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Viral Hepatiti

Control of temporal activation of hepatitis C virus-induced interferon response by domain 2 of nonstructural protein 5A

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Background & Aims: Hepatitis C virus (HCV) nonstructural protein 5A (NS5A) is a multifunctional protein playing a crucial role in diverse steps of the viral replication cycle and perturbing multiple host cell pathways. We showed previously that removal of a region in domain 2 (D2) of NS5A (mutant NS5A$^{D2\Delta}$) is dispensable for viral replication in hepatoma cell lines. By using a mouse model and immune-competent cell systems, we studied the role of D2 in controlling the innate immune response.

Methods: *In vivo* replication competence of NS5A$^{D2\Delta}$ was studied in transgenic mice with human liver xenografts. Results were validated using primary human hepatocytes (PHHs) and mechanistic analyses were conducted in engineered Huh7 hepatoma cells with reconstituted innate signaling pathways.

Results: Although the deletion in NS5A removed most of the interferon (IFN) sensitivity determining-region, mutant NS5A$^{D2\Delta}$ was as sensitive as the wild type to IFN-α and IFN-λ *in vitro*, but severely attenuated *in vivo*. This attenuation could be recapitulated in PHHs and was linked to higher activation of the IFN response, concomitant with reduced viral replication and virus production. Importantly, immune-reconstituted Huh7-derived cell lines revealed a sequential activation of the IFN-response *via* RIG-I (retinoic acid-inducible gene I) and MDA5 (Myeloma differentiation associated factor 5), respectively, that was significantly higher in the case of the mutant lacking most of NS5A D2.

Conclusions: Our study reveals an important role of NS5A D2 for suppression of the IFN response that is activated by HCV *via* RIG-I and MDA5 in a sequential manner.

Keywords: NS5A; HCV; Subversion of interferon response; HCV-mediated MDA5 activation.

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Abbreviations: HCV, hepatitis C virus; RIG-I, retinoic acid-inducible gene 1; TLR-3, Toll-like receptor 3; RLR, RIG-I like receptor; MDA5, Melanoma Differentiation-Associated protein 5; IFN, interferon; IRF-3, IFN regulatory factor-3; ISG, IFN-induced gene; MAVS, mitochondrial antiviral signaling protein; NS, nonstructural protein; LCS, low-complexity sequence; D2, Domain 2; PHHs, primary human hepatocytes; Aa, amino acid residue; ISDR, IFN-sensitivity determining region; $TCID_{50}$, tissue culture infection dose 50%; h-alb, human albumin; RI, replacement index; MOI, multiplicity of infection; SeV, Sendai virus; 2′-CMC, 2′-C-Methylcytidine; PIV5, Parainfluenza virus 5; CARD, caspase activation and recruitment domain; NF-κB, nuclear factor 'kappa-light-chain-enhancer' of activated B-cells; PKR, protein kinase R; TRAF2, tumor necrosis factor receptor-associated factor 2; AH, amphipathic α-helix; DAA, direct acting antivirals; Mengo Zn, Mengovirus Zn mutant.

Introduction

HCV is a positive strand RNA virus affecting ~3% of the world population. HCV is a major causative agent of liver diseases including hepatosteatosis, fibrosis, liver cirrhosis and hepatocellular carcinoma. Chronicity of infection predominates as only ~20% of infected individuals clear the virus spontaneously. This low number illustrates efficient virus escape from the immune response, but the underlying molecular mechanisms are only partially understood.

Innate immunity is the first line of host defense against pathogen infection. Viral RNA can be recognized by pathogen recognition receptors such as Toll-like receptor 3 (TLR3) and RIG-I (retinoic acid-inducible gene I)-like receptors (RLRs), such as RIG-I or MDA5 (Myeloma differentiation associated factor 5). These RLRs induce the activation of the interferon (IFN) regulatory factor-3 (IRF-3) resulting in transcription of type I and type III IFN as well as a subset of IFN-stimulated genes (ISGs) such as ISG56 [1,2]. Both RIG-I and MDA5 have different, but redundant roles in pathogen recognition [3]. Upon RNA binding, they recruit the adaptor protein MAVS (mitochondrial antiviral signaling protein) [4], inducing phosphorylation and nuclear

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translocation of IRF-3. This activation is disrupted by the viral protease activity in nonstructural protein 3 (NS3) cleaving MAVS and abrogating IRF3-dependent induction of the IFN response [5].

In addition to NS3, NS5A has been implicated in counteracting antiviral immune responses [6]. NS5A is composed of an amino-terminal amphipathic α-helix responsible for membrane association and three domains that are separated by low-complexity sequences (LCSs). Domain 1 (D1) is highly structured and responsible for dimerization of NS5A. D3 is essential for assembly of infectious HCV particles [7]. However, the role of NS5A D2 for the viral life cycle is less well understood.

We reported earlier that removing most of NS5A D2 has no effect on RNA replication or virus production in Huh7-derived cells, which are most permissive for HCV [8]. By using a mouse-based *in vivo* model, primary human hepatocytes (PHHs) and engineered cell lines, we show here that NS5A D2 attenuates activation of type I and type III IFN response that is induced by HCV *via* RIG-I and MDA5 in a sequential manner.

Material and methods

Plasmids and cells

The following plasmids have been described elsewhere: pFK Jc1-5A$^{D2\Delta}$ (earlier named pFK Jc1Δ2222-2280 [8]); pFK-J6/C3 (Jc1) [9]; pFK JcR2a [10]. Huh7.5 cells were a kind gift of Dr. C.M. Rice, Rockefeller University, New York.

Primary human hepatocytes

PHHs were purchased from Biopredic (Rennes, France). Infection was performed as described elsewhere [11].

Humanized mice

Livers of two- to four-weeks old male and female PXB-mice (uPA$^{+/+}$/SCID) were engrafted by injection of PHHs into the spleen. Mice with a blood concentration of human albumin (h-alb) >6.0 mg/ml were selected for HCV infection. Each mouse was inoculated with ~10^6 $TCID_{50}$ of Jc1 or Jc1-5A$^{D2\Delta}$ (~10^8 HCV RNA copies per mouse). At different time points, blood was collected to quantify h-alb and HCV RNA.

For further details related to Material and methods see Supplementary materials.

