JFH1 and JFH1ΔGDD replicons shows that active replication increased IFN production. Although WT MEFs did not support detectable JFH1 replication (Figure 1*C*), these cells produced higher amounts of IFN compared to IFNAR$^{-/-}$ and IRF-7$^{-/-}$ cells (Figure 3). These data suggest that replication must occur at the very early stages after transfection in WT cells, but the induced IFN response was sufficient to inhibit replication. Lower IFN expression in IRF-7$^{-/-}$ and IFNAR$^{-/-}$ cells could be explained by the missing amplification loop or by reduced expression of factors involved in IFN induction. Since treatment with type I IFN neutralizing antibodies abolished luciferase expression in the IFN-reporter cell line, we can rule out a significant contribution of type III IFN. Therefore, our results suggest that HCV RNA triggers an antiviral state in fibroblasts characterized by secretion of type I IFN and control of RNA replication.

HCV cleaves murine MAVS and inhibits IFN induction

Several pathogenic viruses including HCV have evolved strategies to inhibit early signaling events that lead to IFN production. NS3-4A, a multifunctional protein of HCV has been shown to block the activation of IRF-3 by cleaving the adaptor protein MAVS[11]. Additionally, cleavage of murine MAVS overexpressed in human cells has been reported[32]. To examine if the type I IFN secreted by MEFs is due to an inability of MAVS cleavage we electroporated the JFH1 replicon in MEFs and determined MAVS cleavage. MAVS cleavage was detectable 48 hours after electroporation of JFH1 RNA, whereas no cleavage was observed after electroporation of JFH1ΔGDD. In addition, expression of

NS3-4A led to cleavage of endogenous MAVS, (Figure 4*A*), indicating that murine endogenous MAVS is cleavable by NS3-4A protein of HCV.

In order to investigate the impact of NS3-4A and JFH1 replicon on IFN induction, we infected cells with NDV and determined secreted IFN in the supernatant of the cells (Figure 4*B/C*). Both NS3-4A and JFH1 replicon transfection lowered the amount of IFN induced by NDV, whereas no inhibition was detectable upon expression of the protease defective mutant NS3-4AS129A. These data indicate that the HCV induced NS3-4A protease partially repressed secretion of type I IFN from MEFs.

Type I IFN independent antiviral responses by IRF-1, IRF-5 and PKR*.*

In spite of successful prevention of IFN induction by several viruses, induction of ISGs is still detectable[33]. Additionally, IRF-1 and IRF-3 dependent early induction of antiviral gene expression and control of viral infection, independent of type I IFN production, has been described [13, 14, 34]. To determine restriction factors of HCV replication that operate independent of type I IFN, we neutralized type I IFN in the supernatant of cells (Figure 5). Notably, depletion of secreted type I IFNs was not sufficient to increase HCV replication in WT MEFs suggesting the presence of additional factor(s) that restrict HCV replication in these cells. In contrast, enhanced HCV replication was observed upon type I IFN neutralization in cells generated from IRF-5, IRF-7, IRF-1 and PKR knock-out mice. These results argue, that lesion of these genes weakens such IFN-independent defense mechanisms to the extent that HCV replication is detectable upon neutralization of secreted IFN. We conclude that in WT MEFs, IFN-dependent and -independent mechanisms contribute towards the control of HCV replication, and that IFN-independent

defenses are mediated through IRF-5, IRF-7, IRF-1 and PKR. Interestingly, no further increase of HCV replication was detectable in IRF-3$^{-/-}$, IFNAR$^{-/-}$ and STAT-1$^{-/-}$ MEFs in the presence of type I IFN depleting antibodies. From this we conclude that the extent of HCV replication cannot be further increased because depletion of type I IFN response affects the same pathway. Taken together, these data show that apart from the effects mediated by the type I IFN response, IFN-independent antiviral effects are involved in restricting HCV replication. The underlying mechanisms are potential targets for HCV treatment.

