JOURNAL OF VIROLOGY, Jan. 2011, p. 596–605 Vol. 85, No. 1
0022-538X/11/$12.00 doi:10.1128/JVI.01592-10
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Neutralizing Antibody-Resistant Hepatitis C Virus Cell-to-Cell Transmission▿†‡

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Received 30 July 2010/Accepted 4 October 2010

Hepatitis C virus (HCV) can initiate infection by cell-free particle and cell-cell contact-dependent transmission. In this study we use a novel infectious coculture system to examine these alternative modes of infection. Cell-to-cell transmission is relatively resistant to anti-HCV glycoprotein monoclonal antibodies and polyclonal immunoglobulin isolated from infected individuals, providing an effective strategy for escaping host humoral immune responses. Chimeric viruses expressing the structural proteins representing the seven major HCV genotypes demonstrate neutralizing antibody-resistant cell-to-cell transmission. HCV entry is a multistep process involving numerous receptors. In this study we demonstrate that, in contrast to earlier reports, CD81 and the tight-junction components claudin-1 and occludin are all essential for both cell-free and cell-to-cell viral transmission. However, scavenger receptor BI (SR-BI) has a more prominent role in cell-to-cell transmission of the virus, with SR-BI-specific antibodies and small-molecule inhibitors showing preferential inhibition of this infection route. These observations highlight the importance of targeting host cell receptors, in particular SR-BI, to control viral infection and spread in the liver.

Hepatitis C virus (HCV) establishes chronic infection in 3% of the world's population, resulting in a progressive liver disease that is one of the leading indications for liver transplantation. HCV has evolved several immune evasion strategies in order to persist within the infected host (15, 20, 40), including genetic escape from humoral immune responses (25, 46). However, functional constraints may restrict antigenic change in some regions of the virally encoded E1E2 envelope glycoproteins, such as the CD81 receptor binding site (9, 11, 33). The observation that glycoprotein-specific antibodies from chronically infected subjects neutralize the infectivity of laboratory prototype HCV strains yet demonstrate a limited ability to control HCV replication *in vivo* (40) suggest that additional means of evading antibody responses may exist.

How virus particles disseminate within an immune-competent host has been a relatively neglected area of study; however, it is becoming increasingly clear that viruses employ multiple strategies to infect new target cells. Diffusion through the pericellular environment or the vascular circulation introduces a rate-limiting step in virus entry and exposes particles to the humoral immune system. Consequently, a number of viruses have evolved direct cell-to-cell modes of transmission that maximize particle delivery, often in a neutralizing antibody (nAb)-resistant manner (reviewed in reference 30).

We (44) and others (48) previously reported that HCV strain JFH-1 could be transmitted via cell-free and cell-to-cell routes *in vitro*. We extend these observations and show that disruption of HCV particle assembly or physical separation of target and producer cells ablates transmission, demonstrating that intact virions are transferred via cell-cell contacts. HCV is readily transmitted in the presence of patient-derived antibodies that are able to neutralize cell-free virus infectivity. However, cell-to-cell transmission of HCV was sensitive to some glycoprotein-specific monoclonal antibodies, notably those targeting the first hypervariable region in E2 (HVR1). A diverse panel of chimeric cell-cultured HCVs (HCVcc) representing the seven major genotypes (12) infect via cell-to-cell contact, demonstrating that this route of transmission is a universal property of HCV.

HCV entry is a complex process that is dependent on host cell molecules: scavenger receptor BI (SR-BI), tetraspanin CD81, and the tight-junction proteins claudin-1 (CLDN1) and occludin (5, 29, 43). Coexpression of human SR-BI, CD81, claudin-1, and occludin renders nonliver cells permissive for HCV entry, suggesting that these four proteins constitute the minimal receptor requirement (34). We demonstrate that CD81 and both tight-junction protein entry factors are required for cell-free and cell-to-cell transmission. However, an-

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† Supplemental material for this article may be found at http://jvi.asm.org/.

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▿ Published ahead of print on 20 October 2010.

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tibodies and small-molecule entry inhibitors targeting SR-BI (41) preferentially inhibit cell-to-cell transmission. Furthermore, increased SR-BI expression in the target cell augments nAb-resistant infection, suggesting that SR-BI expression levels limit cell-to-cell transmission. These findings shed new light on the strategies employed by HCV to evade the humoral immune response; they have major implications for the development of targeted anti-glycoprotein immune therapies; and they highlight the importance of targeting virus receptors, in particular SR-BI, as a method to curtail HCV transmission and immune evasion.