Results

Removal of most of NS5A D2 does not affect HCV replication and IFN sensitivity in Huh7 cells

Although NS5A, most notably D2 containing the IFN-sensitivity determining region (ISDR), has been implicated in resistance to the antiviral activity of IFN, the underlying mechanism is poorly defined [12]. While complete removal of D2 abrogates HCV replication [13], consistent with an earlier report [8] we found that a deletion spanning amino acid (aa) residues 2222 to 2280 of the genotype 2a HCV isolate JFH1 does not affect RNA replication as determined with an intragenotypic chimeric *Renilla* luciferase reporter virus [10] (Fig. 1A and B). Moreover, this mutant (here designated 5A$^{D2\Delta}$) produced amounts of infectious intra- and extracellular virus particles comparable to wild type (Fig. 1C and D). Although at early time points virus titers were slightly higher in case of the mutant, the difference to wild type

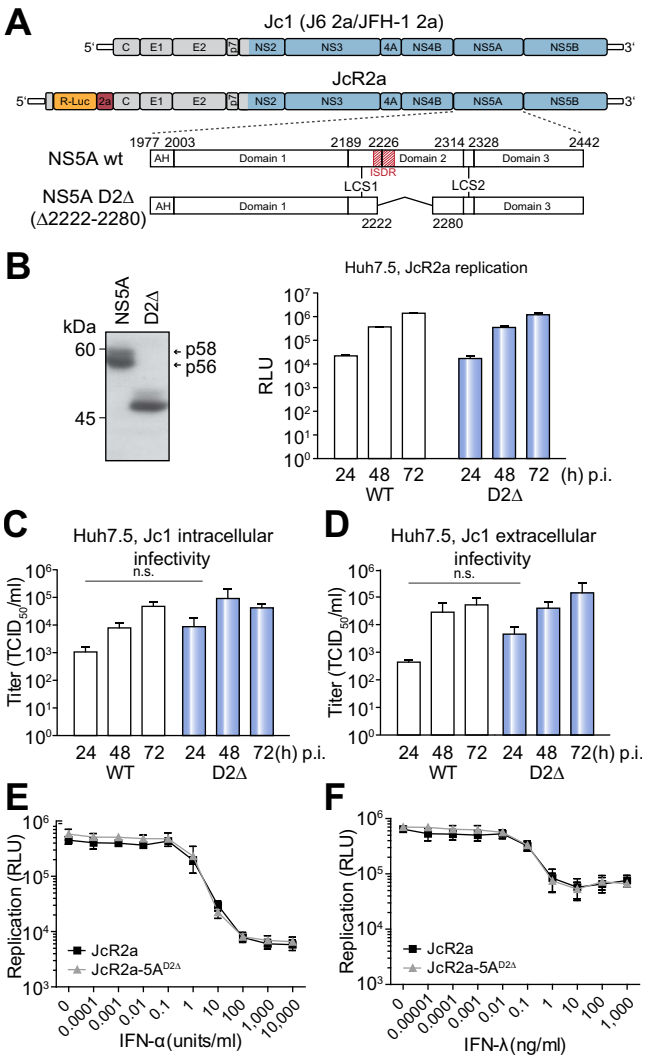

Fig. 1. Replication fitness and IFN sensitivity of NS5A mutant. (A) HCV genomes used in this study (numbers refer to aa positions of JFH-1). (B) Replication of JcR2a or the JcR2a-5A$^{D2\Delta}$ deletion mutant; means of triplicate experiments ± SD. Western blot analysis of NS5A phospho-variants expressed in infected cells see left panel. (C, D) Virus titers in cells and culture supernatants (means of triplicate experiments ± SD); n.s., no statistical significance. (E, F) No effect of D2 deletion on IFN sensitivity. Huh7.5 cells were infected and treated with IFN-α (E) or IFN-λ (F). Shown are means of triplicate measurements ± SD of a representative experiment (n = 2).

was not statistically significant. Importantly, this lack of phenotype was not due to the use of the highly permissive cell clone Huh7.5, because the same results were obtained with high-passage Huh7 cells (Supplementary Fig. 1). These results corroborated our previous observations [8] and suggested that most of NS5A D2 is dispensable for the HCV life cycle in hepatoma cell lines.

Since the deletion that we had introduced removed ~75% of the ISDR (reported for genotype 2a to reside between aa 2213–2248 [12]), we wondered whether the mutant is more sensitive to IFN. To this end, Huh7.5 cells were infected with JcR2a or JcR2a-5A$^{D2\Delta}$ and 24 h later treated for a period of 48 h with increasing concentrations of IFN-α and IFN-λ1 (Fig. 1E and F, respectively). Both wild type and 5A$^{D2\Delta}$ were equally sensitive to these cytokines.

A deletion in NS5A D2 causes strong attenuation of HCV in vivo

Assuming that NS5A D2 might play a role in virus-host interaction, not properly recapitulated in human hepatoma cells, we determined replication fitness of the 5A$^{D2\Delta}$ mutant *in vivo* by using the Alb-uPA$^{+/+}$ xenograft mouse model. These animals suffer from transgene-induced liver damage allowing xenografting with primary human hepatocytes (PHHs) a few weeks after birth. The mouse liver is progressively repopulated by human cells and maintains normal metabolic functions [14]. Purified Jc1 and Jc1-5A$^{D2\Delta}$ virus particles of comparable quality were used for injection of the animals (Fig. 2A). Seven transgenic mice with a replacement index (RI) of PHHs in the mouse liver of >70%, as deduced from serum concentration of human albumin (h-alb) (Fig. 2B) and immunohistological analysis (Fig. 2C), were each injected with ~10^6 $TCID_{50}$ of infectious HCV (corresponding to ~10^8 RNA copies). Three animals were inoculated with Jc1 (mouse # 101, 102, 103) and 4 with Jc1-5A$^{D2\Delta}$ (mouse # 201, 202, 203, and 204). Blood samples were collected periodically to monitor h-alb levels and HCV RNA copy number (Fig. 3A) to correlate viral replication with the RI by normalization to h-alb levels. Already seven days after injection, high viral load was detected in all three mice inoculated with Jc1 (Fig. 3B). On average, RNA load in these animals fluctuated around 5×10^5 to ~10^6 HCV RNA copies per mg h-alb (Fig. 3B; Supplementary Fig. 2). In the case of animals injected with Jc1-5A$^{D2\Delta}$, two major differences were noticed. First, replication kinetic was profoundly delayed in three of the four animals and only in one animal HCV RNA could be detected in serum at day seven post inoculation. Importantly, only at day 28 post inoculation, viremia was detectable in all four animals. Second, on average, viremia in these animals was ~fivefold lower than in wild type