IRFs mediate IFN-independent antiviral responses

The replication of HCV in IRF-1, IRF-5 and IRF-7 knock-out MEFs in the absence of type I IFN response prompted us to examine their anti-HCV activity. The different knock-out cells were reconstituted with cDNAs of IRF-1 and constitutively active IRF-5(4D) and IRF-7(M15). HCV replication was determined in the presence of neutralizing type I IFN antibodies (Figure 6). Expression of IRF-1, IRF-5 and IRF-7 were able to significantly reduce HCV replication in the presence of type I IFN depleting antibodies. These data suggest that IRF-1, IRF-5 and IRF-7 mediated signaling can directly suppress HCV replication, independent of the type I IFN-mediated antiviral response.

MAVS and IRF-1 are crucial restriction factors of HCV replication in hepatocytes

Since HCV is a hepatotropic virus, we decided to test if the IFN-independent responses observed in MEFs were equally active in hepatocytes. To this end, we transfected primary hepatocytes with JFH1 RNA in the absence or presence of type I IFN neutralizing antibodies and determined replication (Figure 7*A*). Replication was detectable in primary

hepatocytes with lesions in MAVS and IRF-1 whereas no replication was observed in the WT and IFNAR$^{-/-}$, IRF-3$^{-/-}$, and IRF-5$^{-/-}$ hepatocytes. Luciferase levels were lower in primary hepatocytes in comparison to fibroblast. To determine the replication efficiency in primary hepatocytes we determined NS5A expression in hepatocytes isolated from MAVS$^{-/-}$ mice (Figure 7B). NS5A expression was detectable in amounts similar to IFNAR$^{-/-}$ MEFs, indicating a low but detectable replication of JFH1 in primary hepatocytes. To determine the impact of MAVS and IRF-1 on HCV replication we reconstituted the cells with cDNA of MAVS and IRF-1 (Figure 7C). Expression of MAVS and IRF-1 abolished HCV replication. These data indicate that adaptor protein MAVS and IRF-1 are critical in determining HCV clearance in hepatocytes in a cell type specific antiviral action.

DISCUSSION

HCV infects approximately 3% of the global population. The chronic sequelae of HCV range from liver fibrosis and cirrhosis to hepatocellular carcinoma. Although the combination therapy of IFN-α and Ribavirin is largely successful, not all patients achieve the desired antiviral response. Variations in treatment outcome have been reported both within and across viral genotypes[35, 36] indicating the role of both viral and host genetics in determining viral clearance. In this study, we reveal IRF-1, IRF-5 and IRF-7 as mediators of IFN-independent antiviral responses which play an important role in cellular restriction of HCV replication in a cell type specific manner.

Factors that are involved in IFN induction and execution can possibly have IFN-independent antiviral action if their activity is directly induced by the virus[37]. This was examined in this study for HCV replication. We used conditionally immortalized MEFs and primary hepatocytes from mice with lesions in genes involved in host innate immunity and IFN signaling, as a powerful system to define the roles of cellular genes in the control of HCV replication.

IRF-3$^{-/-}$ MEFs were shown to be permissive to HCV replication[26] and defective IRF-3 activation in Huh-7.5 cells[38, 39] allows increased permissivity to HCV replication. This indicates that IRF-3 is a restriction factor for HCV replication. Apart from its role in the virus mediated induction of type I IFNs, IRF-3 regulates antiviral responses by directly inducing a subset of antiviral ISGs[40]. Thus, IRF-3 could contribute to HCV permissivity through the IFN system as well as to an IFN-independent mechanism, by directly inducing ISGs. Our data shows that HCV replication in IFNAR$^{-/-}$ cells, which are resistant to the type I IFN response, is comparable to that in IRF-3$^{-/-}$ cells (Figure 1). Although IFNAR knock-out cells

cannot react to type I IFN response, the IRF-3 mediated induction of ISGs is still intact. This leads to the conclusion, that IFN-independent induction of ISGs by IRF-3 plays, if at all, a minor role in restricting HCV replication. STAT1 is a key signaling molecule downstream of the IFN receptor but could be activated through direct viral actions. However, STAT1$^{-/-}$ cells showed replication capacity comparable to IFNAR$^{-/-}$ cells, and thus significant contribution to and IFN-independent antiviral response has not to be assumed. In addition, the absence of IRF-7 does not permit HCV replication indicating that the amplification of type I IFN response by IRF-7 is not important for controlling HCV replication.

