Interferon-Induced Transmembrane Proteins Mediate Viral Evasion in Acute and Chronic Hepatitis C Virus Infection

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Although 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 (nAb) response in clinical cohorts of HCV-infected patients. Using pseudoparticles bearing HCV envelope proteins from acutely infected patients, we show that HCV variants isolated preseroconversion are more sensitive to the antiviral activity of IFITMs than variants from patients isolated during chronic infection postseroconversion. Furthermore, HCV variants escaping nAb responses during liver transplantation exhibited a significantly higher resistance to IFITMs than variants that were eliminated posttransplantation. Gain-of-function and mechanistic studies revealed that IFITMs markedly enhance the antiviral activity of nAbs 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 indicate IFITM proteins as drivers 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. (Hepatology 2019;70:1506-1520).

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

Abbreviations: HCV, hepatitis C virus; HCVcc, cell-culture–derived HCV; HCVpp, HCV pseudoparticle; HIV, human immunodeficiency virus; HMAb, human monoclonal antibody; IFITM, interferon-induced transmembrane protein; IFN, Interferon; IgG, immunoglobulin G; LT, liver transplantation; MLV, murine leukemia virus; nAb, neutralizing antibody; PHHs, primary human hepatocytes; TCID50, tissue culture infectious dose 50%.
countries. Moreover, in the majority of cases, HCV infection remains undiagnosed or is diagnosed at a late stage because of 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) induce virus neutralizing antibodies (nAbs), and be safe in healthy volunteers. (7) Furthermore, broadly virus nAbs 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) Although 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 (IFNs) stimulate the expression of a set of more than 300 IFN-stimulated genes, several of which have been shown to exert antiviral activity against HCV. (11) A family of these genes, the IFN-induced transmembrane (IFITM) proteins, are potent inhibitors of host cell entry of a broad range of enveloped viruses, including HCV. (12-15) Whereas IFITM1 is primarily located at the cytoplasmic membrane and restricts HCV entry by interacting with the HCV coreceptor, 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)

Whereas 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 given that it allows the study of HCV infection and viral escape in a very well-defined time frame 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 nAbs during HCV cell entry. For this, we used HCV pseudoparticles (HCVpp) bearing envelopes from patients with acute infection before seroconversion or patients...
undergoing liver transplantation (LT) because of chronic hepatitis C. Moreover, we utilized neutralizing human monoclonal antibodies (HMAbs) derived from patients with chronic HCV infection.

Materials 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 (DC-2016-2616).

CELL LINES AND PRIMARY HUMAN HEPATOCYTES

HEK293T, Huh7.5.1, Huh7.5.1-NTCP, and HepG2-CD81 cells were isolated and cultured as described. Primary human hepatocytes (PHHs) were isolated from liver resections as described.

ANTIBODIES

The anti-E2 HMAbs (CBH-20, CBH-7, CBH-22, and HC84.26.WH.5DL) and human anti-HCV sera have been described. The antibodies directed against IFITM1, IFITM2/3 (Proteintech, Manchester, UK), and β-actin (Sigma-Aldrich, St. Louis, MO) and the protocols for detection of IFITM proteins by western blotting and immune fluorescence have been described in a previous work.

PLASMIDS

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. The plasmids coding for the envelope proteins and HCVcc chimera bearing envelope proteins isolated from patients undergoing LT were described in a previous work. The plasmids encoding the envelope proteins of the chronic variants are described in Lavillette et al. (UKN1A14.38 and UKN3A1.28), Kato et al. (HCV-J), and in Colpitts et al. (gt3SXBl). The full-length chimeric clone incorporating the UKN1.5.3 E1E2 genes was generated in the H77/JFH-1 virus background, using described methods.

STATISTICAL ANALYSIS

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 one-tailed Student t test was used for single comparisons. A P value of <0.05 was considered statistically significant.

VECTOR PRODUCTION, TRANSDUCTION, AND SELECTION OF STABLE CELLS

Retroviral vectors for transduction were generated by transfection of HEK293T cells as described, 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. For transduction, cells were seeded at subconfluent density and spin-inoculated with the retroviral vectors at 4,000 g for 30 minutes. 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 of puromycin.