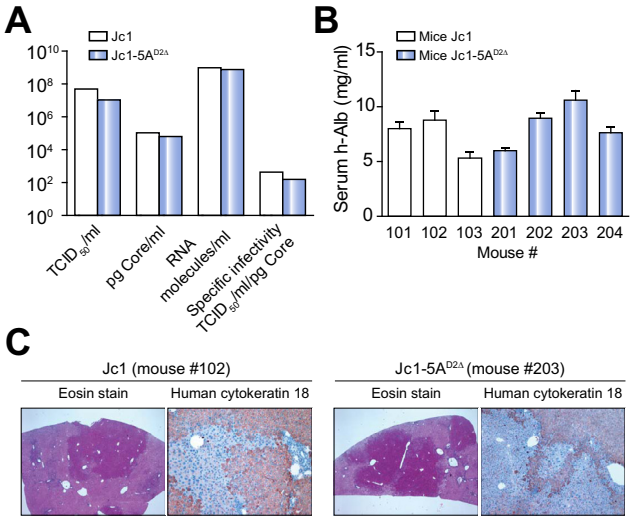

Fig. 2. Characterization of virus inoculum and xenografted mice used for *in vivo* test. (A) Quantification of virus stocks used for inoculation of mice. Amounts of infectious virus particles ($TCID_{50}$/ml), Core protein and viral RNA were determined. Specific infectivity corresponds to the ratio of virus titer and amount of Core protein ($TCID_{50}$/pg Core). (B) Quantification of average human serum human albumin (h-alb) amounts in each animal serum. (C) Repopulation of mouse livers with PHHs. Shown are histological analyses of liver sections of mouse #102 (Jc1) and #203 (Jc1-5A$^{D2\Delta}$). PHHs were visualized by Eosin staining (dark red) or by immunohistology using an antibody recognizing human cytokeratin 18 (blue stain).

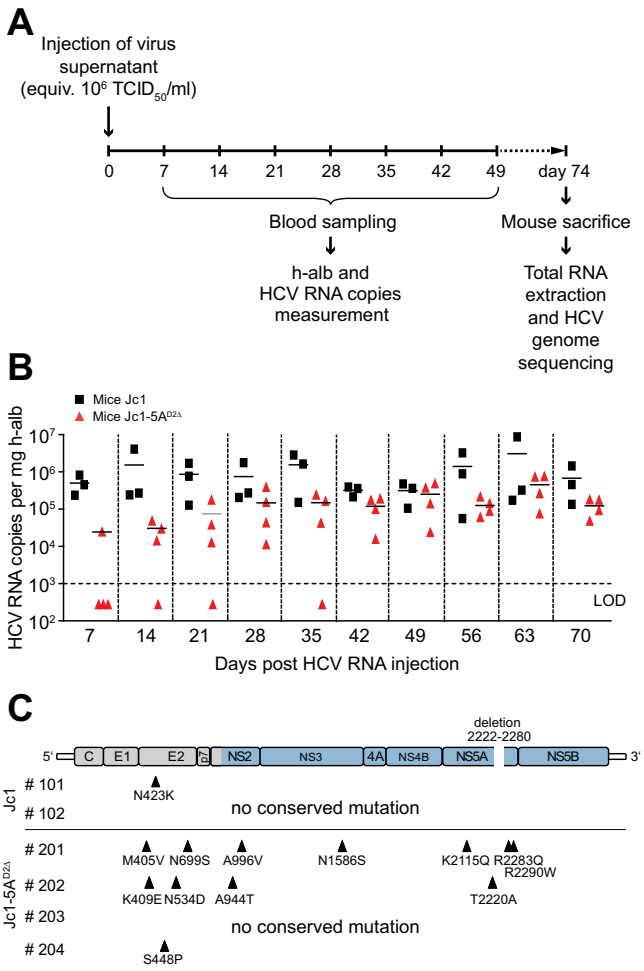

Fig. 3. Mutant Jc1-5A$^{D2\Delta}$ is severely attenuated *in vivo*. (A) Schematic outline of the experimental approach. (B) Scatter plot representing the time course of HCV viremia in serum of mice injected with Jc1 (black squares) or Jc1-5A$^{D2\Delta}$ (red triangles). HCV RNA amounts were normalized to h-alb levels of the respective mouse. The dotted line (10^3 RNA copies per mg h-alb) indicates our limit of detection. (C) Summary of the conserved nucleotide substitutions detected in mice at day 74.

virus-inoculated mice (~2×10^5 HCV RNA copies per mg h-alb; Fig. 3B and Supplementary Fig. 2). While in Jc1-infected mice, viremia was rather stable throughout the experiment, in all Jc1-5A$^{D2\Delta}$ virus-inoculated animals viremia steadily increased up to day 42–49 (Fig. 3B and Supplementary Fig. 2). This was not due to higher cytopathogenicity of the mutant virus, because immune histological analysis revealed comparable growth of transplanted human hepatocytes (Fig. 2C), consistent with the comparable RI in wild type and mutant virus-inoculated animals. These results suggest that Jc1-5A$^{D2\Delta}$ is strongly attenuated *in vivo*, arguing that NS5A D2 is an important fitness determinant. This is not due to replication or virus production *per se*, as the attenuation was not found in Huh7 cells.