PKR is an ISG and a direct role of PKR in the suppression of HCV RNA replication has been shown by over expression studies[41-43]. PKR$^{-/-}$ cells showed HCV replication but our data indicated that the extent of HCV replication is lower in comparison to that of IRF-3$^{-/-}$ and IFNAR$^{-/-}$ MEFs. The initial HCV replication followed by a subsequent decrease (Figure 5) could be explained by a mechanism wherein HCV uses PKR to inhibit the IFN pathway at the translational level[44].

Previous studies have shown antiviral responses of IRF-1 and IRF-5[45, 46]. Over expression of IRF-1 results in the induction of IFN and the ensuing IFN-mediated antiviral responses inhibit HCV replication[47, 48]. However, infection studies in IRF-1$^{-/-}$ mice indicated that endogenous IRF-1 is not involved in IFN induction by virus infection[49]. Our results showed that HCV replication is prevented in IRF-1$^{-/-}$ fibroblasts (Figure 1), indicating no impact in antiviral response in the presence of an intact IFN response. However, while IRF-1 is not required for the IFN response, it can induce ISGs during VSV infections in the absence of type I IFNs[13]. This was confirmed in the present study in fibroblasts where IRF-1 controls

IFN-independent signaling events and antiviral immunity[13]. Dixit and colleagues described a mechanism by which IRF-1 induces an antiviral response by regulating MAVS-dependent signaling from peroxisomes in the absence of type I IFN[14]. To test this, we performed HCV replication assays in MEFs in the presence of IFN-neutralizing antibodies. Surprisingly, inhibition of type I IFNs had no impact on HCV replication in WT MEFs, indicating that block of IFN response alone is not sufficient to facilitate HCV replication (Figure 5). These findings seem to be in contrast to IFNAR$^{-/-}$ MEFs, where HCV replication is detectable (Figure 1). This discrepancy is explained by the lower endogenous levels of ISGs and IRFs in IFNAR$^{-/-}$ cells as shown earlier[37]. Neutralizing type I IFN results in HCV replication in IRF-1, IRF-5 and IRF-7 deficient MEFs (Figure 5) suggesting IRF mediated induction of ISGs as a possible IFN-independent antiviral response.

To analyze the contribution of non-IFN restriction in a HCV relevant system we used primary mouse hepatocytes. While MAVS knock-out cells were permissive to HCV replication, no detectable replication was observed in IFNAR$^{-/-}$ hepatocytes (Figure 7). Interestingly, permissivity of immortalized MAVS$^{-/-}$ and IFNAR$^{-/-}$ hepatocytes has been reported[50], but if immortalized cells reproduce a physiological response is debatable because expression of oncogenes used for immortalization could influence signaling pathways such as those involved in IFN response[51]. These data reveal non-IFN mediated restriction of HCV by IRF-1 in hepatocytes although replication is low, while the observed function of IRF-5 and IRF-7 is not relevant or extremely low in hepatocytes. Novel data showed that IRF-5 is sufficient to induce IFN-β production in response to viral infection, but does not induce ISGs directly[52]. Therefore, a redundant function of IRF-5 in hepatocytes or a mechanism, by which HCV specifically inhibits IRF-mediated antiviral response, is not

excluded. Because HCV replication is detectable in primary hepatocytes isolated from IRF-$1^{-/-}$ mice, a pivotal role of IRF-1 in restriction of HCV replication in MEFs and hepatocytes is likely.