HCVpp PRODUCTION, INFECTION, AND NEUTRALIZATION

HCVpp were generated by transfection of HEK293T cells as described. To study HCV entry, HCVpp were added to IFITM-transduced Huh7.5.1, Huh7.5.1-NTCP cells, or PHHs in triplicate and incubated for 72 hours at 37°C. HCV entry was determined by analysis of luciferase reporter gene expression as described. For the study of antibody-mediated neutralization, HCVpp were mixed with autologous anti-HCV serum, control serum,
anti-E2 HMAbs, or irrelevant isotype control immunoglobulin G (IgG), preincubated for 30 minutes at 37°C, and added to Huh7.5.1, Huh7.5.1-NTCP cells, or PHHs in triplicate for 72 hours at 37°C. To assess the effect of amphotericin B on the cooperative inhibition of HCV entry by IFITM proteins and nAbs, Huh7.5.1 cells were treated with 5 µmol/L of amphotericin B (Sigma-Aldrich) for 1 hour at 37°C before infection with antibody-treated HCVpp.

**HCVcc PRODUCTION, INFECTION, AND NEUTRALIZATION**

Plasmids for HCVcc production of Jc1 and Con1 chimera with luciferase reporter (Luc-Jc1 and Con1-R2A) have been described. HCVcc were produced in Huh7.5.1 cells as described. Infectivity was quantified by luciferase activity or by determining the tissue culture infectious dose 50% (TCID50). HCVcc neutralization, using patient serum, IgG, and monoclonal antibodies, was analyzed as described.

**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 because NTCP has been described to have a functional role in regulation of IFN-stimulated gene expression. Given that 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 (Fig. 1A). Pseudoparticles bearing the envelope protein of the IFITM-resistant retrovirus, MLV, were used as a negative control (Fig. 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 because of an altered subcellular localization of IFITM1 in hepatocytes, as reported by Wilkins et al., 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 noted for nonpolarized Huh7.5.1 cells (Fig. 1C and Supporting Fig. 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 (Supporting Fig. S2) or distribution (Supporting Fig. S3). We next assessed the effect of IFITM proteins on HCV entry in the context of authentic virus using infectious HCVcc. 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 (Fig. 1D). Expression of IFITM proteins was confirmed by immunoblotting (Fig. 1E).

Finally, we studied the subcellular localization of IFITMs in Huh7.5.1 cells. Immunohistochemistry studies (Fig. 1F and Supporting Fig. S3) showed that IFITM1 was located at the plasma membrane, as shown by colocalization with SYFP tagged with a membrane-targeting signal, whereas IFITM2 and 3 were found in endosomal compartments, as shown by partial colocalization with the endosomal marker, Rab7a (Supporting Fig. S3). These observations are similar to previous results observed in Huh7 cells. 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 ANTIVIRAL ACTIVITY OF IFITMs THAN VARIANTS FROM CHRONIC INFECTION**

To understand the role of IFITMs in the acute phase of infection, we analyzed IFITM sensitivity...
of HCVpp expressing viral envelopes of three HCV variants isolated from the same patient at three different time points postinfection (UKNP1.5.1 preseroconversion; UKNP1.5.2 acute phase, 2 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) (Fig. 2E). HCVpp expressing envelopes of all variants were comparably susceptible to inhibition by all three tested IFITM proteins (Fig. 2A and Supporting Fig. S4). Interestingly, the analysis of the HCVpp bearing sequential HCVpp envelope proteins revealed a marked and significant decrease of IFITM sensitivity over time (Fig. 2A). This decrease was not attributed to differences in the relative infectivities of the HCVpp (Fig. 4A). Thus, infection mediated by the envelope proteins of variant UKNP1.5.3, which was isolated 6 months after seroconversion during the chronic phase of infection, was inhibited by only 60% upon directed expression of IFITM proteins (Fig. 2A). The results obtained for UKNP1.5.3 were confirmed using an HCVcc chimera (Fig. 2B), whereas 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 preseroconversion and during chronic infection could be confirmed with a larger panel of samples. For this, we analyzed the envelope proteins from 6 different early-acute HCV patients and four variants derived from chronically infected HCV patients. Among the preseroconversion isolates, the highest susceptibility was observed for UKNP1.3.1 with more than 98%-99% inhibition of entry upon IFITM protein expression (Supporting Fig. S4). UKNP1.6.1 was the most resistant with around 85% inhibition (Supporting Fig. S4), which correlated with the sensitivity to nAbs that was published. (31) Neutralization sensitivity of the
E1E2 proteins of this cohort was shown to be consistent in HCVpp and HCVcc models of infection, indicating that the same holds true for their IFITM-sensitivity. When we compared the entry of HCVpp expressing preseroconversion 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 postseroconversion were much more resistant to inhibition by IFITM proteins (Fig. 2C,D and Supporting Fig. 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 LT ARE MORE RESISTANT TO INHIBITION BY IFITM PROTEINS THAN NONESCAPE VARIANTS

