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Complete List of Authors:	Wrensch, Florian; Inserm U1110, University of Strasbourg Ligat, Gaetan; Inserm U1110, University of Strasbourg Heydmann, Laura; Inserm U1110, University of Strasbourg Schuster, Catherine; Inserm U1110, University of Strasbourg Zeisel, Mirjam; Inserm U1110, University of Strasbourg; Inserm U1052, CNRS UMR 5286, Cancer Research Center of Lyon (CRCL) Pessaux, Patrick; Inserm U1110, University of Strasbourg Habersetzer, François; Inserm U1110, University of Strasbourg King, Barnabas; The University of Nottingham, School of Molecular Medical Sciences Tarr, Alexander; The University of Nottingham, School of Molecular Medical Sciences Ball, Jonathan; The University of Nottingham, School of Molecular Medical Sciences Pöhlmann, Stefan; German Primate Centre Leibniz Institute for Primate Research, Infection Biology Unit; University of Göttingen Faculty of Biology and Psychology Winkler, Michael; German Primate Centre Leibniz Institute for Primate Research, Infection Biology Unit Keck, Zhenyong; Stanford University, Department of Pathology Foung, Steven; Stanford University, Department of Pathology Baumert, Thomas; Inserm U1110, University of Strasbourg
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IFITMs mediate viral evasion in acute and chronic hepatitis C virus infection

Florian Wrensch[1,2], Gaëtan Ligat [1,2], Laura Heydmann[1,2], Catherine Schuster[1,2], Mirjam B. Zeisel[1,2,3], Patrick Pessaux[1,2,4], François Habersetzer[1,2,4], Barnabas J. King[5,6], Alexander W. Tarr[5,6], Jonathan K. Ball[5,6], Michael Winkler[7], Stefan Pöhlmann[7,8], Zhen-yong Keck[9], Steven K.H. Foung[9], Thomas F. Baumert[1,2,4,10]

[1]Inserm, U1110, Institut de Recherche sur les Maladies Virales et Hépatiques, 67000 Strasbourg, France; [2]Université de Strasbourg, 67000 Strasbourg, France; [3]Inserm U1052, CNRS UMR 5286, Cancer Research Center of Lyon (CRCL), Université de Lyon (UCBL), 69373 Lyon, France; [4]Institut Hospitalo-Universitaire, Pôle Hépato-digestif, Hôpitaux Universitaires de Strasbourg, 67000 Strasbourg, France; [5]School of Life Sciences, The University of Nottingham, Nottingham NG7 2UH, UK ; [6]NIHR Nottingham BRC, Nottingham University Hospitals NHS Trust and the University of Nottingham, Nottingham NG7 2UH, UK; [7]Infection Biology Unit, German Primate Center–Leibniz Institute for Primate Research, 37077 Göttingen, Germany, [8]Faculty of Biology and Psychology, University of Göttingen, 37073 Göttingen, Germany; [9]Department of Pathology, Stanford University School of Medicine, Stanford, CA 94305; [10]Institut Universitaire de France, 75231 Paris, France

Key words: neutralizing antibody, cell entry, HCV, escape, vaccine

Contact information: Prof. Thomas F. Baumert, Inserm U1110, Institut de Recherche sur les Maladies Virales et Hépatiques, 3 rue Koeberlé, 67000 Strasbourg, France, Tel: +33368853703, Fax: +33368853724, Email: thomas.baumert@unistra.fr

List of abbreviations: HCV, hepatitis C virus; IFITM, interferon-induced transmembrane protein; HCVpp, HCV pseudoparticle; HMAb, human monoclonal antibody; HCVcc, cell culture derived HCV; MLV, murine leukemia virus ; $TCID_{50}$, tissue culture infectious dose 50 % ; nAb, neutralizing antibody.

Author contributions. FW designed, performed and analyzed experiments. LH and GL performed and analyzed experiments. TFB, MBZ and CS designed and analyzed experiments and edited the MS and figures. BK, AT and JB helped design and carry out the study through generation and testing of novel E1E2 clones and HCV chimeras and provided important critique and intellectual content to the manuscript, MW and SP provided IFITM expression plasmids, analyzed IFITM expression and localization by IF and edited the MS. ZK and SKHF provided anti-HCV HMAbs and edited the MS text. PP provided liver tissue resections for isolation of primary human hepatocytes, FH provided serum from chronically infected patients. FW and TFB wrote the MS. TFB initiated and coordinated the study.

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Abstract

While adaptive immune responses against hepatitis C virus (HCV) infection have been studied in great detail, the role of innate immunity in protection against HCV infection and immune evasion is only partially understood. Interferon-induced transmembrane proteins (IFITMs) are innate effector proteins restricting host cell entry of many enveloped viruses, including HCV. However, the clinical impact of IFITMs on HCV immune escape remains to be determined. Here, we show that IFITMs promote viral escape from the neutralizing antibody response in clinical cohorts of HCV-infected patients. Using pseudoparticles bearing HCV envelope proteins from acutely infected patients, we show that HCV variants isolated pre-seroconversion are more sensitive to the antiviral activity of IFITMs than variants from patients isolated during chronic infection post-seroconversion. Furthermore, HCV variants escaping neutralizing antibody responses during liver transplantation exhibited a significantly higher resistance to IFITMs than variants that were eliminated post-transplantation. Gain-of-function and mechanistic studies revealed that IFITMs markedly enhance the antiviral activity of neutralizing antibodies and suggest a cooperative effect of human monoclonal antibodies and IFITMs for antibody-mediated neutralization driving the selection pressure in viral evasion. Perturbation studies with the IFITM antagonist amphotericin B revealed that modulation of membrane properties by IFITM proteins is responsible for the IFITM-mediated blockade of viral entry and enhancement of antibody-mediated neutralization. **Conclusion:** Our results identify IFITM proteins as a previously unknown driver of viral immune escape and antibody-mediated HCV neutralization in acute and

chronic HCV infection. These findings are of clinical relevance for the design of urgently needed HCV B cell vaccines and might help to increase the efficacy of future vaccine candidates.

Introduction

It is estimated that more than 71 million patients are chronically infected with hepatitis C virus (HCV) (1). HCV infection is a leading cause of liver disease and cancer worldwide. The development of direct acting antivirals markedly improved the outcome of antiviral treatment with cure of the majority of treated patients (2). However, several challenges remain (3). High treatment costs prevent or limit access of patients to therapy in resource-poor countries and may lead to selective use even in industrialized countries. Moreover, in the majority of cases HCV infection remains undiagnosed or is diagnosed at a late stage due to the limited efficacy of current HCV screening programs. Furthermore, direct acting antivirals will not cure virus-induced end-stage liver disease such as hepatocellular carcinoma and certain patient subgroups do not respond to or cannot tolerate direct acting antiviral-based treatment strategies (4, 5). Finally, reinfection remains possible, making control of HCV infection difficult in people at risk, such as drug abusers. These unmet medical needs warrant the development of an effective vaccine, protecting from chronic HCV infection as a means to impact the epidemic on a global scale (3).

Both cellular and humoral immune responses have been suggested to play a key role in protection against infection in humans and nonhuman primates. Thus, vaccine development has focused on eliciting both B and T cell responses (3). Indeed, a B cell vaccine consisting of recombinant E1E2 viral envelope glycoprotein was shown to provide partial protection against

chronic HCV infection (6), to induce virus neutralizing antibodies and to be safe in healthy volunteers (7). Furthermore, broadly virus neutralizing antibodies have been shown to confer protection against HCV in humanized mouse models (8, 9) and are considered a promising strategy to fight emerging infectious diseases (10). While adaptive immune responses have been studied in great detail, the role of innate immune responses in HCV infection is only partially understood.

The innate immune response constitutes the first line of defense against viral infections. Interferons stimulate the expression of a set of more than 300 interferon-stimulated genes, several of which have been shown to exert antiviral activity against HCV (11). A family of these genes, the interferon-induced transmembrane (IFITM) proteins are potent inhibitors of host cell entry of a broad range of enveloped viruses, including HCV (12-15). While IFITM1 is primarily located at the cytoplasmic membrane and restricts HCV entry by interacting with the HCV co-receptor CD81 (13), IFITM2 and 3 localize to endosomal compartments and potentially restrict viral infection by blocking virus entry at the stage of hemifusion (16) or fusion pore formation (17).

While the antiviral activity of the IFITM proteins against HCV has been studied in cell culture models (13, 14, 18), the role of the IFITM proteins in viral pathogenesis during clinical HCV infection is unknown. It is unclear whether inhibition of virus entry by IFITM proteins contributes to viral clearance, whether IFITM-HCV interactions impact viral persistence in chronic infection and whether IFITM proteins and antibodies cooperate to inhibit viral entry.

Clinical cohorts for the study of acute and chronic HCV infection have been a valuable tool to investigate the mechanisms of HCV persistence and escape. These include cohorts comparing early and late stage infection (19, 20). Furthermore, liver graft infection is a unique model since it allows the study of HCV infection and viral escape in a very well defined timeframe and detailed patient material (21-23).

To address the clinical role of IFITMs for viral escape and B cell responses, we investigated virus-host interactions of IFITM proteins and neutralizing antibodies during HCV cell entry. For this, we used HCV pseudoparticles (HCVpp) bearing envelopes from patients with acute infection prior to seroconversion or patients undergoing liver transplantation due to chronic hepatitis C. Moreover, we employed neutralizing human monoclonal antibodies (HMAbs) derived from patients with chronic HCV infection.

Material and Methods

Human material. Human material, including sera and liver tissues from patients undergoing surgical resection for isolation of human hepatocytes and followed at Strasbourg University Hospital, was obtained with informed consent from all patients. The protocol was approved by the Ethics Committee of Strasbourg University Hospital (CPP 10-17).

Cell lines and primary human hepatocytes. 293T cells, Huh7.5.1, Huh7.5.1-NTCP and HepG2-CD81 cells were isolated and cultured as described previously (18, 19, 24). Primary human hepatocytes (PHH) were isolated from liver resections as described previously (25).