MATERIALS AND METHODS

Cell lines and antibodies. Huh-7.5 cells (C. Rice, Rockefeller University, New York, NY) and Huh-7 Lunet cells (T. Pietschmann, Twincore, Hanover, Germany [4]) were propagated in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% nonessential amino acids. Huh-7.5 cells were transduced to overexpress SR-BI as previously described (13). Rat anti-E2 monoclonal antibodies (MAbs) (9/27, 3/11, and 11/20) and a control MAb (10/76b) were generated as previously described (16). IgG was isolated from the serum samples of six chronically infected HCV patients by use of protein G-conjugated Sepharose beads and was pooled (GE Healthcare, United Kingdom). Anti-CD81 MAbs were generated by immunizing mice with full-length purified CD81, and anti-SR-BI MAbs were a gift from Pfizer Ltd. Anti-CLDN1 serum was raised by genetic immunization of Wistar rats using a human CLDN1 cDNA expression vector, as previously described (22). Anti-occludin was purchased from Invitrogen. Lentiviral short hairpin RNA (shRNA) vectors (pLK01) specific for occludin were purchased from Open Biosystems (Huntsville, AL).

Infectious coculture assay. Huh-7.5 cells were electroporated with *in vitro*-transcribed full-length HCV RNA 72 h prior to their use in the assay (24, 47). Unlabeled naïve target cells were seeded in a collagen-coated 12-well plate (1.25×10^5 cells/well) and allowed to rest for 1 h at 37°C in the presence of either control or neutralizing anti-glycoprotein specific antibodies. HCV-infected producer cells were labeled with 5-chloromethylfluorescein diacetate (CMFDA) (Invitrogen, CA) by incubating the cells at 37°C with 5 μM CMFDA (DMEM–3% FBS) for 30 min. Cells were washed and trypsinized. An equal number of CMFDA-labeled producer cells were seeded into coculture with the naïve target cells (total, 2.5×10^5 cells in 1 ml DMEM–3% FBS). "Indirect" coculture assays were performed by seeding 2.5×10^5 cells in 6-well plates on either side of a 0.1-μm transwell insert (BD Falcon, CA). After 48 h, cocultured cells were trypsinized, harvested, and fixed, and the culture medium was collected to allow quantification of infectious cell-free virus. *De novo* transmission events were determined by staining for HCV nonstructural protein NS5A and were quantified by flow cytometry (see Fig. S1 in the supplemental material). To investigate the role of receptors in HCV coculture transmission, receptor antagonists were added to cocultures alongside anti-glycoprotein nAbs. Inhibition by each antagonist was calculated by comparison of transmission in treated and control cells. For confocal imaging of viral transmission, the target and producer cells were seeded onto collagen-coated 13-mm-diameter glass coverslips at a 1:20 ratio at 0.75× standard seeding density (1.75×10^5 cells/well).

Cell-free infectivity. To assess the infectivity of cell-free particles generated in the coculture assay, the culture supernatant was titrated in a standard infectious assay. Briefly, Huh-7.5 cells were seeded at 0.75×10^4/well of a 96-well plate and, the following day, infected with a serially diluted sample. After 48 h, the cells were stained for NS5A, foci were counted, and infectivity was expressed as the number of focus-forming units (FFU) per milliliter.

Flow cytometry. For CD81 staining, 2×10^5 cells were incubated in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 0.01% sodium azide (PBA) for 20 min at 37°C. The CD81-specific MAb 2.s131 or an irrelevant IgG control was incubated with cells in PBS for 30 min (2 μg/ml) at room temperature (RT), and unbound antibody was removed by washing. An Alexa 488-conjugated anti-mouse secondary antibody (dilution, 1/1,000; Invitrogen, CA) was incubated for a further 30 min at RT, and the cells were washed and fixed in 1% paraformaldehyde. To detect infection, cells were fixed with 1% paraformaldehyde and permeabilized in a buffer containing PBS plus 1% BSA and 0.5% saponin, and either an anti-NS5A 9E10 primary antibody (C. Rice, Rockefeller University, New York, NY) or an irrelevant IgG control was added for 30 min at RT. Unbound antibody was removed by washing, and the cells were incubated for a further 30 min at RT with an Alexa Fluor RPE-conjugated anti-mouse IgG2a isotype-specific secondary antibody (dilution, 1/1,000; Invitrogen, CA), followed by a buffer wash. Bound antibody was detected by flow cytometry using a FACSCalibur instrument (BD Biosciences) and analyzed with FlowJo software (TreeStar, Ashland, OR).