Our findings indicate that IFN-independent antiviral pathways might be involved in direct antiviral responses against HCV. We speculate that ISGs are executing the antiviral function of the IRFs, since such activities have been reported earlier and could be explained by their direct binding to the respective promoters. In contrast to type I IFN, IRFs are not secreted from the cells indicating that an IRF-mediated antiviral response is intracellular and therefore limited to infected cells. Nevertheless, IRF-mediated IFN-independent antiviral response could serve as a “fail-safe” mechanism, in the event that the virus circumvents the IFN response. The importance of IRF-1 in antiviral response against HCV is underscored by the finding that chronically infected patients with an increased induction of IRF-1 show better outcome and responsiveness to treatment[53]. This suggests that stimulation of IRF-1 could be a new target to improve therapies to restrict HCV replication in acute and chronic hepatitis C.

Figure legends:

Figure 1.The IFN system restricts HCV replication

Schematic representation of (A) HCV subgenomic replicon luciferase reporter (JFH1) and (B) the lentiviral plasmid harboring miR-122 expression cassette. A. The subgenomic replicon consists of the non translated regions (NTR), the N-terminal 12 amino-acid coding sequence of core fused in frame with the firefly luciferase gene (F-Luc), the EMCV-IRES (EI), the non structural proteins NS3-NS5B. B. Specific miR-122 sequences are embedded within sequences derived from miR-30 and driven by the spleen focus formation virus (SFFV) promoter. In addition, the construct harbor a green fluorescent protein (GFP) gene associated with miR-122 expression by an EMCV-IRES (EI) and the woodchuck hepatitis post-transcriptional regulatory element (WPRE). C. Transient replication assay using RNA from JFH1 (filled diamond) or JFH1ΔGDD (open circle). MEFs stably expressing miR-122 were electroporated with RNA of JFH1 or JHF1ΔGDD, harvested at the indicated time points (hours post electroporation (Hpe)), and assayed for luciferase activity indicative of replication. Values measured at 4 hours post electroporation were used as read-out for transfection efficiency. Data represent the mean value of three independent experiments, with error bars showing SD.

Figure 2. HCV protein detection in knock-out MEFs

A. Detection of NS5A expression from JFH1 and JFH1ΔGDD replication in Huh7.5 cells and $IFNAR^{-/-}$ MEFs by Western blotting 48 hours post electroporation. B. Immunoflourescence detection of NS3 expression. The indicated MEFs stably expressing miR-122 (GFP^{+}) were electroporated with JFH1 or JFHΔGDD RNA. Fixed cells were

stained with a primary antibody against NS3, followed by a secondary anti-mouse Cy5 (red) conjugated antibody. Coverslips were mounted on Fluoroshield containing DAPI (cyan).

Figure 3. HCV RNA induces type I IFN

WT, IFNAR$^{-/-}$ and IRF-7$^{-/-}$ cells were electroporated with RNA for JFH1 and JFH1ΔGDD or without RNA (mock). IFN were detected in supernatants harvested 0, 4, 24 and 48 hours post electroporation by Mx2luc reporter cells stimulated with the supernatants of electroporated cells for 24h. To determine the IFN species, cells were incubated with 2 µg/ml IFNα (4EA1) and IFN-β (7FD3) antibodies neutralizing 250 U of type I IFNs. Data represent the mean value of three independent experiments, with error bars showing SD.

Figure 4. Cleavage of MAVS and inhibition of IFN response in mouse cells by NS3/4A and JFH1

MEFs were transfected with NS3-4A or NS3-4AS129A expression plasmids or RNA for JFH1 and JFH1ΔGDD. A. MAVS cleavage was detected by Western blotting 48 hours post transfection. B/C. Upon transfection, cells were infected with NDV. Type I IFN levels in the supernatants were tested using Mx2luc reporter cells. Data represent the mean value of two independent experiments, with error bars showing SD.

Figure 5. Type I IFN independent antiviral response by IRF-1, IRF-5, IRF-7 and PKR

Indicated MEFs expressing miR-122 were electroporated with RNA from JFH1 in the presence (open circle) or absence (filled diamond) of neutralizing antibodies directed

against IFNα and IFNβ. Values measured at 4 hours post electroporation were used as read-out for transfection efficiency. Data represent the mean value of three independent experiments. Error bars indicate SD.