Antibodies. The anti-E2 HMAbs (CBH-20, CBH-7, CBH-22, HC84.26.WH.5DL) and human anti-HCV sera have been described previously (19, 24, 26-29). The antibodies directed against IFITM1, IFITM2/3 (Proteintech) and β-actin (Sigma) and the protocols for detection of IFITM proteins by western blot and immune fluorescence have been described in (30).

Plasmids. The plasmids for the generation of HCVpp and cell culture derived HCV (HCVcc) (Jc1 (genotype 2a) chimera Luc-Jc1 and Con1 (genotype 1b) chimera Con1R2A) have been described in (21), The plasmids coding for the envelope proteins and HCVcc chimera bearing envelope proteins isolated from patients undergoing liver transplantation were described in (21). The plasmids encoding the envelope variants of acute patients have been described in (31) (accession numbers KU285163, KU285164 and KU285165). Plasmid UKN1a.16.16 (accession number MK124622) was generated as described in (32). The plasmids encoding the envelope proteins of the chronic variants are described in (32) (UKN1A14.38 and UKN3A1.28.), (33) (HCV-J) and in (34) (gt3SXB1). The full-length chimeric clone incorporating the UKN1.5.3 E1E2 genes was generated in the H77/JFH-1 virus background, using previously described methods (35).

Statistics. Data are shown as mean ± SEM if n ≥3. Representative experiments are shown as mean ± SD. Normality was assessed using the Shapiro-Wilk test. The 1-tailed Student's t test was used for single comparisons. A p-value of less than 0.05 was considered statistically significant.

Vector production, transduction and selection of stable cells. Retroviral vectors for transduction were generated by transfection of 293T cells as described previously (30) using the

CMV-Gag-Pol MLV (mouse leukemia virus) packaging construct, a vesicular stomatitis virus-G-encoding plasmid and plasmids coding for the IFITM proteins (pQCXIP) or empty vector as a control (30). For transduction, cells were seeded at subconfluent density and spin-inoculated with the retroviral vectors at 4000 x g for 30 min. Cells were then incubated at 37°C for 48 hours to allow efficient transgene expression. Cells stably expressing IFITM proteins were subsequently selected with 1.8 µg/mL puromycin.

HCVpp production, infection, and neutralization. HCVpp were generated by transfection of 293T cells as described previously (19), To study HCV entry, HCVpp were added to IFITM-transduced Huh7.5.1, Huh7.5.1-NTCP cells or PHH in triplicate and incubated for 72 h at 37°C. HCV entry was determined by analysis of luciferase reporter gene expression as described previously (24). For the study of antibody-mediated neutralization, HCVpp were mixed with autologous anti-HCV serum, control serum, anti-E2 HMAbs or irrelevant isotype control IgG, preincubated for 30 min at 37°C and added to Huh7.5.1, Huh7.5.1-NTCP cells or PHH in triplicate for 72 h at 37°C (21, 24). To assess the effect of amphotericin B on the cooperative inhibition of HCV entry by IFITM proteins and neutralizing antibodies, Huh7.5.1 cells were treated with 5 µmol/L amphotericin B (Sigma Aldrich) for 1 hour at 37 °C prior to infection with antibody-treated HCVpps.

HCVcc production, infection, and neutralization. Plasmids for cell culture-derived HCV (HCVcc) production of Jc1 and Con1 chimera with luciferase reporter (Luc-Jc1 and Con1-R2A) have been described previously (22, 36-39). HCVcc were produced in Huh7.5.1 cells as described previously (39). Infectivity was quantified by luciferase activity, or by determining the

tissue culture infectious dose 50% (TCID50) (22). HCVcc neutralization using patient serum, IgG, and mAbs was analyzed as described previously (22).

Results

IFITM proteins inhibit cell entry of HCVpp and HCVcc. To characterize the role of IFITMs in clinical HCV infection, we first investigated inhibition of viral entry into cells. For this, we transduced Huh7.5.1 or Huh7.5.1-NTCP cells with retroviral vectors encoding the antivirally active IFITM proteins (IFITM1, IFITM2 and IFITM3) and then infected the cells with HCVpp bearing the envelope proteins of HCV genotype 1b. Huh7.5.1-NTCP cells were used since NTCP has been described to have a functional role in regulation of interferon stimulated gene expression (18). Since no differences in IFITM antiviral activity on HCV entry and infection were observed between Huh7.5.1 and Huh7.5.1-NTCP cells, when IFITMs were exogenously expressed, Huh7.5.1 cells were then used for all subsequent experiments. Entry of HCVpp was restricted by all three IFITMs, with IFITM2 and 3 showing a slightly higher restriction than IFITM1 (Figure 1A). Pseudoparticles bearing the envelope protein of the IFITM resistant retrovirus MLV (40) were used as negative control (Figure 1B). Entry driven by the MLV-envelope protein was not modulated by IFITM proteins, as expected. To analyze the impact of cell polarization, which might affect IFITM activity due an altered subcellular localization of IFITM1 in hepatocytes, as reported by Wilkins et al (13), we studied the effect of IFITMs on HCVpp entry in polarized HepG2-CD81 cells in side-by-side experiments. The inhibition pattern observed upon IFITM expression was very similar to that seen for nonpolarized Huh7.5.1 cells (Figure 1C and Figure

S1), suggesting that polarization appears not to modulate the ability of IFITM proteins to block HCV entry. This is in line with the finding that IFITM-expression did not alter CD81 surface expression (Figure S2) or distribution (Figure S3). We next assessed the effect of IFITM proteins on HCV entry in the context of authentic virus using infectious HCVcc. The sensitivity of HCVcc infection to inhibition by IFITM proteins was assessed in Huh7.5.1 cells stably expressing IFITM1, 2 or 3. Similar to results observed for HCVpp, infection of Huh7.5.1 cells by HCVcc was inhibited by all three IFITMs (Figure 1D). Expression of IFITM proteins was confirmed by immunoblot (Figure 1E).

Finally, we studied the subcellular localization of IFTMs in Huh7.5.1 cells. Immunohistochemistry studies (Figure 1F, Figure S3) showed that IFITM1 was located at the plasma membrane, as shown by colocalization with SYFP tagged with a membrane-targeting signal, while IFITM2 and 3 were found in endosomal compartments, as shown by partial colocalization with the endosomal marker Rab7a (Figure S3). These observations are similar to previous results observed in Huh7 cells (14). In summary, these results demonstrate that infection of Huh7.5.1 with HCVpp or HCVcc is a suitable model to study the molecular mechanisms of inhibition of HCV infection by IFITM proteins.

Clinical variants isolated during acute HCV infection before seroconversion were more sensitive to anti-viral activity of IFITMs than variants from chronic infection.

To understand the role of IFITMs in the acute phase of infection, we analyzed the IFITM-sensitivity of HCVpp expressing viral envelopes of three HCV variants isolated from the same patient at three different time points post infection (UKNP1.5.1 pre-seroconversion; UKNP1.5.2

acute phase, two months later; UKNP1.5.3 chronic phase, 7 months later; Table 1). These variants vary at key residues, including residues near or within the CD81 binding sites (aa312; 439; 500; 536; 626; 742) (31) (Figure 2E). As shown in Figure 2A and Figure S4, HCVpp expressing envelopes of all variants were comparably susceptible to inhibition by all three tested IFITM proteins. Interestingly, the analysis of the HCVpp bearing sequential HCVpp envelope proteins revealed a marked and significant decrease of IFITM-sensitivity over time (Figure 2A). This decrease was not due to differences in the relative infectivities of the HCVpp (Figure 4A). Thus, infection mediated by the envelope proteins of variant UKNP1.5.1, isolated before seroconversion, and variant UKNP1.5.2, isolated right after seroconversion, was inhibited by 98% and 85% respectively. In contrast, transduction driven by the envelope proteins of variant UKNP1.5.3, which was isolated six months after seroconversion during the chronic phase of infection, was inhibited by only 60% upon directed expression of IFITM proteins (Figure 2A). The results obtained for UKNP1.5.3 were confirmed using an HCVcc chimera (Figure 2B), whereas the infectivity of HCVcc derived from the two other strains was too low to obtain conclusive results. Next, we investigated whether the differential IFITM-sensitivity of HCV envelope proteins obtained pre-seroconversion and during chronic infection could be confirmed with a larger panel of samples. For this, we analyzed the envelope proteins from six different early acute HCV patients and four variants derived from chronically infected HCV patients. Among the pre-seroconversion isolates, the highest susceptibility was observed for UKNP1.3.1 with more than 98-99 % inhibition of entry upon IFITM protein expression (Figure S4). UKNP1.6.1 was the most resistant with about 85 % inhibition (Figure S4), which correlated with the sensitivity to

neutralizing antibodies that was published previously (31). The neutralization sensitivity of the E1E2 proteins of this cohort was shown to be consistent in HCVpp and HCVcc models of infection (31), indicating the same holds true for their IFITM-sensitivity. When we compared the entry of HCVpp expressing pre-seroconversion envelope glycoproteins to entry of HCVpp bearing envelope glycoproteins derived from independent chronic samples of the same genotypes, we observed a significant and unexpected difference in IFITM-susceptibility. The HCVpp bearing envelopes from variants isolated from chronic infection post-seroconversion were much more resistant to inhibition by IFITM proteins (Figure 2C, 2D and Figure S4), independent of the genotype of the variants. Taken together, these results suggest that IFITMs may pose significant selective pressure on HCV during the acute phase of infection that can result in viral evasion. The identification of mutations unique to variants during chronic infection suggest their possible involvement in these interactions.