Laser scanning confocal microscopy. Control and shRNA-transduced Huh-7.5 cells were grown on glass coverslips and fixed with ice-cold methanol (occludin and claudin-1) or 3% paraformaldehyde (CD81) 24 h postseeding. Primary antibodies were applied for 1 h at room temperature. After two washes with PBS, Alexa Fluor 488-conjugated anti-mouse, anti-rabbit, or anti-rat (Invitrogen, CA) secondary antibody was applied for 1 h at room temperature. For imaging of infectious coculture transmission, cells were fixed with ice-cold methanol and stained for NS5A using 9E10 as the primary antibody and Alexa Fluor 594-conjugated anti-mouse IgG2a as the secondary antibody. Cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen) for visualization of nuclei and were mounted with ProLong Gold antifade reagent (Invitrogen). Cells were viewed by laser scanning confocal microscopy on a Zeiss META head confocal microscope with a 40× (coculture) or 60× (receptor expression) water immersion objective.

RESULTS

HCV transmission in coculture is resistant to the neutralizing effects of anti-glycoprotein antibodies. We assessed the sensitivity of HCV strain H77/JFH coculture transmission to a panel of anti-E2 glycoprotein antibodies with diverse specificities: rodent MAb 9/27 is specific for amino acids 396 to 407 within the first hypervariable region (HVR1); rodent MAbs 3/11 and 11/20 recognize linear epitopes comprising amino acids 412 to 423 and 436 to 447, respectively, within the discontinuous CD81 binding site (16); and human MAbs CBH-4G, HC-1, HC-11, and CBH-23 are specific for conformation-dependent epitopes (19). In the majority of cases, cell-free virus infectivity decreased with increasing nAb concentration; >95% of infectious cell-free particles were neutralized with rodent MAbs and polyclonal HCV^+ IgG (Fig. 1). In contrast, the frequency of newly infected target cells was only modestly reduced by the MAbs and polyclonal IgG. Although MAbs 9/27 and 11/20 exhibited the greatest activity against coculture transmission, neither treatment was able to ablate transmission at the maximum concentration tested, reaching ~80 and ~70% inhibition, respectively (Fig. 1). Similar results were obtained with the human MAbs (see Fig. S2 in the supplemental material). These data demonstrate that HCV coculture transmission is relatively resistant to a wide variety of glycoprotein-specific antibodies, consistent with a role for direct cell-to-cell transmission in nAb evasion and persistence *in vivo*.

nAb-resistant transmission requires cell contact and particle assembly. To examine the processes of transmission, we used two methods to segregate target and producer cells within the coculture (Fig. 2A). Assays were performed where the cell seeding density was lowered to reduce the number of cell-cell contacts. Alternatively, producer cells were grown on the lower face of a transwell insert directly above the target cells to prevent cell contact and to optimize the diffusion of cell-free particles (Fig. 2A). In direct coculture, both cell-free and cell-cell modes of transmission can occur; however, upon segregation of producer and target cells, HCV can be transmitted only via the extracellular medium. In the standard assay, ~40% of target cells became infected, with an approximately equal ratio of nAb-resistant and -sensitive routes of viral transmission (Fig. 2A). However, nAb-resistant transmission was significantly reduced at the lower seeding density and was abrogated when the cells were separated by a transwell insert (Fig. 2A).