Figure 6. Reconstitution of IRF-1, IRF-5 and IRF-7 inhibits HCV replication independent of type I IFNs

Indicated MEFs were electroporated with RNA from JFH1 and DNA from IRF-1 or the constitutively active IRF-5(4D) and IRF-7(M15) in the presence or absence of neutralizing antibodies directed against IFNα and IFNβ. Cells were harvested and luciferase values were measured.

Figure 7. MAVS and IRF-1 are crucial in limiting JFH1 replication in primary hepatocytes

A. Indicated primary hepatocytes were transfected with RNA from JFH1 in the absence (filled diamond) or presence (filled circle) of antibodies neutralizing type I IFN, harvested at the indicated time points (hours post electroporation (Hpe)), and assayed for luciferase activity. B. IFNAR$^{-/-}$ MEFs and MAVS$^{-/-}$ primary hepatocytes were transfected with JFH1 or JFH1ΔGDD RNA. NS5A expression was determined by Western blot 48 hours post transfection. C. MAVS$^{-/-}$ and IRF-1$^{-/-}$ primary hepatocytes were transfected with RNA from JFH1 and MAVS or IRF-1 expression plasmids in the presence of antibodies neutralizing type I IFN. Cells were harvested and assayed for luciferase activity. Values measured at 4 hours post electroporation were used as read-out for transfection efficiency. Data

represent the mean value of two independent experiments with transfections performed in triplicates. Error bars indicate SD.

References

1. Global surveillance and control of hepatitis C. Report of a WHO Consultation organized in collaboration with the Viral Hepatitis Prevention Board, Antwerp, Belgium. J Viral Hepat 1999;6:35-47.
2. Vogel W. Treatment of acute hepatitis C virus infection. J Hepatol 1999;31 Suppl 1:189-92.
3. He XS. Regulation of Adaptive Immunity by HCV. In: Tan SL, ed. Hepatitis C Viruses: Genomes and Molecular Biology. Norfolk (UK), 2006.
4. Binder M, Kochs G, Bartenschlager R, et al. Hepatitis C virus escape from the interferon regulatory factor 3 pathway by a passive and active evasion strategy. Hepatology 2007;46:1365-74.
5. Saito T, Owen DM, Jiang F, et al. Innate immunity induced by composition-dependent RIG-I recognition of hepatitis C virus RNA. Nature 2008;454:523-7.
6. Sumpter R, Jr., Loo YM, Foy E, et al. Regulating intracellular antiviral defense and permissiveness to hepatitis C virus RNA replication through a cellular RNA helicase, RIG-I. J Virol 2005;79:2689-99.
7. Honda K, Takaoka A, Taniguchi T. Type I interferon [corrected] gene induction by the interferon regulatory factor family of transcription factors. Immunity 2006;25:349-60.
8. Heim MH, Moradpour D, Blum HE. Expression of hepatitis C virus proteins inhibits signal transduction through the Jak-STAT pathway. J Virol 1999;73:8469-75.

9. Li K, Foy E, Ferreon JC, et al. 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 2005;102:2992-7.

10. **Li XD, Sun L**, Seth RB, et al. Hepatitis C virus protease NS3/4A cleaves mitochondrial antiviral signaling protein off the mitochondria to evade innate immunity. Proc Natl Acad Sci U S A 2005;102:17717-22.

11. Meylan E, Curran J, Hofmann K, et al. Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. Nature 2005;437:1167-72.

12. Garaigorta U, Chisari FV. Hepatitis C virus blocks interferon effector function by inducing protein kinase R phosphorylation. Cell Host Microbe 2009;6:513-22.

13. Stirnweiss A, Ksienzyk A, Klages K, et al. IFN regulatory factor-1 bypasses IFN-mediated antiviral effects through viperin gene induction. J Immunol 2010;184:5179-85.