Clinical HCV variants associated with viral immune escape during liver transplantation are more resistant to inhibition by IFITM proteins than non-escape variants. To investigate the contribution of IFITM proteins to viral escape in chronic HCV infection, we took advantage of a well-characterized clinical cohort of patients undergoing liver transplantation with *de novo* infection of the liver graft (21, 22) (Table 1). In this cohort, variants selected post-transplantation are characterized by more efficient viral entry and escape from neutralizing antibodies (21, 22). We produced HCVpp bearing the full length E1/E2 proteins of variants differing in sensitivity to neutralizing antibodies and subsequently infected transiently transduced IFITM-expressing Huh7.5.1 cells. We observed that all patient-derived envelope

proteins were sensitive to inhibition by IFITM proteins. However, variants that were characterized by escape from the neutralizing antibody (nAb) response were less affected by expression of IFITM proteins than those that were sensitive to neutralizing antibodies, as shown for variants derived from two different patients (Figure 3A). This was confirmed by $TCID_{50}$ analyses on IFITM2 and 3 expressing cells using HCVcc chimeras expressing the envelope proteins of two representative variants (variant VL with nAb escape phenotype and variant VA with nAb sensitivity) isolated from the same patient (Figure 3B) (22). Next, we extended our analysis to 19 envelope variants (nine non-nAb escape and ten nAb escape-variants (Figure S5)) derived from five different patients and observed a significantly higher sensitivity to inhibition by IFITM2 (Figure 3C) and IFITM3 (Figure 3D) for non-nAb escape variants and a significantly higher resistance of nAb escape variants to inhibition by IFITM proteins. The direct comparison of entry efficiency and IFITM-sensitivity revealed no apparent correlation of these two variables (Figure 4B). This shows that indeed selection for IFITM-sensitivity post-liver transplantation and not just more efficient entry of the escape variants is responsible for the differential inhibition by IFITM proteins.

In summary, these results suggest that IFITM proteins are important determinants for viral escape and that escape from IFITM proteins is associated with resistance to antibody-mediated neutralization.

Neutralizing antibodies and IFITM proteins cooperatively block HCV entry. The differential inhibition of antibody escape and non-escape HCV strains by IFITM proteins in chronic infection as well as the enhanced IFITM-sensitivity of viral strains in the acute phase

prior to antibody development prompted us to analyze the interplay between the antiviral activities of IFITM proteins and the neutralizing B cell response in detail. For this, HCVpp were incubated with low concentrations of neutralizing sera prior to infection of transiently transduced IFITM2 expressing cells. Treatment with low concentrations of neutralizing patient serum (1:200) did not significantly reduce viral entry into IFITM-negative control cells (Figure 5A). IFITM2 expression reduced virus entry in the absence of neutralizing sera by ten-fold for the non-escape variant and five-fold for the escape variant, respectively (Figure 5A). When serum-treated HCVpp were used to infect IFITM2-expressing cells, we observed a marked increase in neutralization. The neutralization was about three- to four-fold higher compared to the control-treated HCVpp, although the same serum-treatment had no effect on IFITM-negative control cells. Furthermore, the increase in neutralization was significantly and markedly higher for the non-escape variants compared to the escape variants (Figure 5A), suggesting a potential role of the IFITM-mediated enhancement of neutralization as a determinant for viral escape. Titration of the neutralizing serum corroborated our finding that neutralizing antibodies and IFITM proteins cooperatively block virus entry. Inhibition correlated with the concentration of the neutralizing serum, as shown by the slope of the regression curves for neutralization on control or IFITM2-expressing cells (Figure 5B). The slope on IFITM2-expressing cells was more than ten-fold higher as compared to the IFITM-negative control cells confirming a marked enhancement of neutralization by IFITM2. Furthermore, the IFITM-mediated enhancement of neutralization was confirmed using HCVcc of genotype 1b (Con1). As shown in Figure 5C, expression of IFITM2

enhanced the neutralization of HCVcc Con1 by a weakly neutralizing heterologous serum (1:100 dilution) from less than two-fold to 60-fold (Figure 5C).

To assess which serum component was responsible for the enhanced neutralizing capacity of the sera in the presence of IFITM proteins we used HMAbs directed against different epitopes of the HCV E2 protein, some of which overlap with the polymorphic sites in acute patient variants. Similar as in experiments using sera, the HMAbs were used at sub-neutralizing concentrations (15 µg/mL) that in our model only had a low effect on virus entry inhibition (at maximum about 40 % or 1.67 fold inhibition by HC84.26.WH.5DL, lower for the other HMAbs) (Figure 5D). When IFITM2-expressing cells where infected with the HMAb-treated HCVpp we again observed a marked cooperative effect that directly correlated with the neutralizing properties of the antibody. Indeed, while the non-neutralizing HMAb CBH-20 did not exert a cooperative effect, the affinity matured anti-E2 antibody HC84.26.WH.5DL with potent neutralizing properties increased the inhibition of virus entry following IFITM2 expression to more than 100-fold (Figure 5D). The antibodies CBH-7, HC11 and CBH-2 had intermediate effects (Figure 5D).

Next, we confirmed these findings in the most physiologically relevant cell culture system: infection of PHH (Figure 5E). Similar to the Huh7.5.1 cells, treatment of the HCVpp with low concentrations of antibodies only had a minor influence on virus entry into naive PHH. As shown for Huh7.5.1 cells, IFITM2 expression blocked HCV entry into PHH, with the non-escape variant being more susceptible. Treatment with the neutralizing antibodies HC-11 and CBH-2 increased the neutralization of the non-escape variant on IFITM-expressing cells by about 3-fold. Again,

the increase in neutralization was markedly lower in the escape variants (Figure 5E), confirming the results that were obtained with Huh7.5.1 cells. Taken together, these data show that innate and adaptive immune responses targeting viral entry cooperate to inhibit HCV infection and drive viral immune evasion in acute and chronic HCV infection.

Cooperative inhibition of HCV entry by IFITM proteins and neutralizing antibodies is attenuated by treatment with amphotericin B. It is known that IFITM proteins restrict virus entry at the stage of hemifusion (16) or fusion pore formation (17) by altering curvature and fluidity of host cell membranes through direct or indirect mechanisms, which render virus-host cell fusion less energetically favorable (41, 42). Notably, the antiviral effect of IFITM2 and 3 on influenza virus infection is attenuated by incubation of host cells with amphotericin B, an amphiphilic antifungal drug that integrates into endosomal membranes (43), which can be regarded as an IFITM antagonist. Mechanistic studies revealed that the compound decreases the curvature and increases the fluidity of the endosomal membrane, which counteracts the IFITM-mediated antiviral effects within the endosomal membrane in an indirect manner (43), as illustrated in Figure 6D. We thus used amphotericin B to analyze whether IFITM-mediated modulation of membrane properties and the resulting inhibition of viral entry is required for the cooperative antiviral activity of antibodies and IFITM proteins. Treatment of cells with amphotericin B reduced HCVpp entry into the host cells by about three-fold (Figure 6A) and markedly reduced the antiviral activity of all IFITM proteins (Figure 6B). Moreover, amphotericin B treatment largely abrogated the cooperation of IFITMs and neutralizing sera in inhibition of HCVpp cellular entry (Figure 6C), indicating that IFITM-mediated modulation of cellular

membranes and the resulting antiviral activity are responsible for the cooperative inhibition of virus entry by IFITM proteins and antibodies (illustrated in Figure 6D). Interestingly, a similar enhancement of neutralization was observed using interferon-treatment of Huh7 cells (Figure S6) supporting our conclusion that the IFITM-mediated antiviral effect and not a direct interaction with the IFITM proteins is responsible for the enhancement of neutralization.

Discussion

In this study we provide conclusive evidence that IFITM proteins are important determinants of viral escape from antiviral B cell responses in patients. This is supported by our finding that viral envelope proteins obtained from acute pre-seroconversion patients showed an increased IFITM-sensitivity as compared to envelope proteins obtained from chronic patients. Furthermore, the functional analysis of HCV variants from patients escaping viral neutralizing responses during liver transplantation compared with variants that are eliminated post-transplantation revealed a direct correlation of escape from neutralizing responses and resistance to inhibition by IFITM proteins.

Our finding that HCV variants of acute patients isolated pre-seroconversion were more susceptible to inhibition by IFITM proteins than variants derived from chronically-infected patients indicates that IFITM proteins drive immune evasion. This is supported by our analysis of sequentially isolated envelope proteins from one patient. IFITM resistance increased over time, with the envelope proteins isolated during chronic infection showing the highest IFITM resistance. Furthermore, the acquisition of mutations within epitope II and the CD81 binding

domain that are targeted by neutralizing antibodies suggests that the IFITMs might modulate interactions with the adaptive immune response. Indeed, the analysis of post-transplant variants revealed a direct association of resistance to inhibition by IFITM proteins and escape from the nAb response. Escape of the virus from host responses is critical for viral spread and survival (44). In part, these findings could explain the low efficacy of innate immune activation in chronic HCV-infected patients (45). Escape from innate responses does not only prevent the immune system from clearing the viral infection but also limits the response to interferon-based therapies (44). Furthermore, the finding that variants selected post-liver transplantation and characterized by viral escape were significantly more resistant to inhibition by IFITM proteins than variants that were eliminated post-transplantation (with sensitivity to antibody-mediated neutralization) could also explain the rapid selection of these resistant variants. In addition, the distribution of the IFITM-sensitivity of the escape variants appeared to be less dispersed than that of the non-escape variants, potentially reflecting a bottleneck during the selection process. Differences in IFITM expression levels between host and graft tissue might drive the selection of highly infectious IFITM resistant variants, subsequently leading to reinfection of the graft, as universally observed.