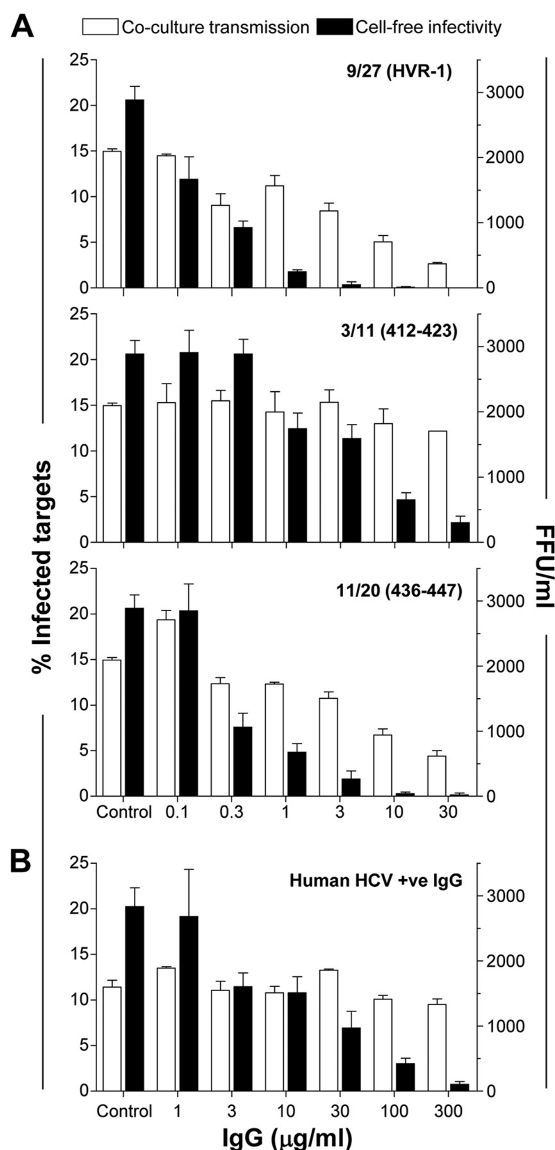

FIG. 1. Effects of anti-glycoprotein antibodies on HCV H77/JFH cell-free infectivity and coculture transmission. Anti-E2 MAbs 9/27, 3/11, and 11/20 (A) and pooled IgG isolated from 6 HCV-infected individuals (B) were titrated in an HCV strain H77/JFH coculture assay. Coculture transmission (open bars), expressed as the percentage of target cells that were infected (left *y* axis), and the infectivity of cell-free virus (filled bars), expressed as focus-forming units per milliliter (right *y* axis), were measured. All titrations were performed in duplicate. Error bars indicate standard deviations. The data shown are representative of 3 experiments.

These observations suggest that cell-free virus does not contribute to nAb-resistant transmission, consistent with a direct cell-to-cell mechanism of nAb evasion.

It is possible that HCV may evade anti-glycoprotein antibody responses via the direct transfer of RNA genomes between infected and naïve cells, thus negating the role of virions in transmission. Indeed, the exosome secretion pathway represents an attractive target for localized spread of intracellular pathogens (37). To test this model, we used a J6/JFH virus encoding a deleted NS5A (domain III-del B) (42) that expresses all of the viral proteins but lacks a critical phosphorylation event in NS5A required for particle assembly, providing an ideal tool to investigate whether the nonencapsidated HCV genome is capable of cell-to-cell spread. For the two genomic constructs, the numbers of infected ($NS5A^+$) producer cells were comparable [20.6% for J6/JFH and 24.6% for J6/JFH/(del B)] (Fig. 2B). J6/JFH cocultures efficiently transmitted infection to 21% of target cells after 48 h; however, no target cells became infected when cocultured with J6/JFH/(del B)-expressing cells, showing that nonencapsidated HCV genomes are not transferred between cells (Fig. 2B). These observations suggest that particle assembly is essential for HCV coculture transmission.

nAb-resistant cell-to-cell transmission of diverse HCV genotypes. We performed infectious coculture assays with a panel of chimeric JFH viruses bearing the structural proteins of genotype 1a to 7a viruses (12). The different viral strains generated various levels of cell-free infectious virus (Fig. 3A) that were generally predictive of transmission efficiency (Fig. 3C), in agreement with our earlier results demonstrating that coculture transmission is dependent on infectious particles. To neutralize cell-free particle infectivity, cross-reactive pooled HCV patient IgG (300 μg/ml) was added to the cocultures (Fig. 3B). All of the viral strains demonstrated nAb-resistant and -sensitive coculture transmission (Fig. 3C); however, differences were noted in the relative mode of transmission between viral strains. These data suggest that nAb evasion by direct cell-to-cell transfer of virions is a feature common to all HCV genotypes.