Gain of fitness of Jc1-5A$^{D2\Delta}$ in vivo does not result from adaptive mutations

Since RNA copy numbers detected in Jc1-5A$^{D2\Delta}$-inoculated mice steadily increased during the observation period and reached

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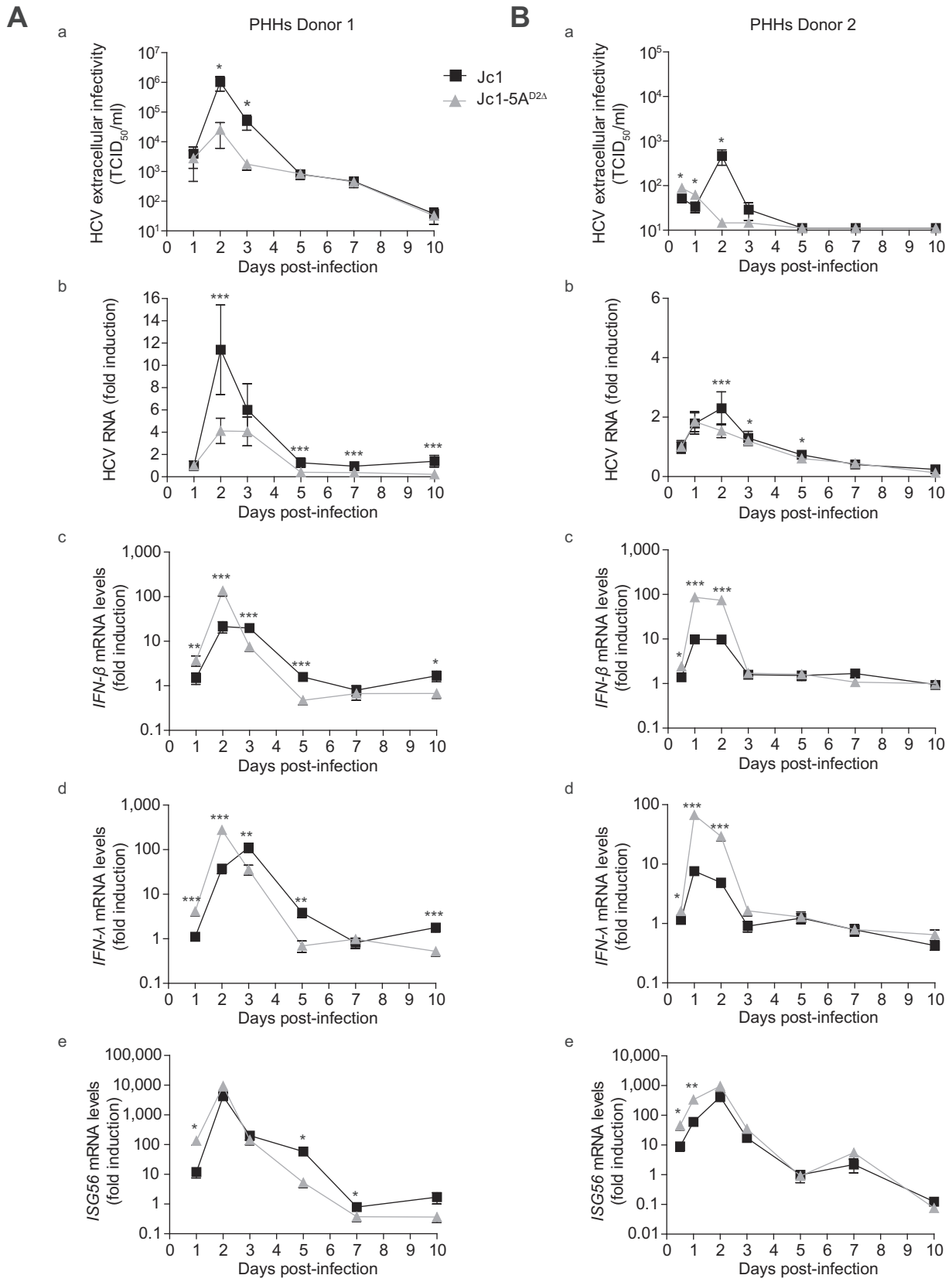

wild type levels at later time points, we reasoned that adaptive mutations restoring fitness might have emerged. Therefore, total RNA was isolated from the serum of the animals, HCV cDNAs were amplified in two overlapping fragments and cloned as described previously (note that no amplicon was obtained for mouse #103) [15]. The first fragment spanned the coding region of Core to the end of NS3; the second fragment the region encoding most of NS3 to the end of NS5B. For each amplicon derived from a given animal, at least three cDNA clones were analyzed (Supplementary Fig. 3). Only nucleotide substitutions conserved in all three cDNA clones were considered. Substitutions present in both Jc1 and Jc1-5A$^{D2\Delta}$ genomes were disregarded, because they reflected genetic variability independent from specific adaptation of the mutant. As summarized in Fig. 3C, three of the four mutant virus-inoculated animals (# 201, 202, and 204) contained HCV variants with aa substitutions in both structural and non-structural protein coding regions. Importantly, no substitution was found in HCV genomes extracted from mouse #203. Although we cannot exclude that minor HCV variants or mutations conserved in less than three of the analyzed cDNA clones conferred growth advantage in the animals, these results suggested that the increase of Jc1-5A$^{D2\Delta}$ replication over time might be independent from specific adaptive mutations in the HCV coding regions. The data therefore argued for a complex virus – host cell co-adaptation.

NS5A D2 is important to suppress the interferon response in primary human hepatocytes

Since an adaptive immune response is lacking in the Alb-uPA$^{+/+}$SCID mouse model, we reasoned that differences in the cytokine response, most notably the IFN system, might have been the cause for the attenuation of mutant Jc1-5A$^{D2\Delta}$. Since this difference was not observed in Huh7 cells, we tested our assumption by using PHHs that are IFN-competent. Cells derived from two donors were infected with Jc1 or Jc1-5A$^{D2\Delta}$ at a MOI of 1 $TCID_{50}$ per cell (Fig. 4; for details of donors see Supplementary Fig. 4). Supernatants and cells were harvested at regular intervals and amounts of infectious extracellular virus particles were determined by limiting dilution assay (Fig. 4Aa and Ba). Intracellular HCV RNA levels (Fig. 4Ab and Bb) and mRNA levels of three ISGs, *ISG56*, *IFN-β*, and *IFN-λ*, were quantified by qRT-PCR (Fig. 4Ac-e and Bc-e). In spite of profound donor-to-donor variation, *ISG56* and *IFN* mRNA levels were strongly induced by Jc1 infection reaching maximal levels at day two or three post-infection (p.i.). This induction correlated with amplification kinetic of HCV RNA and virus production, reaching peak levels already at day two p.i., and was followed by a sharp decline of viral RNA and virus amounts, arguing for potent suppression of HCV replication by the induced antiviral response. Importantly, Jc1-5A$^{D2\Delta}$ infection induced a significantly stronger IFN response in both donors, correlating well with rapid suppression of viral RNA replication and virus production. Variability in the magnitude of ISG and IFN induction observed with PHHs from donor 1 and 2 correlated with HCV replication level (Fig. 4A and B). Thus, NS5A D2 plays an important role in controlling kinetics and magnitude of the IFN response against HCV infection, thus providing an explanation for the attenuation of the NS5A deletion mutant *in vivo*.