How can mutations present in HCV in chronic patients confer relative IFITM resistance? For one, HCV variants that escape immune control frequently exhibit enhanced binding to the HCV co-receptor CD81 (22) and IFITM1 has been suggested to exert antiviral activity in part by interacting with CD81 (13). However, there are no reports of direct interactions of IFITM2 and 3 with CD81 and other HCV (co-)receptors (14), although IFITM3 seems to partially colocalize with

CD81 (14). Additionally, IFITM resistant HCV, like IFITM resistant influenza A viruses (46), might exhibit an altered pH-optimum for virus entry thereby avoiding the need to fuse with IFITM-rich internal membranes. Finally, IFITM-sensitivity might by linked to the number of viral glycoproteins incorporated into the viral membrane, as previously demonstrated for simian immunodeficiency virus (47) or to the composition of the viral membrane itself, however our previous characterization of HCV envelope glycoprotein variants proteins in liver transplantation indicate that an increased amount of incorporation of envelope proteins is most likely not responsible for the observed changes in IFITM-sensitivity (21). It remains to be determined whether IFITM-induced changes in membrane composition or altered interaction with lipoproteins contribute to the IFITM-mediated escape from the nAb response.

The proteins of the IFITM family are potent inhibitors of host cell entry of a wide range of enveloped viruses (11), including HCV (13, 14, 18). A single-cell analysis of clinical human liver samples by laser capture microdissection and qRT-PCR revealed that IFITM3 expression and HCV RNA were largely mutually exclusive (48), indicating an important role of the IFITM proteins in HCV cell tropism. A very recent publication indicates that stem cells, that do not respond to interferon, express high levels of interferon stimulated genes, including IFITM3, to protect them from viral infection (49). Constitutive expression of interferon stimulated genes is lost upon differentiation into hepatocyte-like cells (49) but becomes interferon-inducible, highlighting an important contribution of the IFITM proteins to the innate defenses against HCV and other pathogens.

Perturbation studies with amphotericin B that acts as an IFITM-antagonist in the context of influenza virus infections (43) revealed that modulated membrane-properties are responsible for the IFITM-mediated enhancement of neutralization, indicating that other innate entry effectors, as shown for pretreatment with interferon-alpha, might exploit similar inhibitory mechanisms to block virus entry.

Interestingly, a recent study has suggested a different role for IFITMs in clinical HIV-1 infection: While transmitted founder viruses where almost resistant to inhibition by IFITM proteins, the virus became more susceptible over time, as it escaped from the nAb response (50). This observation could reflect different roles of innate immune responses in HCV and HIV infections with our findings supporting a much more prominent role of the IFITMs during acute HCV infection compared to HIV. Furthermore, the increased IFITM-sensitivity could in part explain the high susceptibility of acute HCV infection to interferon treatment. On a mechanistic point of view, IFITM-sensitivity of HIV was associated with receptor-usage, with CCR5-tropic viruses being generally more resistant to IFITM proteins than CXCR4-tropic viruses (50). This suggests that the differential sensitivity of HIV to inhibition by the IFITM proteins might be due to receptor-mediated targeting to subcellular compartments with differential IFITM expression, or due to changes in envelope structure and electrochemical properties due to the switch of receptor tropism from CCR5 to CXCR5, which was not observed for HCV infection.

Taken together, our findings show that IFITMs are important drivers of viral immune evasion in acute and chronic HCV infection by enhancing antibody—mediated neutralization. Harnessing these effects will help to facilitate the design of protective B cell HCV vaccines.

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Author names in bold designate shared first authorship

Figure Legends

Figure 1: Directed IFITM expression inhibits HCV infection in cell culture. Huh7.5.1 (A and B) or HepG2-CD81 (C) cells were transduced with vectors encoding the indicated IFITM proteins or were transduced with empty vector as control. Transduced cells were then infected with HCVpp of GT1b (A), GT1a (C) or with MLVpp (B). Infection was assessed after 72 h by measuring luciferase activity. Individual representative experiments are shown. Error bars represent SD. Similar results were obtained in more than three independent experiments. (D) Huh7.5.1 cells stably expressing IFITMs were infected with HCVcc Luc-Jc1. Infection efficiency was assessed after 72 h by measuring luciferase activity. Shown is a representative of three independent experiments, error bars indicate SD. (E) Expression of IFITM proteins in Huh7.5.1 cells was assessed by immunoblot using IFITM-specific antibodies. β-Actin is used as loading control. One representative western blot is shown (F). Huh7.5.1 cells transduced with IFITM1-3 or a control vector were stained with a mouse anti-IFITM1 or a rabbit anti-IFITM2 antibody (IFITM2 and 3).

Figure 2: Differential sensitivity of acute and chronic HCV variants to inhibition by IFITM proteins. Huh7.5.1 cells were transduced to express the indicated IFITM proteins and then infected with HCVpp bearing envelope proteins derived from acute or chronic patients. (A) Analysis of the IFITM-sensitivity of sequential envelope variants (UKNP1.5.1 pre-seroconversion; UKNP1.5.2 acute phase, two months later; UKNP1.5.3 chronic phase, 7 months later), isolated from a single HCV patient. (B) $TCID_{50}$ analysis of UNKP1.5.3 HCVcc infection of

transduced IFITM-expressing Huh7.5.1 cells. Shown are means of two experiments performed in sextuplicates. Error bars represent SD. (C) and (D) E1E2 patient variants isolated from patients with acute or chronic HCV infection of genotype 1A and 3. Each dot represents the result for a single envelope variant and IFITM2- (C) or IFITM3-expressing (D) Huh7.5.1 cells. Shown are the means of three experiments conducted in triplicates. Error bars represent SEM. Control was set to 100%. *P < 0.05, ** P < 0.01 using one-sided students T-test. (E) Clustal O Alignment of the protein sequences of H77c, UKNP1.5.1, UKNP1.5.2 and UKNP1.5.3. The sequences were obtained from GenBank. Changes highlighted in yellow are unique for UKNP1.5.1, changes in green are only present in UKNP1.5.2 and changes highlighted in red are unique for UKNP1.5.3. HVR1 and 2 are indicated in blue, red letters mark key positions for CD81-binding.

Figure 3: IFITMs differentially restrict HCV variants isolated from patients undergoing liver transplantation. Huh7.5.1 cells were transduced to express the indicated IFITM proteins and then infected with HCVpp pseudotyped with HCV E1E2 patient variants isolated from patients undergoing liver transplantation. Infection was assessed after 72 h by measuring luciferase activity. (A) Results for variants from two patients are expressed as means ± SEM percentage HCVpp infection compared to control cells (set at 100%) from three independent experiments performed in triplicate. (B) TCID50 analysis of HCVcc infection of IFITM-expressing Huh7.5.1 cells. Shown are means of three experiments performed in sextuplicates. Error bars represent SEM. Control was set to 100 %. (C and D) Each dot represents the result for a single

variant. Results for % Infection of IFITM2 (C) and IFITM3 (D) positive cells compared to the control of HCV variants from five different patients. *P < 0.05; ** P < 0.01 one-sided Students T-test. Shown are the means of three experiments performed in triplicates.

Figure 4: The antiviral effect of the IFITMs is independent of virus infectivity. Huh7.5.1 cells were transduced to express IFITM proteins and infected with HCVpp bearing the envelope proteins of the indicated variants. (A) Shown are three individual datasets of the experiments that are featured in figure 2A. (B) Correlation of relative infectivity (highest infectivity set to 1) and relative IFITM-sensitivity (highest IFITM-sensitivity set to 1, lowest to 0). Each dot represents one of the escape variants shown in Figure 3C and 3D. Shown are the results of one representative experiment performed in triplicate (n=9).

Figure 5: IFITM2 enhances antibody-mediated neutralization of HCV cell entry. Huh7.5.1 cells (A, B, C, D) or primary human hepatocytes (E) were transduced by retroviral vectors coding for IFITM2 or empty vector as control. Forty-eight hours after transduction cells were infected with HCVpps expressing the envelope of variants selected during liver graft infection associated or not-associated with viral escape (A,B,D,E), or with HCVcc of genotype 1b (Con1) (C). Before infection, the particles were coincubated with serum derived from chronically HCV infected patients (A, B, C) or 15 µg/ml of the patient-derived HMAbs CBH-20 CBH-7, HC11, CBH-2 and HC84.26.WH.5DL or control antibody R04 (D, E) at 37 °C for 1 h. Entry of HCVpp was assessed 72 h post infection by measuring luciferase activity. Results are shown as fold inhibition of virus

entry. Inhibition of entry by control vector in combination with the control antibody R04 or with control serum was set to 1. (B) The equation of the regression curve of anti-HCV serum and IFITM2 expressing cells was calculated as 48748 x^2+ 640 x + 1.9 The corresponding curve for anti-HCV serum on naïve cells was 4380 x^2+ 149 x + 1.1 (C) Infection with HCVcc was analyzed 72 h post infection by measuring luciferase activity. The graph represents means of three (D) or four (A, B) experiments that were performed in triplicates. Error bars represent SEM. (C) Shows a representative experiment performed in triplicates (n=6). (E) Represents a single experiment performed in PHH. Error bars show SD.

Figure 6: Cooperative inhibition of HCV entry by IFITM proteins and neutralizing antibodies can be attenuated by treatment with amphotericin B. Huh7.5.1 cells were transduced by retroviral vectors coding for an empty control vector (A), with vectors coding for IFITM1, 2 and 3 and empty vector as control (B) or only IFITM2 plus control (C) Forty-eight h after transduction cells were treated with vehicle control or 5 µg/ml amphotericin B for 1 h. Afterwards, the cells were infected with HCVpp expressing the envelope of a variant not associated with viral escape and sensitive to antibody-mediated neutralization (P1VA). (C) Cells were infected with HCVpp pretreated with serum derived from chronically HCV infected patients or with control serum at 37 °C for 1 h. Entry of HCVpp was assessed 72 h post infection by measuring luciferase activity. (A) Results are shown in RLU. (B,C) Results were normalized for the vector control and are shown as fold inhibition compared to the respective controls. Shown are the means of representative experiments performed in triplicates (n=6) ± SD. (D) Model of

cooperative inhibition of HCV entry by IFITMs and neutralizing antibodies and the antagonistic effect of amphotericin B. The interaction between infectious particles and cell surface receptors triggers endocytosis. Entry is blocked by IFITM proteins and neutralizing antibodies. Amphotericin B (AmphoB) is believed to rescue virus entry by antagonizing the IFITM-mediated increase of membrane rigidity and curvature.