14. Dixit E, Boulant S, Zhang Y, et al. Peroxisomes are signaling platforms for antiviral innate immunity. Cell 2010;141:668-81.

15. May T, Hauser H, Wirth D. Transcriptional control of SV40 T-antigen expression allows a complete reversion of immortalization. Nucleic Acids Res 2004;32:5529-38.

16. Surdziel E, Cabanski M, Dallmann I, et al. Enforced expression of miR-125b affects myelopoiesis by targeting multiple signaling pathways. Blood 2011;117:4338-48.

17. **Steinmann E, Brohm C**, Kallis S, et al. Efficient trans-encapsidation of hepatitis C virus RNAs into infectious virus-like particles. J Virol 2008;82:7034-46.

18. Haid S, Windisch MP, Bartenschlager R, et al. Mouse-specific residues of claudin-1 limit hepatitis C virus genotype 2a infection in a human hepatocyte cell line. J Virol 2010;84:964-75.

19. van den Hoff MJ, Moorman AF, Lamers WH. Electroporation in 'intracellular' buffer increases cell survival. Nucleic Acids Res 1992;20:2902.

20. Lindenbach BD, Evans MJ, Syder AJ, et al. Complete replication of hepatitis C virus in cell culture. Science 2005;309:623-6.

21. Cheng TF, Brzostek S, Ando O, et al. Differential activation of IFN regulatory factor (IRF)-3 and IRF-5 transcription factors during viral infection. J Immunol 2006;176:7462-70.

22. Caillaud A, Hovanessian AG, Levy DE, et al. Regulatory serine residues mediate phosphorylation-dependent and phosphorylation-independent activation of interferon regulatory factor 7. J Biol Chem 2005;280:17671-7.

23. Langereis MA, Feng Q, van Kuppeveld FJ. MDA5 localizes to stress granules, but this localization is not required for the induction of type I interferon. J Virol 2013;87:6314-25.

24. Schwerk J, Köster M, Hauser H, et al. Generation of Mouse Small Intestinal Eithelial Cell Lines that Allow the Analysis of Specific Innate Immune Functions. PLoS One 2013, 8: e72700

25. Kato T, Date T, Miyamoto M, et al. Efficient replication of the genotype 2a hepatitis C virus subgenomic replicon. Gastroenterology 2003;125:1808-17.

26. Lin LT, Noyce RS, Pham TN, et al. Replication of subgenomic hepatitis C virus replicons in mouse fibroblasts is facilitated by deletion of interferon regulatory factor 3 and expression of liver-specific microRNA 122. J Virol 2010;84:9170-80.

27. Long G, Hiet MS, Windisch MP, et al. Mouse hepatic cells support assembly of infectious hepatitis C virus particles. Gastroenterology 2011;141:1057-66.

28. **Chang J, Guo JT**, Jiang D, et al. Liver-specific microRNA miR-122 enhances the replication of hepatitis C virus in nonhepatic cells. J Virol 2008;82:8215-23.

29. **Barnes BJ, Richards J,** Mancl M, et al. Global and distinct targets of IRF-5 and IRF-7 during innate response to viral infection. J Biol Chem 2004;279:45194-207.

30. Yanai H, Chen HM, Inuzuka T, et al. Role of IFN regulatory factor 5 transcription factor in antiviral immunity and tumor suppression. Proc Natl Acad Sci U S A 2007;104:3402-7.

31. Pulverer JE, Rand U, Lienenklaus S, et al. Temporal and spatial resolution of type I and III interferon responses in vivo. J Virol 2010;84:8626-38.

32. Ahlen G, Derk E, Weiland M, et al. Cleavage of the IPS-1/Cardif/MAVS/VISA does not inhibit T cell-mediated elimination of hepatitis C virus non-structural 3/4A-expressing hepatocytes. Gut 2009;58:560-9.

33. Stojdl DF, Lichty BD, tenOever BR, et al. VSV strains with defects in their ability to shutdown innate immunity are potent systemic anti-cancer agents. Cancer Cell 2003;4:263-75.