Taking advantage of this system, we tested the possible impact of some of the mutations detected in HCV genomes replicating in the infected mice on replication fitness. Although the absence of mutations in animal #203 argued against adaptation of the virus (Fig. 3C), we generated a Jc1-5A$^{D2\Delta}$ variant containing all four mutations present in HCV of animal #202 (Fig. 3C). These mutations resided in E2 (K410E, N534D), NS2 (A944T), and NS5A (T2220A). This variant, designated Jc1-5A$^{D2\Delta}$mut202, was used to inoculate PHHs from a third donor, in parallel to Jc1 and the parental Jc1-5A$^{D2\Delta}$ mutant. As shown in Supplementary Fig. 5, highest replication was again found with the wild type. Although IFN response obtained with PHHs of this donor were rather low, both the parental NS5A deletion mutant and the variant Jc1-5A$^{D2\Delta}$mut202 induced a higher IFN-β and ISG56 response than wild type. Importantly, replication and virus production of these variants were comparable, but significantly lower than wild type. These results corroborate the important role of NS5A D2 in attenuating the IFN response and support further the absence of adaptive mutations in Jc1-5A$^{D2\Delta}$ genomes replicating in infected mice.

Activation of the interferon response by HCV via MDA5

Although results obtained with PHHs provided first insights into the mechanism of attenuation, this cell system is limited by poor availability, high donor-to-donor variation and technical difficulties to manipulate the cells. Therefore, we attempted to establish Huh7-based cell systems allowing the recapitulation of phenotypes observed in PHHs. However, Huh7 cells induce poor ISG expression upon virus infection and have a very low IFN production [16]. Moreover, Huh7.5 cells that are most permissive to HCV infection, express very low amounts of MDA5 and have a defective RIG-I gene [5]. To overcome these impairments, we used previously described Huh7.5 cells stably expressing functional RIG-I [17] and generated in addition analogous cell lines expressing ectopically MDA5 (Huh7.5 MDA5 cells) or the empty expression vector (Huh7.5 Ctrl cells) (Fig. 5A).

To determine restoration of RNA sensing and activation of the IFN response, cells were co-transfected with a reporter plasmid containing the ISG56 promoter sequence upstream of the *Firefly* luciferase gene and a control plasmid encoding *Renilla* luciferase to normalize for transfection efficiency. Six hours post transfection, cells were stimulated with the synthetic double-stranded RNA (dsRNA) analogue poly(I:C) for 16 h. ISG56 promoter activity was induced ~15-fold in both RIG-I and MDA5 overexpressing cell lines relative to non-stimulated cells (Fig. 5B). As alternative stimulus, we chose Sendai virus (SeV) infection that was shown to be sensed exclusively by RIG-I [3]. Indeed, SeV infection stimulated exclusively ISG56 promoter activity in Huh7.5 RIG-I cells,

Fig. 4. Attenuation of Jc1-5A$^{D2\Delta}$ replication in PHHs correlates with enhanced activation of the IFN response. PHHs of two donors (A, Donor 1; B, Donor 2) were infected with Jc1 (black lines) or the Jc1-5A$^{D2\Delta}$ mutant virus (grey lines). At given time points after infection, supernatants were harvested and extracellular virus titers were determined. HCV RNA as well as *IFN-β*, *IFN-λ1*, and *ISG56* mRNA levels were determined by qRT-PCR. Results were normalized to values obtained with day one samples. Shown are fold induction of gene expression relative to non-infected cells. Statistical significance is shown in the top.

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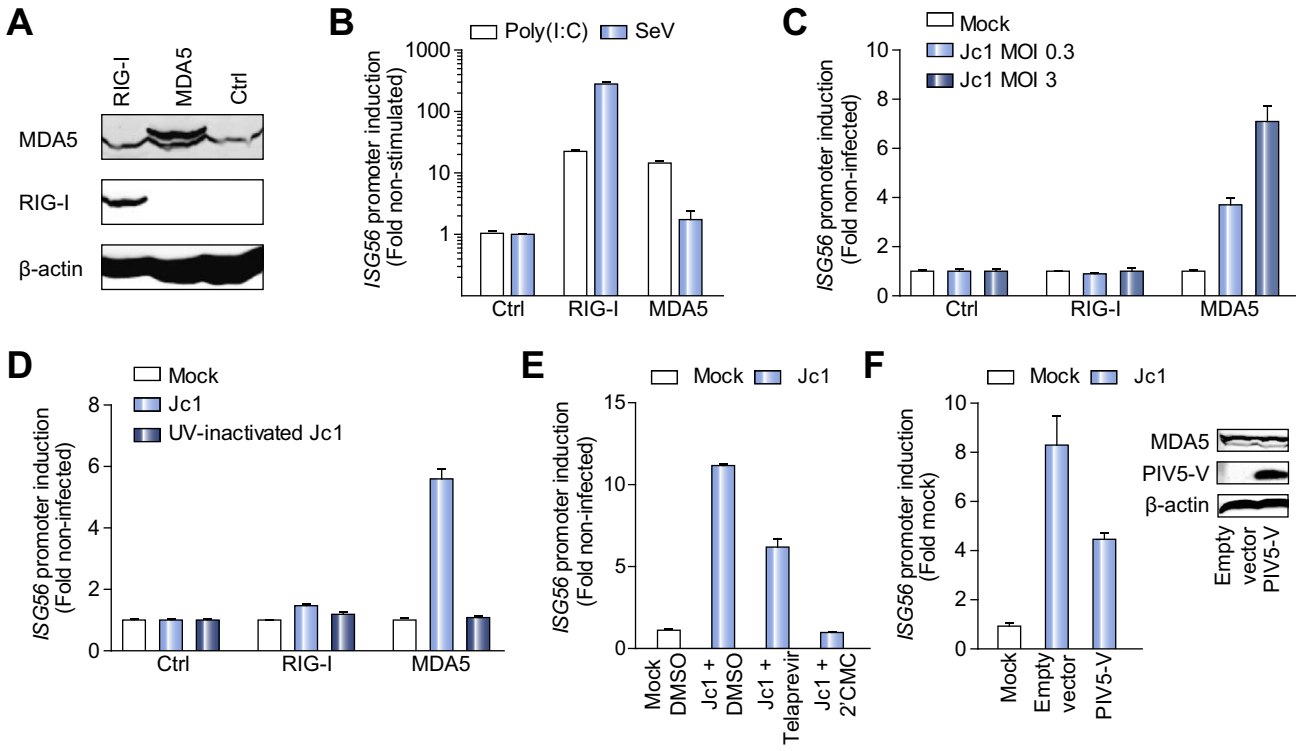