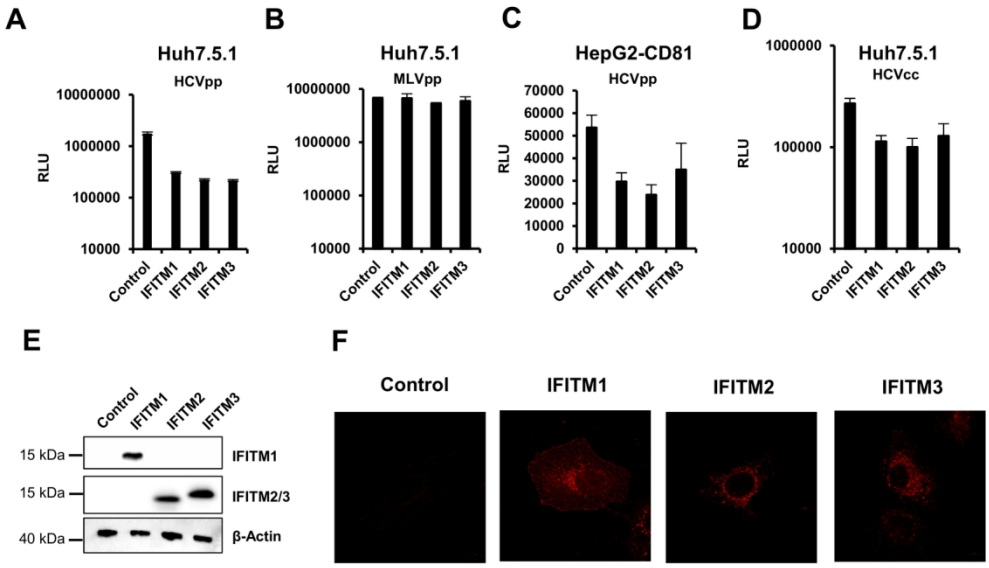

Figure 1: Directed IFITM expression inhibits HCV infection in cell culture.

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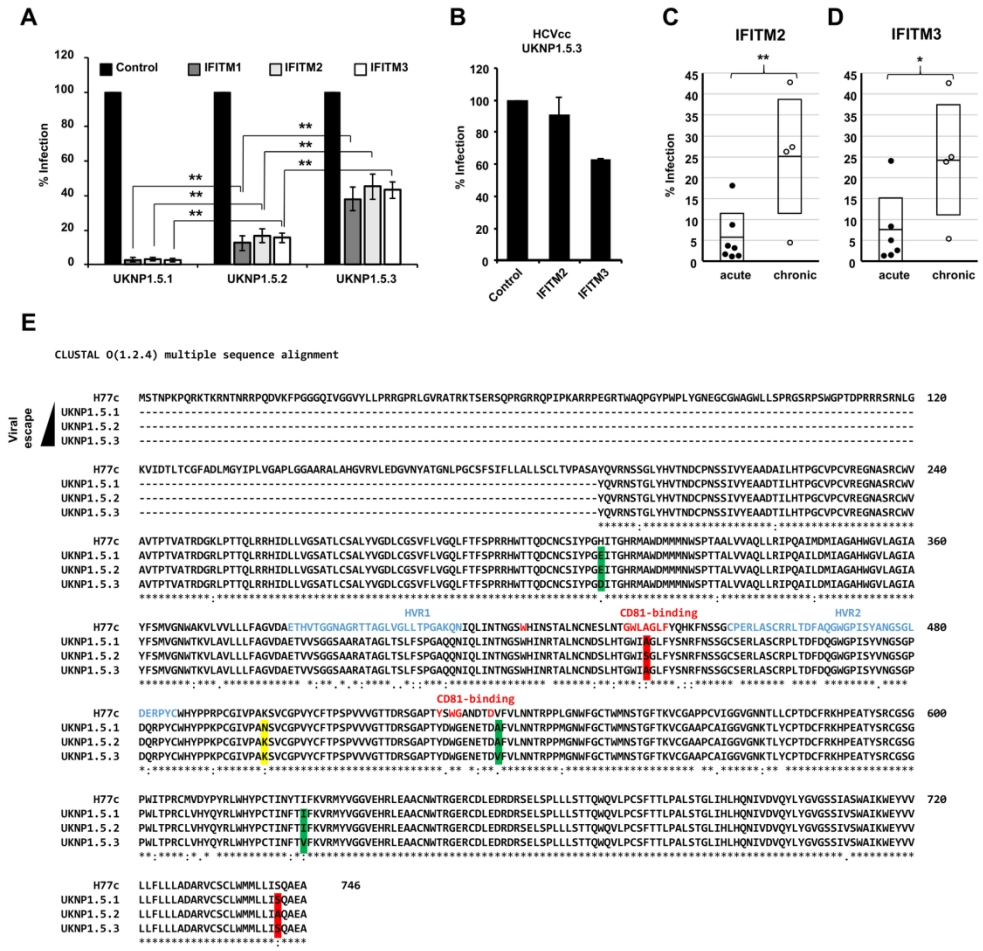

Figure 2: Differential sensitivity of acute and chronic HCV variants to inhibition by IFITM proteins.

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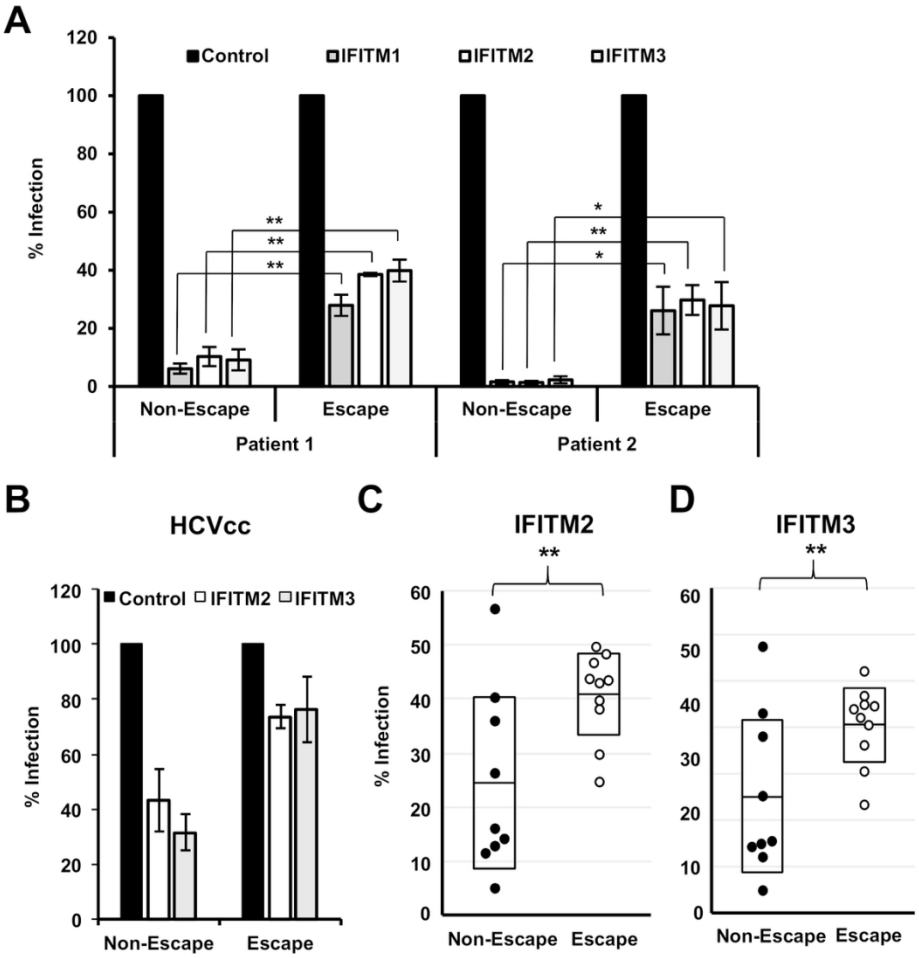

Figure 3: IFITMs differentially restrict HCV variants isolated from patients undergoing liver transplantation.

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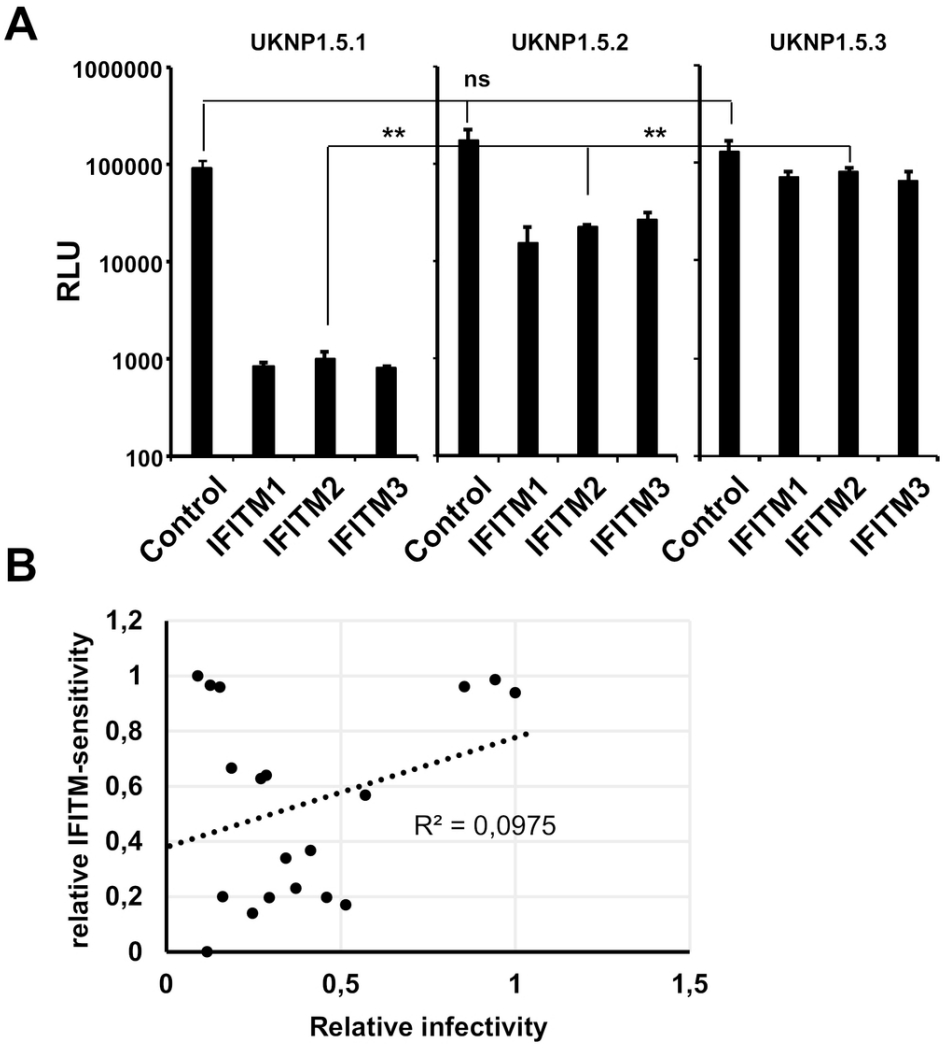

Figure 4: The antiviral effect of the IFITMs is independent of virus infectivity.