To investigate the contribution of IFITM proteins to viral escape in chronic HCV infection, we took...
FIG. 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 preseroconversion; UKNP1.5.2 acute phase, 2 months later; UKNP1.5.3 chronic phase, 7 months later), isolated from a single HCV patient. (B) TCID₅₀ analysis of UKNP1.5.3 HCVcc infection of transduced IFITM-expressing Huh7.5.1 cells. Shown are means of two experiments performed in sextuplicates. Error bars represent SD. (C,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-tailed Student 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. Abbreviations: HVR1, hypervariable region 1; HVR2, hypervariable region 2.

advantage of a well-characterized clinical cohort of patients undergoing LT with de novo infection of the liver graft (21,22) (Table 1). In this cohort, variants selected posttransplantation are characterized by more-efficient viral entry and escape from nAbs (21,22). We produced HCVpp bearing the full-length E1E2 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 nAb response were less affected by expression of IFITM proteins than those that were sensitive to nAbs, as shown for variants derived from 2 different patients (Fig. 3A). This was confirmed by TCID\textsubscript{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 (Fig. 3B).\textsuperscript{(22)} Next, we extended our analysis to 19 envelope variants (nine non-nAb escape and 10 nAb escape variants (Supporting Fig. S5) derived from 5 different patients and observed a significantly higher sensitivity to inhibition by IFITM2 (Fig. 3C) and IFITM3 (Fig. 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 (Fig. 4B). This shows that indeed selection for IFITM sensitivity post-LT, and not just more efficient entry of the escape variants, is responsible for the differential inhibition by IFITM proteins.

FIG. 3. IFITMs differentially restrict HCV variants isolated from patients undergoing LT. 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 LT. Infection was assessed after 72 hours by measuring luciferase activity. (A) Results for variants from 2 patients are expressed as means ± SEM percentage HCVpp infection compared to control cells (set at 100%) from three independent experiments performed in triplicate. (B) TCID\textsubscript{50} 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,D) Each dot represents the result for a single variant. Results for percentage infection of IFITM2- (C) and IFITM3-positive (D) cells compared to the control of HCV variants from 5 different patients. *\(P < 0.05\), **\(P < 0.01\), one-tailed Student \(t\) test. Shown are the means of three experiments performed in triplicates.
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.

**IFITM PROTEINS AND nAbs COOPERATIVELY BLOCK HCV ENTRY**

The differential inhibition of antibody escape and nonescape HCV strains by IFITM proteins in chronic infection as well as the enhanced IFITM sensitivity of viral strains in the acute phase preceding 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 before 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 (Fig. 5A). IFITM2 expression reduced virus entry in the absence of neutralizing sera by 10-fold for the nonescape variant and 5-fold for the escape variant, respectively (Fig. 5A). When serum-treated HCVpp were used to infect IFITM2-expressing cells, we observed a marked increase in neutralization. Neutralization was approximately 3- to 4-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 nonescape variants compared to the escape variants (Fig. 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 nAbs 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 (Fig. 5B). The slope on IFITM2-expressing cells was more than 10-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). Expression of IFITM2 enhanced the neutralization of HCVcc Con1 by a weakly neutralizing heterogeneous serum (1:100 dilution) from less than 2-fold to 60-fold (Fig. 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 around 40% or 1.67-fold inhibition by HC84.26.WH.5DL, lower for the other HMAbs; Fig. 5D). When IFITM2-expressing cells where infected with the HMAb-treated HCVpp, we again

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**FIG. 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 data sets of the experiments that are featured in Fig. 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 Fig. 3C,D. Shown are the results of one representative experiment performed in triplicate (n = 9). **P < 0.01, one-tailed Student t test. Abbreviation: RLU, relative light units.**
observed a marked cooperative effect that directly correlated with the neutralizing properties of the antibody. Indeed, whereas 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 (Fig. 5D). The antibodies CBH-7, HC11, and CBH-2 had intermediate effects (Fig. 5D).