Fig. 5. HCV activates MDA5 in reconstituted Huh7.5 cells. (A) Stable expression of RIG-I, MDA5 or control vector (Ctrl) in Huh7.5 cells. ISG56 promoter-assay in stable Huh7.5 cells after stimulation with poly(I:C) or infection with Sendai virus (SeV) (B) or Jc1 (C–F). Values were normalized to non-stimulated or non-infected cells; mean values ± SD of three independent experiments. No activation of MDA5 by UV-inactivated HCV (D) or in DAA-treated Jc1-infected cells (E). (F) PIV5 V protein blocks Jc1-induced activation of MDA5 signaling pathway. Expression of MDA5 and V protein was determined by Western blot (right panel).

but not in Huh7.5 MDA5 cells (Fig. 5B). These results show that RNA sensing and activation of IRF-3 dependent signaling are effective in Huh7.5 RIG-I and MDA5 cells.

We next determined whether also HCV infection is recognized by either RLR. Cells were infected with Jc1 and 48 h later, ISG56 promoter activation was determined. Consistent with our earlier report, HCV infection did not activate the ISG56 reporter in Huh7.5 RIG-I cells [17]. Surprisingly, however, a MOI-dependent activation of ISG56 promoter was observed in Huh7.5 MDA5 cells (Fig. 5C). This activation was specific and dependent on viral replication, because infection of these cells with UV-inactivated Jc1 did not induce an IFN response (Fig. 5D). Moreover, suppression of HCV replication by the NS3 protease inhibitor telaprevir or the nucleoside analogue 2′-C-methylcytidine (2′-CMC) potently suppressed activation of the IFN response (Fig. 5E). Finally, we found that transient expression of the V protein from parainfluenza virus 5 (PIV5), a well-described inhibitor of MDA5 [18], in Huh7.5 MDA5 cells impaired HCV-induced ISG56 promoter activation (Fig. 5F). Altogether these results demonstrate that HCV is sensed by MDA5 which required viral replication.

HCV activates the interferon response via RIG-I and MDA5 in a sequential manner

Taking advantage of the Huh7.5 cell lines with restored HCV sensing, we compared activation of the RIG-I and MDA5 pathway between Jc1 and the Jc1-5A$^{D2\Delta}$ mutant. To include possible differences in activation kinetics, we conducted a time course experiment. In addition to Huh7.5 control (Ctrl) cells, we included a Huh7.5-derived cell line stably expressing a MDA5 mutant lacking the caspase activation and recruitment domain (CARD; Huh7.5 MDA5ΔCARD cells). Cells were infected at a MOI of 10 $TCID_{50}$ per cell and ISG56 promoter activity was determined. We observed a temporal activation of the RIG-I mediated IFN response that was detectable 10 h p.i., lasted until 24 h p.i. and was no longer measurable 40 h p.i. (Fig. 6A). This result was consistent with our earlier report and indicated rapid and efficient control of RIG-I signaling by HCV [17]. Interestingly, the decrease in activation of RIG-I was concomitant with an activation of the MDA5 signaling pathway that was strongly detectable 40 h p.i. (Fig. 6A). This activation was specific and not found with Huh7.5 MDA5ΔCARD cells. Moreover, this sequential activation of RIG-I and MDA5 was also obtained with poly(I:C) arguing that this kinetic is intrinsic to these RLRs (Supplementary Fig. 6). Most strikingly, although Jc1-5A$^{D2\Delta}$ infection led to a similar sequential activation of RLRs, the kinetic was faster leading to higher levels of ISG56 promoter activation 10 h and 40 h p.i. in case of RIG-I and MDA5, respectively (Fig. 6A). Jc1 and Jc1-5A$^{D2\Delta}$ infectivity titers of supernatants collected from the infected cell lines were comparable, thus excluding the possibility that the observed differences were caused by different amounts of viral RNA inducer (Fig. 6B).

Although Huh7.5 RIG-I cells have an impaired type I IFN production, they can release type III IFN by using a strong stimulus such as SeV infection (data not shown). Thus, it was possible that the observed ISG56 activation was caused by IFN released from infected cells rather than by direct HCV-mediated IRF3 activation.

To differentiate between these possibilities, we characterized the supernatants collected from HCV infected cell lines for the presence of IFN by using a highly sensitive HCV-based bioassay [19]. However, in spite of high sensitivity of this assay, no antivirally active IFN could be detected (Supplementary Fig. 7) arguing against IFN-mediated amplification of the ISG56-based read-out.

The sequential activation of RIG-I and MDA5 that both signal via MAVS was perplexing. On one hand, both Jc1 and Jc1-5A$^{D2\Delta}$ efficiently control RIG-I signaling by NS3-mediated MAVS cleavage; on the other hand, we observed robust activation of MDA5 at late time points after infection when the amounts of NS3 are clearly higher than at early time points (Supplementary Fig. 8). This raised the question whether MDA5 activation was indeed mediated by MAVS or some other, undefined pathway. To address this possibility, we generated Huh7.5 MDA5 cell lines stably expressing the NS3/4A protease efficiently cleaving and inactivating MAVS. Huh7.5 MDA5 cells expressing a Ctrl plasmid and MDA5ΔCARD cells were used as control. Upon infection of these cells with Jc1 or Jc1-5A$^{D2\Delta}$, MDA5-mediated signaling was severely impaired by the protease showing that HCV-induced activation of the IFN system by MDA5 was also mediated by MAVS (Fig. 6C).