nAb-resistant cell-to-cell transmission is CD81 dependent. We have shown that cell-free virus infectivity is sensitive to the neutralizing effect(s) of antibodies targeting the viral glycoproteins (Fig. 1); consequently, the addition of nAb to HCV cocultures enables us to monitor cell-to-cell transmission. We employed an H77/JFH coculture assay in the presence or absence of neutralizing anti-E2 MAb 9/27 to assess the receptor dependency of cell-to-cell transmission. We first examined CD81 dependency using a panel of anti-receptor antibodies. Prior to coculture, target cells were treated for 1 h either with anti-CD81 MAbs specific for a range of conformation-dependent epitopes or with a control antibody. All of the anti-CD81 MAbs inhibited nAb-sensitive and nAb-resistant transmission by more than 90%, suggesting similar CD81 dependency for the two routes of infection (Fig. 4A). Indeed, titration of anti-CD81 MAb 2.s131 demonstrated comparable inhibition of nAb-sensitive and nAb-resistant transmission (Fig. 4B). To confirm that HCV coculture transmission is CD81 dependent, we used a Huh-7-derived Lunet cell line that expresses low levels of CD81 as measured by flow cytometry (Fig. 4E). Parental Lunet cells were resistant to HCV pseudoparticle and cell-free HCVcc infection (data not shown), as previously reported (21). H77/JFH- and SA13/JFH-infected producer cells were cocultured with parental Huh-7 Lunet cells or those transduced to express human CD81. Robust target cell infection was detected only in Huh-7 Lunet-CD81 cells, consistent with CD81 being a critical HCV entry factor (Fig. 4C). However, a low number of SA13/JFH-infected Huh-7 Lunet cells were detected, which may represent CD81-independent infection or could be attributed to the small number of Huh-7 Lunet cells expressing low-level CD81 noted by flow cytometry (Fig.

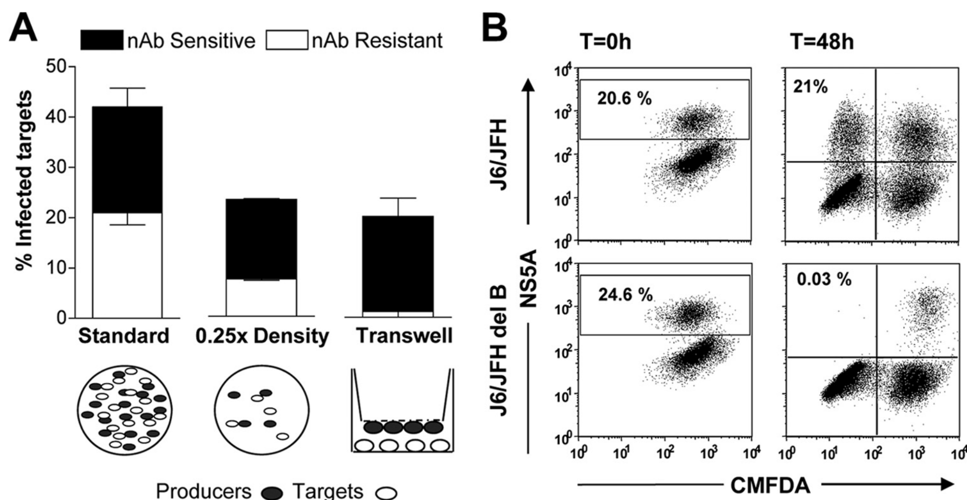

FIG. 2. nAb-resistant HCV transmission requires cell contact and infectious HCV particles. (A) The standard H77/JFH coculture assay was modified either by performing the coculture at a low (0.25×) seeding density or by seeding the target and producer cells on opposing faces of a culture well and transwell insert. In both cases, a 1:1 target/producer ratio was maintained. The frequency of infected target cells in the absence (nAb sensitive) or presence (nAb resistant) of MAb 9/27 at 4 μg/ml is shown. The experiments were performed in duplicate, and error bars indicate standard deviations. The data set is representative of 4 experiments. (B) J6/JFH and J6/JFH del B RNA were electroporated into Huh-7.5 cells. After 72 h, the cells were labeled with CMFDA, and NS5A expression was measured either immediately ($T = 0$ h) (left) or 48 h after coculture with Huh-7.5 target cells ($T = 48$ h) (right).