Next, we confirmed these findings in the most physiologically relevant cell-culture system: infection of PHHs (Fig. 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 naïve PHHs. As shown for Huh7.5.1 cells, IFITM2 expression blocked HCV entry into PHHs, with the nonescape variant being more susceptible. Treatment with the neutralizing antibodies, HC-11 and CBH-2, increased the neutralization of the nonescape variant on IFITM-expressing cells by approximately 3-fold. Again, the increase in neutralization was markedly lower in the escape variants (Fig. 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.

FIG. 5. IFITM2 enhances antibody-mediated neutralization of HCV cell entry. Huh7.5.1 cells (A-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 HCVpp 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-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 hour. Entry of HCVpp was assessed 72 hours postinfection 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 $48748 \times x^2 + 640 \times x + 1.9$. The corresponding curve for anti-HCV serum on naïve cells was $4380 \times x^2 + 149 \times x + 1.1$. (C) Infection with HCVcc was analyzed 72 hours postinfection 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) A representative experiment performed in triplicates (n = 6). (E) Represents a single experiment performed in PHHs. Error bars show SD.

**COOPERATIVE INHIBITION OF HCV ENTRY BY IFITM PROTEINS AND nAbs IS ATTENUATED BY TREATMENT WITH AMPHOTERICIN B**

It is known that IFITM proteins restrict virus entry at the stage of hemifusion or fusion pore formation 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 Fig. 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 approximately 3-fold (Fig. 6A) and markedly reduced the antiviral activity of all IFITM proteins (Fig. 6B). Moreover, amphotericin B treatment largely abrogated the cooperation of IFITMs and neutralizing sera in inhibition of HCVpp cellular entry (Fig. 6C), indicating that IFITM-mediated modulation of cellular membranes and the resulting antiviral activity are responsible for

**FIG. 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 hours after transduction, cells were treated with vehicle control or 5 μg/mL of amphotericin B for 1 hour. Afterward, 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 hour. Entry of HCVpp was assessed 72 hours postinfection 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 nAbs 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. Abbreviations: CLDN1, claudin-1; EGFR, epidermal growth factor receptor; EphA2, ephrin type-A receptor 2; OCLN, occludin; RLU, relative light units.
the cooperative inhibition of virus entry by IFITM proteins and antibodies (illustrated in Fig. 6D). Interestingly, a similar enhancement of neutralization was observed using IFN treatment of Huh7 cells (Supporting Fig. 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 preseroconversion 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 LT compared with variants that are eliminated posttransplantation 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 preseroconversion 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 1 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 posttransplant 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. In part, these findings could explain the low efficacy of innate immune activation in chronic HCV-infected patients. Escape from innate responses does not only prevent the immune system from clearing the viral infection, but also limits the response to IFN-based therapies. Furthermore, the finding that variants selected post-LT and characterized by viral escape were significantly more resistant to inhibition by IFITM proteins than variants that were eliminated posttransplantation (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 nonscape 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 coreceptor, 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 be 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 LT 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 IFN, express high levels of IFN-stimulated genes, including IFITM3, to protect them from viral infection. Constitutive expression of IFN-stimulated genes is lost upon differentiation into hepatocyte-like cells, but becomes IFN 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, revealed that modulated membrane properties are responsible for the IFITM-mediated enhancement of neutralization, indicating that other innate effector cells, as shown for pretreatment with IFN-alpha, might exploit similar inhibitory mechanisms to block virus entry.

Interestingly, a recent study has suggested a different role for IFITMs in clinical human immunodeficiency virus (HIV)-1 infection: Whereas 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. 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 IFN treatment. On a mechanistic point of view, IFITM sensitivity of HIV was associated with receptor usage, with C-C chemokine receptor type 5 (CCR5)-tropic viruses being generally more resistant to IFITM proteins than C-X-C chemokine receptor type 4–tropic viruses. This suggests that the differential sensitivity of HIV to inhibition by the IFITM proteins might be attributed to receptor-mediated targeting to subcellular compartments with differential IFITM expression, or attributed to changes in envelope structure and electrochemical properties because of the switch of receptor tropism from CCR5 to C-X-C chemokine receptor type 5, 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.

Acknowledgment: We thank F. Chisari (The Scripps Research Institute, La Jolla, CA) for the gift of Huh7.5.1 cells and R. Bartenslager (University of Heidelberg), C. Rice (Rockefeller University), and T. Wakita (University of Tokyo) for plasmids for HCVcc and HCVpp production. We thank Sarah Durand (Inserm U1110) and Sabine Gärtner (German Primate Center) for excellent technical support. We thank Dr. Eloi Verrier (Inserm U1110) for helpful discussions.

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