In an attempt to determine the impact of NS5A on IFN signaling more directly, we generated Huh7.5 RIG-I and Huh7.5 MDA5 cells stably overexpressing NS5A or 5A$^{D2\Delta}$. To this end the NS5A proteins were expressed out of the polyprotein context to exclude confounding effects that might be exerted by other HCV proteins such as the NS3 protease (Fig. 7A). Induction of ISG56 and IFN-λ1 mRNAs was measured upon infection with SeV or a mengovirus Zn mutant (Mengo Zn) [20] triggering RIG-I or MDA5, respectively (Fig. 7B and C). Interestingly, wild type NS5A repressed ISG induction by either RLR, but this repression was reduced in the case of NS5A$^{D2\Delta}$. Importantly, the degree of reduction was in the range of the difference observed between NS5A and NS5A$^{D2\Delta}$ in the ISG56 promoter assay.

In conclusion, our results show that HCV activates RIG-I and MDA5 signaling pathways in a sequential and MAVS-dependent manner. Importantly, this activation is suppressed by D2 of NS5A, thus providing an explanation for the attenuation of the mutant lacking part of D2 both in PHHs and *in vivo*.

Discussion

We report that HCV activates RIG-I- and MDA5-mediated signaling in a sequential manner. The observation that poly(I:C) activates both RLRs with similar kinetics argues for an intrinsic property of these sensors and the corresponding signaling pathway. Nevertheless, in HCV-infected cells strand-specificity of RLRs might be of relevance with RIG-I detecting preferentially incoming (single-strand) RNA whereas MDA5 likely recognizes dsRNA replication products generated at later stages of the viral

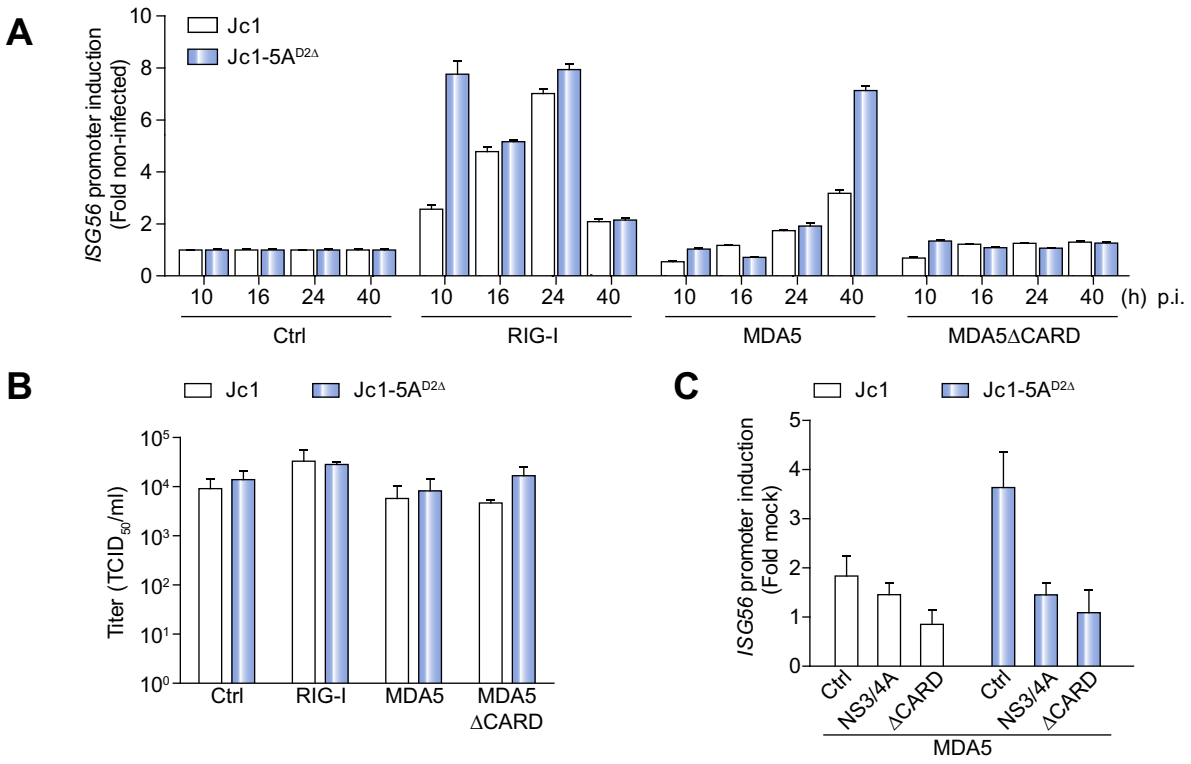

Fig. 6. Sequential activation of RIG-I and MDA5 by HCV in a MAVS-dependent manner. (A) ISG56 promoter assay using reconstituted HCV-infected cells. MDA5ΔCARD served as negative control (fold induction relative to non-infected cells); means of triplicate measurements ± SD (representative experiment; n = 3). (B) Overexpression of RIG-I, MDA5 or MDA5ΔCARD does not affect HCV replication. Virus titers in supernatants harvested 40 h p.i. were determined. (C) Activation of the IFN response *via* MDA5 requires MAVS. ISG56 reporter assay using Huh7.5 MDA5 cells overexpressing NS3/4A or a control vector (Ctrl) and Huh7.5 MDA5ΔCARD (ΔCARD) cells infected with Jc1 or Jc1-5A$^{D2\Delta}$; means of triplicate measurements ± SD (representative experiment; n = 2).