90x100mm (300 x 300 DPI)

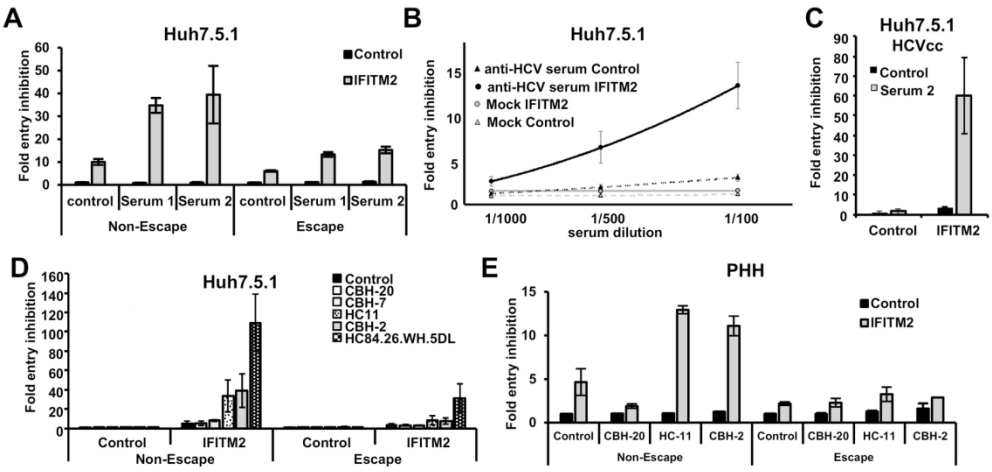

Figure 5: IFITM2 enhances antibody-mediated neutralization of HCV cell entry.

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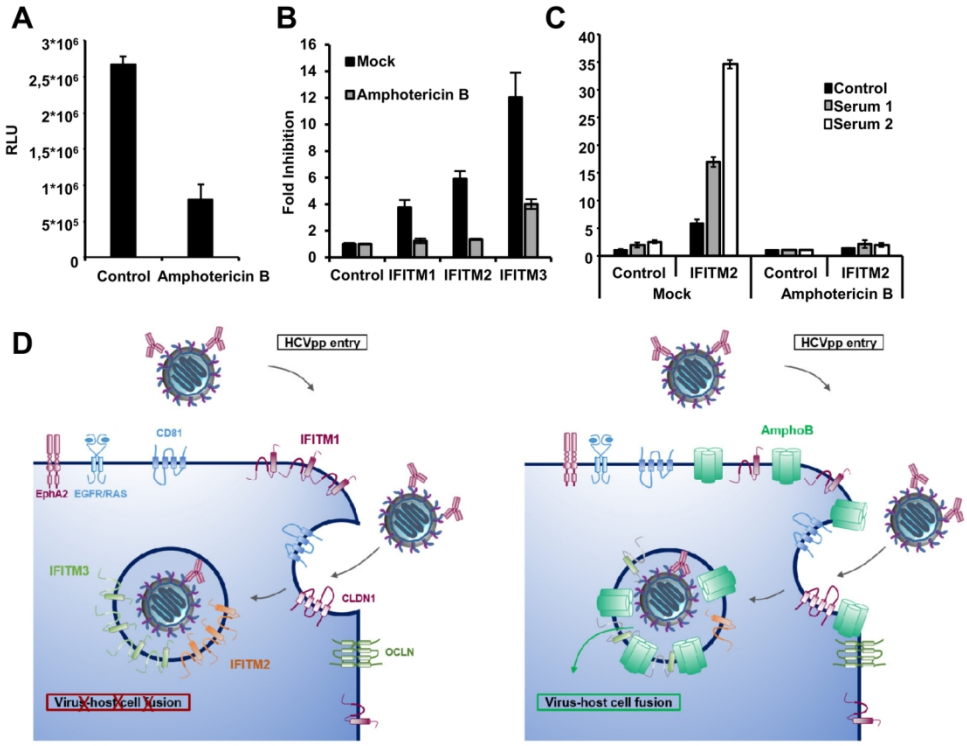


Figure 6: Cooperative inhibition of HCV entry by IFITM proteins and neutralizing antibodies can be attenuated by treatment with amphotericin B.

139x106mm (300 x 300 DPI)

Table 1: **HCV strains used in the study.** Strain name, genotype, clinical setting, phenotype and reference are shown. Abbreviations: LT – liver transplantation. N. T. not tested.

Name	Genotype	Acute/chronic	Neutralization Escape	Citation
P1VA	1b	chronic/LT	-	(22)
P1VC	1b	chronic/LT	-	(22)
P1VI	1b	chronic/LT	-	(22)
P1VK	1b	chronic/LT	-	(22)
P1VL	1b	chronic/LT	+	(22)
P2VA	1b	chronic/LT	-	(22)
P2VD	1b	chronic/LT	-	(22)
P2VH	1b	chronic/LT	+	(22)
P2VI	1b	chronic/LT	+	(22)
P2VJ	1b	chronic/LT	+	(22)
P3VA	1b	chronic/LT	-	(22)
P3VB	1b	chronic/LT	-	(22)
P3VC	1b	chronic/LT	+	(22)
P5VD	1b	chronic/LT	+	(22)
P5VE	1b	chronic/LT	+	(22)
P5VF	1b	chronic/LT	+	(22)
P6VD	1b	chronic/LT	-	(22)
P6VI	1b	chronic/LT	+	(22)
P6VH	1b	chronic/LT	+	(22)
UKNP1.3.1	1a	acute	N. T.	(32)
UNKP1.4.1	1a	acute	N. T.	(32)
UKNP1.5.1	1a	acute	N. T.	(32)
UKNP1.5.2	1a	acute	N. T.	(32)
UKNP1.5.3	1a	chronic	N. T.	(32)

UKNP1.6.1	1a	acute	N. A,	(32)
UKN1a.16.16	1a	acute	N. T.	This study
UKNP3.2.1	3	acute	N. T.	(32)
HCV-J	1b	chronic	N. T.	(33)
UKN1A14.38	1a	chronic	N. T.	(34)
UKN3A1.22	3a	chronic	N. T.	(34)
3a SXB	3a	chronic	N. T.	(35)

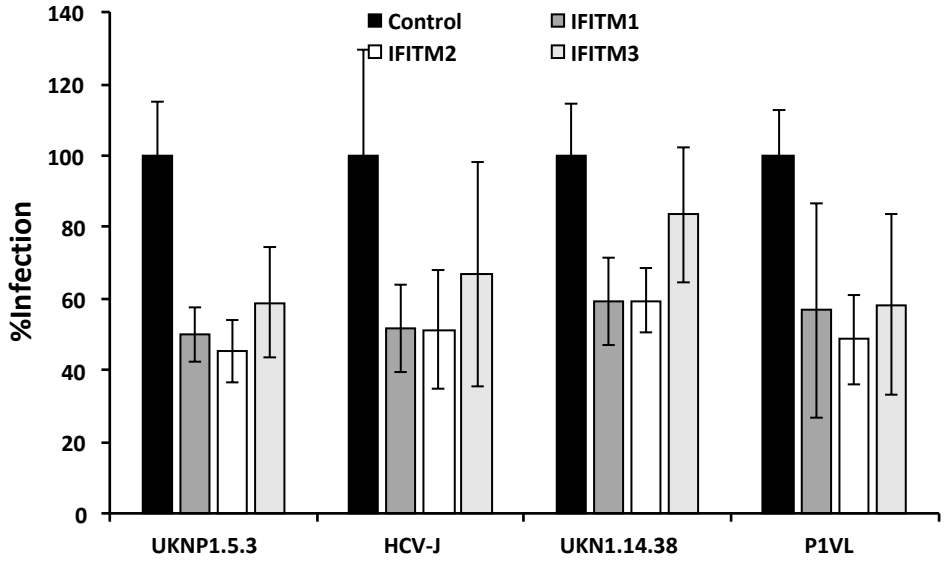
IFITMs mediate viral evasion in acute and chronic hepatitis C virus infection

Florian Wrensch, Gaëtan Ligat, Laura Heydmann, Catherine Schuster, Mirjam B. Zeisel, Patrick Pessaux, François Habersetzer, Barnabas J. King, Alexander W. Tarr, Jonathan K. Ball, Michael Winkler, Stefan Pöhlmann, Zhen-yong Keck, Steven K.H. Foung, Thomas F. Baumert