34. Noyce RS, Taylor K, Ciechonska M, et al. Membrane perturbation elicits an IRF3-dependent, interferon-independent antiviral response. J Virol 2011;85:10926-31.

35. Hadziyannis SJ, Sette H, Jr., Morgan TR, et al. Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. Ann Intern Med 2004;140:346-55.

36. Manns MP, McHutchison JG, Gordon SC, et al. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. Lancet 2001;358:958-65.

37. Paladino P, Cummings DT, Noyce RS, et al. The IFN-independent response to virus particle entry provides a first line of antiviral defense that is independent of TLRs and retinoic acid-inducible gene I. J Immunol 2006;177:8008-16.

38. Foy E, Li K, Sumpter R, Jr., et al. Control of antiviral defenses through hepatitis C virus disruption of retinoic acid-inducible gene-I signaling. Proc Natl Acad Sci U S A 2005;102:2986-91.

39. Yamashiro T, Sakamoto N, Kurosaki M, et al. Negative regulation of intracellular hepatitis C virus replication by interferon regulatory factor 3. J Gastroenterol 2006;41:750-7.

40. Sato M, Suemori H, Hata N, et al. Distinct and essential roles of transcription factors IRF-3 and IRF-7 in response to viruses for IFN-alpha/beta gene induction. Immunity 2000;13:539-48.

41. Chang KS, Cai Z, Zhang C, et al. Replication of hepatitis C virus (HCV) RNA in mouse embryonic fibroblasts: protein kinase R (PKR)-dependent and PKR-independent mechanisms for controlling HCV RNA replication and mediating interferon activities. J Virol 2006;80:7364-74.

42. Rivas-Estilla AM, Svitkin Y, Lopez Lastra M, et al. PKR-dependent mechanisms of gene expression from a subgenomic hepatitis C virus clone. J Virol 2002;76:10637-53.

43. Wang C, Pflugheber J, Sumpter R, Jr., et al. Alpha interferon induces distinct translational control programs to suppress hepatitis C virus RNA replication. J Virol 2003;77:3898-912.

44. Arnaud N, Dabo S, Akazawa D, et al. Hepatitis C virus reveals a novel early control in acute immune response. PLoS Pathog 2011;7:e1002289.

45. Mamane Y, Heylbroeck C, Genin P, et al. Interferon regulatory factors: the next generation. Gene 1999;237:1-14.

46. Barnes B, Lubyova B, Pitha PM. On the role of IRF in host defense. J Interferon Cytokine Res 2002;22:59-71.

47. Pflugheber J, Fredericksen B, Sumpter R, Jr., et al. Regulation of PKR and IRF-1 during hepatitis C virus RNA replication. Proc Natl Acad Sci U S A 2002;99:4650-5.

48. Schoggins JW, Wilson SJ, Panis M, et al. A diverse range of gene products are effectors of the type I interferon antiviral response. Nature 2011;472:481-5.

49. Kimura T, Nakayama K, Penninger J, et al. Involvement of the IRF-1 transcription factor in antiviral responses to interferons. Science 1994;264:1921-4.

50. Aly HH, Oshiumi H, Shime H, et al. Development of mouse hepatocyte lines permissive for hepatitis C virus (HCV). PLoS One 2011;6:e21284.

51. Schlee M, Holzel M, Bernard S, et al. C-myc activation impairs the NF-kappaB and the interferon response: implications for the pathogenesis of Burkitt's lymphoma. Int J Cancer 2007;120:1387-95.

52. Lazear HM, Lancaster A, Wilkins C, et al. IRF-3, IRF-5, and IRF-7 coordinately regulate the type I IFN response in myeloid dendritic cells downstream of MAVS signaling. PLoS Pathog 2013;9:e1003118.

53. Wietzke-Braun P, Maouzi AB, Manhardt LB, et al. Interferon regulatory factor-1 promoter polymorphism and the outcome of hepatitis C virus infection. Eur J Gastroenterol Hepatol 2006;18:991-7.