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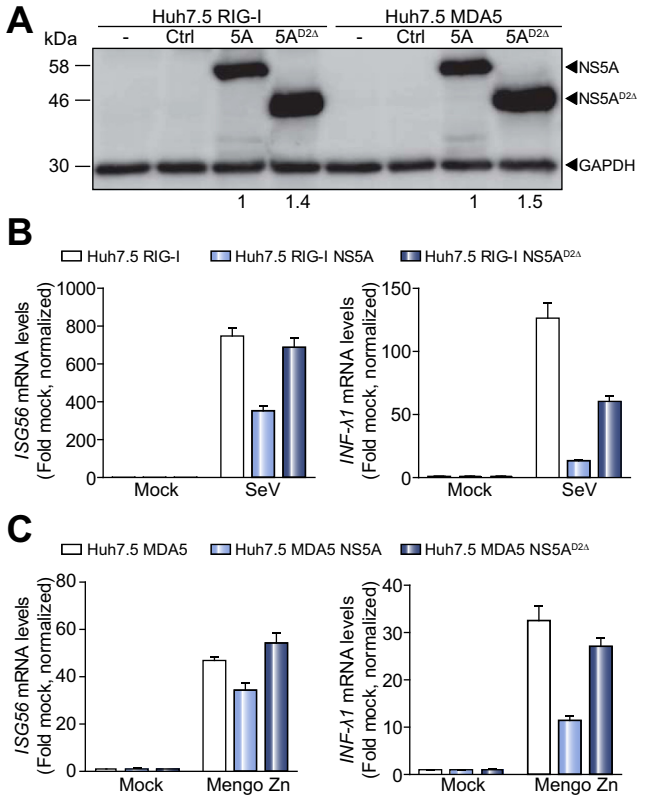

Fig. 7. Inhibition of IFN activation by wild type NS5A but not NS5A$^{D2\Delta}$. (A) Stable expression of NS5A, NS5A$^{D2\Delta}$ and a control plasmid (Ctrl) in Huh7.5 RIG-I and Huh7.5 MDA5 cells. Abundance of HCV proteins was determined by Western blot. GAPDH served as loading control. Amounts of NS5A$^{D2\Delta}$ relative to NS5A are given below the lanes. (B, C) Induction of endogenous ISG56 and IFN-λ1 mRNA levels upon SeV and Mengo Zn infection as determined by qRT-PCR. Shown are fold induction relative to mock cells, normalized to NS5A and NS5A$^{D2\Delta}$ protein abundance. Means of triplicate measurements ± SD (representative experiment; n = 3). Cells specified in the top were infected with SeV for 24 h or Mengo Zn for 8 h (B and C, respectively).

replication cycle. We note that such a complementary role of RIG-I and MDA5 and the temporal detection of PAMPs was previously reported for West Nile Virus [21,22].

Our results are in perfect agreement with a very recent study by Cao and colleagues also showing that MDA5 senses HCV RNA [23]. Moreover, these authors found that MDA5 is more relevant than RIG-I with respect to activation of the IFN response by HCV. Thus far it is unclear what is recognized by MDA5 and how MDA5-mediated signaling is achieved at such late time points after infection when most of MAVS is cleaved by the viral NS3 protease. At least two possibilities can be envisaged: first, MDA5 is activated by a subpopulation of MAVS poorly accessible to the protease such as peroxisomal MAVS [24]; second, MAVS cleavage is not complete and a small fraction, not detectable by our assays, might suffice to activate the IFN response, particularly via MDA5.

In agreement with previous reports [25,26], we observed an activation of the IFN response by HCV *via* TLR3 (Supplementary Fig. 9). Importantly the peak of activation occurred only at 72 h p.i. and no difference in the level of ISG56 promoter activation could be observed between Jc1 and Jc1-5A$^{D2\Delta}$ arguing that NS5A D2 preferentially affects cytosolic and not endosomal RNA sensors.

NS5A was proposed to perturb IFN and NF-κB signaling pathways through interaction with several factors, most notably PKR and TRAF2 [6]. However, no evidence for such interactions or differential activation of PKR by wild type and 5A$^{D2\Delta}$ was found (Supplementary Fig. 10A–C). Apart from a possible modulation of PKR- and NF-κB-dependent pathways, the NS5A deletion mutation might have lower control of IFN-activation because of altered RNA secondary structure or shorter RNA length, rather than exerting an effect at the protein level. However, when we transfected our engineered Huh7.5 cells with Jc1 or Jc1-5A$^{D2\Delta}$ RNA, the IFN response was stimulated to the same extent (Supplementary Fig. 10D). Thus, it is unlikely that the deletion introduced into NS5A altered RNA structure in a way to make it a better ligand for RIG-I and MDA5. Alternatively, NS5A is a RNA binding protein with all three domains contributing to nucleic acid binding, albeit to different extents [27]. Thus, removal of D2 might affect NS5A binding affinity to viral RNA, resulting in inefficient shielding of the genome, and thereby better detection by RIG-I and MDA5, leading to higher activation of the IFN system.

Although 5A$^{D2\Delta}$ lacks two-thirds of the C-terminal of ISDR, a region suggested to be involved in the outcome of IFN treatment [28], we found that this deletion did not affect HCV sensitivity to IFN-α and IFN-λ1. Moreover, neither NS5A nor NS5A$^{D2\Delta}$ stably expressed in the context of the polyprotein NS3-5A affected JAK-STAT signaling pathway as determined by the degree of STAT1 phosphorylation and ISG induction upon IFN treatment (Supplementary Fig. 11). These results corroborate previous findings showing that ISDR mutations found in HCV-infected individuals and associated with IFN sensitivity do not influence HCV IFN response [29]. Importantly, this was found both with the JFH-1 isolate that we also used in our study, and with chimeric genomes containing NS5A sequences of other genotypes [30], arguing that IFN sensitivity *per se* is not directly linked to the ISDR.

It has been shown that HCV induces ISGs in the liver of infected patients [31]. Paradoxically, the level of induction correlates inversely with response to IFN-based therapy [31]. Although no experimental data are available, based on the results reported here it is tempting to speculate that higher ISG pre-activation might be due to distinct mutations in NS5A D2, thus providing a possible explanation for the correlation of ISDR signatures with outcome of IFN therapy [12,32].

In conclusion, we report a sequential activation of the IFN response upon HCV infection *via* RIG-I and MDA5 and a suppression of this response in a NS5A D2-dependent manner. This inhibition might contribute to the high rate of HCV persistence.

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Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Author's contributions

Conception and design of the study: MS.H., O.B., A.R., and R.B.; generation, collection, assembly, analysis and interpretation of data: MS.H., O.B., M.Z., H.R., Y.T., P.S., J.W., O.G., S.B., M.B., V.L., V.L., A.R., and R.B.; drafting of the manuscript: MS.H., A.R., and R.B.; approval of the final version of the manuscript: MS.H., O.B., M.Z., H.R., Y.T., P.S., J.W., O.G., S.B., M.B., V.L., A.R., and R.B.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2015.04.015.

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