Supplementary Information

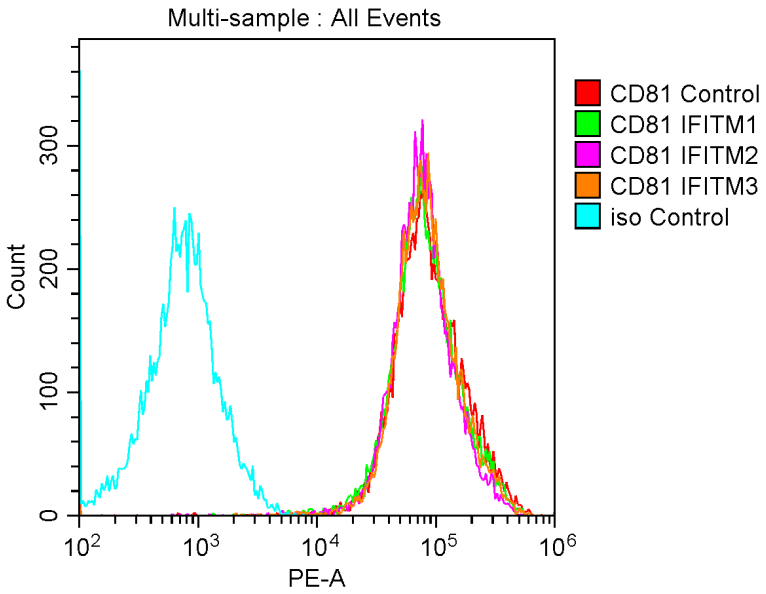
Supplementary Figures

Figure S1: Antiviral activity of IFITM proteins in polarized HepG2-CD81 cells.



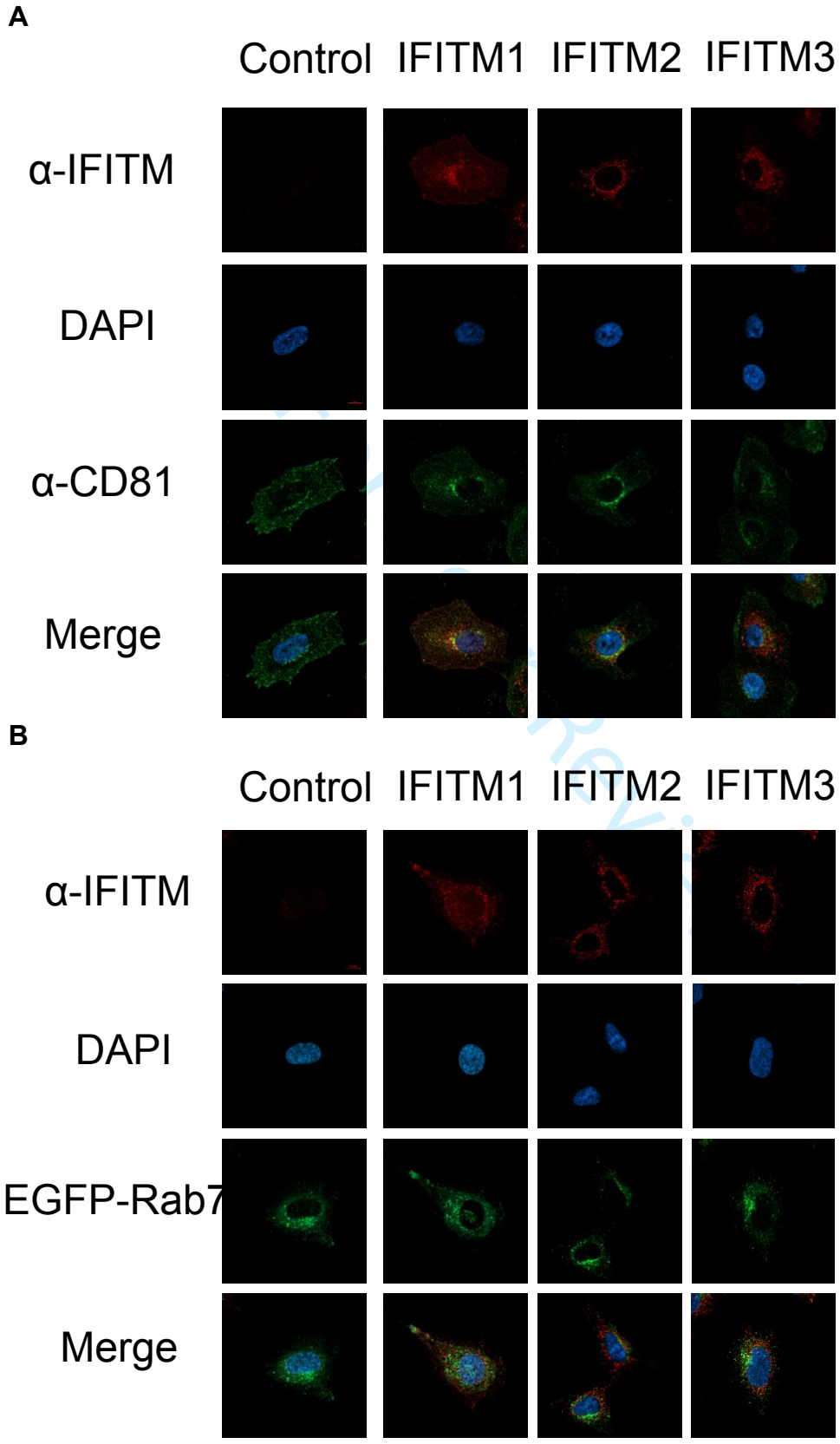
HepG2 cells stably expressing CD81 were transduced with to express IFITM1, 2 or 3, or empty vector as control for 2 days. Transduced cells were then infected for 3 days with HCVpp pseudotyped with HCV E1E2 patient variants isolated from chronic patients. Infection was assessed after 72 h by measuring luciferase activity. Results are displayed as % Infection compared to the empty vector control. Shown are the means of three experiments performed in triplicates.

Figure S2. Unchanged CD81 expression in IFITM-expressing cells.



Huh7.5.1 cells were transduced to express IFITM proteins or with an empty plasmid as control. Cells were harvested 48 h after transduction, washed with PBS and CD81 expression was detected with a mouse anti-CD81 antibody (BD Pharmingen) at 5 µg/ml for 45 min. Staining was done with a secondary goat anti mouse-PE antibody.

Figure S3: Intracellular localization of IFITM proteins.



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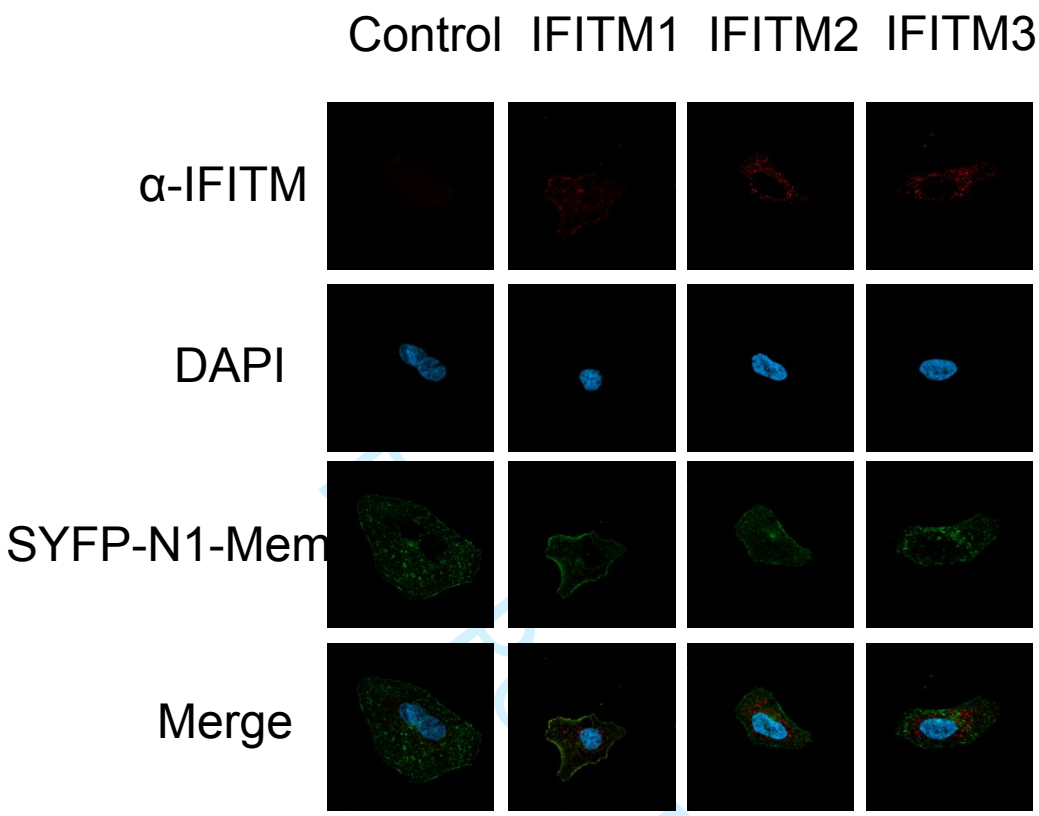
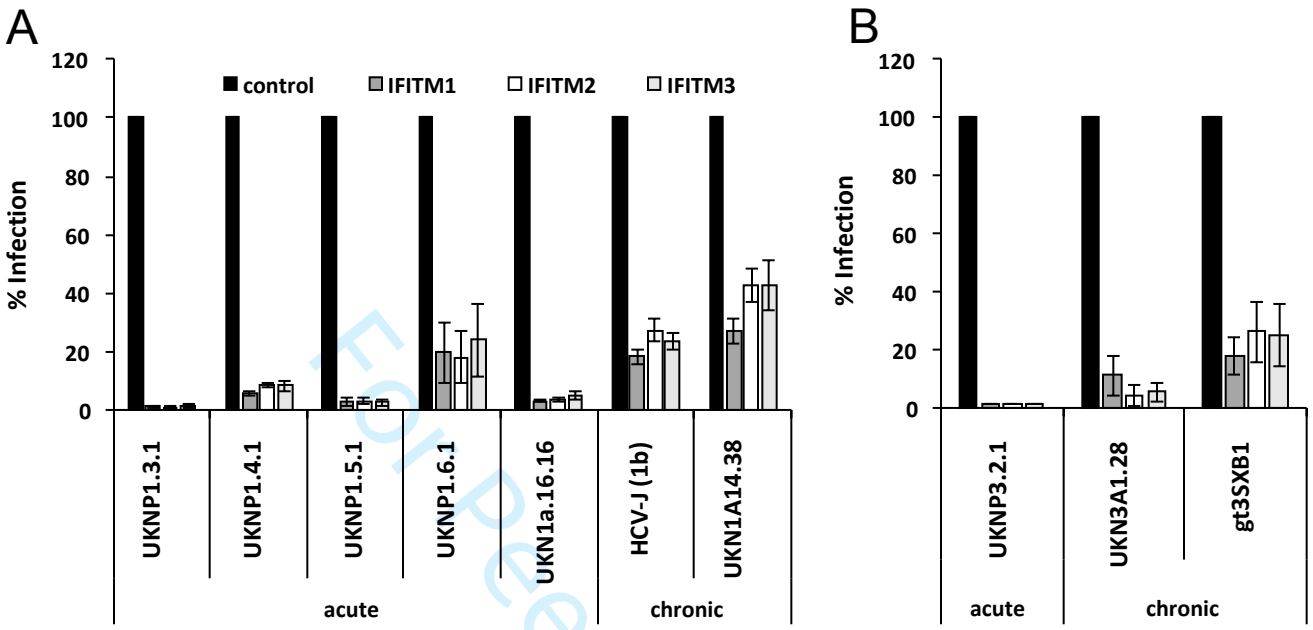


Huh7.5.1 Cells were seeded in 24 well plates at 50.000 cells per well and transduced to express IFITM proteins or a control vector on the next day. Cells in (B) and (C) were cotransduced with vectors coding for EGFP-Rab7a or SYFP-N1-Mem (SYFP with a membrane targeting signal) respectively. After 3 days, cells were detached and seeded into 24-well plates equipped with coverslips (12 mm diameter) at 10-fold dilution. After overnight growth incubation, cells were fixed in 4% paraformaldehyde for 10 min and subsequently permeabilized in PBS/0.2 % triton for 5 min followed by washing in PBS/0.1% tween. To block unspecific binding, cells were incubated for 1 h at room temperature in PBS containing 1% bovine serum albumine and 10% human or bovine serum. The cells were thereafter incubated for 1 h at room temperature with primary antibodies diluted in blocking solution followed by three wash steps in PBS/0.1% tween. As primary antibody we used mouse anti-IFITM1 (1:200 dilution), rabbit anti-IFITM2 (1:200 dilution) (both from Proteintech) or rat anti-CD81. Subsequently, cells were incubated 1 h at room temperature with secondary antibodies diluted in blocking solution

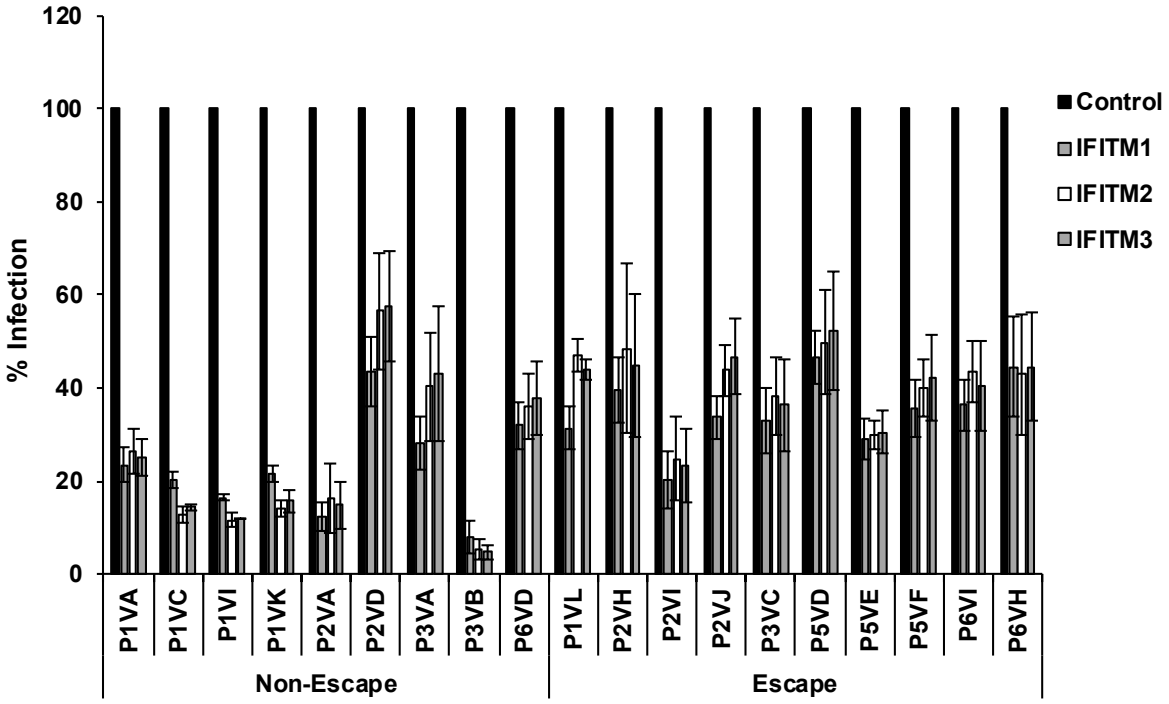
followed by three wash steps in PBS/0.1% tween. As secondary antibodies we used highly cross-adsorbed anti-mouse AlexaFluor488 (1:2000 dilution), anti-rat AlexaFluor488 (1:2000 dilution), anti-mouse AlexaFluor546 (1:500 dilution) or anti-rabbit AlexaFluor546 (1:500 dilution) (all from ThermoFisher). DAPI or Hoechst 33342 were added in the last wash step before coverslips were mounted in Mowiol/DABCO onto glass slides. Imaging was performed on a LSM800 Airyscan (Zeiss) equipped with solid state 405 nm, 488 nm and 561 nm laser lines, GaAsP detectors and a 63x/1.4 NA oil immersion objective. Filter settings were set with the ZEN 2.3 software (Zeiss) to minimize cross talk. Images were taken at the same laser power and gain for a whole image series and were only adjusted to avoid overexposure. Images were recorded at 2048x2048 px with 4-fold averaging. Processing of images for publication was done with ZEN lite.

Figure S4: Differential sensitivity of acute and chronic HCV variants of Gt1a and Gt3 to inhibition by IFITM proteins.



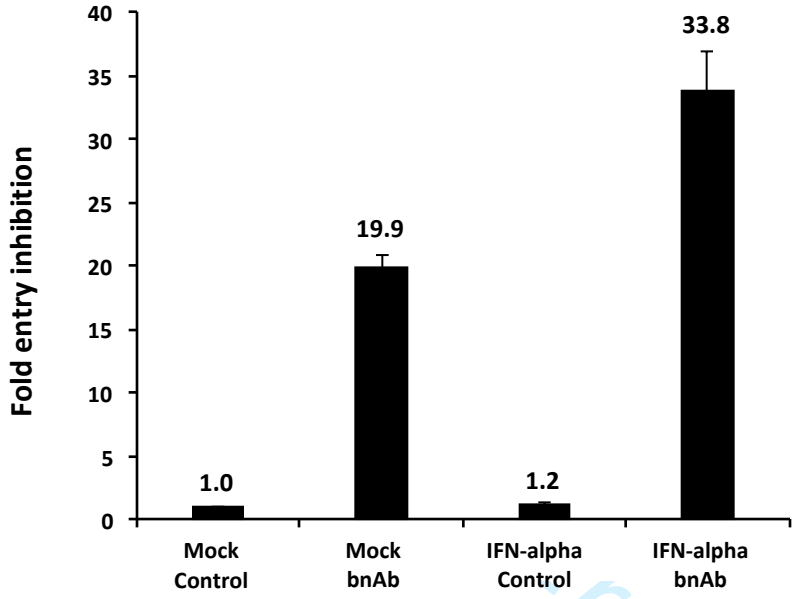
Huh7.5.1 cells were transduced to express the IFITM proteins or an empty control vector and subsequently infected with HCVpp expressing the envelope of variants isolated during acute or chronic HCV infection of genotype 1 (A) or genotype 3 (B). Entry of HCVpp was assessed 72 h post infection by measuring luciferase activity. Results are shown as % infection compared to empty vector control

Figure S5: IFITMs differentially restrict HCV patient variants isolated from patients undergoing liver transplantation.



Huh7.5.1 cells were transduced with an empty vector, pQCXIP-hIFITM1, pQCXIP-hIFITM2 or pQCXIP-hIFITM3 for 2 days. Transduced cells were then infected for 3 days with HCVpp pseudotyped with HCV E1E2 patient variants isolated from patients undergoing liver transplantation. Infection was assessed after 72 h by measuring luciferase activity. Results are displayed as % Infection compared to the empty vector control.

Figure S6: Interferon-alpha enhances neutralization by broadly neutralizing antibodies in Huh7 cells.



Huh7 cells were preatreated with 1000 U/mL of Interferon-alpha for 2 h. Huh7 cells were infected HCVpp bearing an envelope variant associated with viral escape. Before infection, the particles were coincubated with 5 µg/ml of the bnAb HC84.26.WH.5DL or with control antibody R04 at 37 °C for 1 h. Entry of HCVpp was assessed 72 h post infection by measuring luciferase activity. Results are shown as fold inhibition of virus entry. Inhibition of entry by control vector in combination with the control antibody R04 was set to 1. The graph represents the means of two experiments that were performed in triplicates.