4E). Importantly, addition of anti-CD81 MAb 2.s131 ablated SA13/JFH infection of Huh-7 Lunet cells, indicating that CD81-independent infection did not occur. The ability to detect SA13/JFH-infected Huh-7 Lunet cells may represent an increased affinity of SA13 glycoproteins for CD81 or may simply reflect the higher infectivity of this chimeric virus (Fig. 3A). We previously reported that HCV cell-to-cell transmission could occur in the absence of CD81 (44); however, this is most likely attributable to the previous experimental design. In our earlier study, naïve target cells rather than producer cells were CMFDA labeled; consequently, multicell aggregates of infected producer and naïve target cells could be wrongly registered as positive transmission events (an example of this can be found in Fig. S3 in the supplemental material). The current experimental design eliminates these false-positive results. To corroborate these findings, we imaged HCV-infected producer cells cocultured with Huh-7 Lunet cells and found no evidence for CD81-independent transmission (Fig. 4D).

nAb-resistant cell-to-cell transmission is dependent on tight-junction proteins claudin-1 and occludin. The tight-junction proteins claudin-1 and occludin are thought to act during the late stages of HCV entry, and there is limited evidence for direct glycoprotein interaction(s) (10). We assessed the dependency of HCV cell-to-cell transmission on claudin-1 by using a using a recently reported rat polyclonal antiserum that can inhibit cell-free virus infectivity (22). Target cells were treated for 1 h with anti-claudin-1 or a control antiserum prior to coculturing with Huh-7.5 cells infected by chimeric viruses bearing the structural proteins of genotypes 1a, 1b, and 4a (H77/JFH, J4/JFH, and ED43/JFH, respectively) (Fig. 5A). Anti-claudin-1 inhibited nAb-sensitive and -resistant cell-to-cell transmission of all three viruses, demonstrating a somewhat higher efficiency for nAb-sensitive transmission, suggesting that both modes of transmission are claudin-1 dependent. Due to the lack of antibodies targeting extracellular occludin epitopes, we used shRNA to silence protein expression in Huh-7.5 cells (Fig. 5B). Confocal imaging of transduced cells showed a significant reduction in occludin expression, with no observable effects on claudin-1, CD81, or SR-BI expression levels (data not shown). Silencing of occludin in Huh-7.5 target cells reduced both nAb-sensitive and nAb-resistant routes of transmission (Fig. 5C). Thus, the two tight-junction proteins have comparable roles in cell-free and cell-to-cell modes of HCV transmission.

nAb-resistant cell-to-cell transmission is SR-BI dependent. SR-BI is a receptor for high-density lipoprotein and is reported to be involved in the early stages of HCV attachment and entry (35, 39). We (14) and others (6, 49) have reported that anti-SR-BI antibodies can inhibit cell-free HCV infectivity. To assess the role of SR-BI in H77/JFH cell-free and cell-to-cell transmission, we treated Huh-7.5 target cells with increasing concentrations of an anti-SR-BI MAb. Interestingly, anti-SR-BI demonstrated significantly greater inhibition of nAb-resistant cell-to-cell transmission than of nAb-sensitive transmission (Fig. 6A). To further understand the role of SR-BI in cell-to-cell transmission, we employed a cell culture-adapted JFH-1 mutant (the G451R mutant) that is relatively SR-BI independent (14, 51). Coculture of JFH-1- and JFH-1 G451R-infected producer and target cells confirmed the adapted phenotype of the G451R virus, with a greater capacity to transmit than the parental virus (Fig. 6B). However, the G451R virus demonstrated a significantly reduced nAb-resistant cell-to-cell mode of transmission compared to wild-type JFH-1 (Fig. 6B). Furthermore, anti-SR-BI significantly reduced JFH-1 cell-to-cell transmission compared to nAb-sensitive cell-free infection yet had minimal effect on JFH G451R transmission (Fig. 6C). We previously reported that SR-BI overexpression increased the susceptibility of Huh-7.5 cells to HCVcc infection (14); this phenotype is accompanied by an increase in the size of JFH-1-infected cell foci, indicative of enhanced cell-to-cell trans-

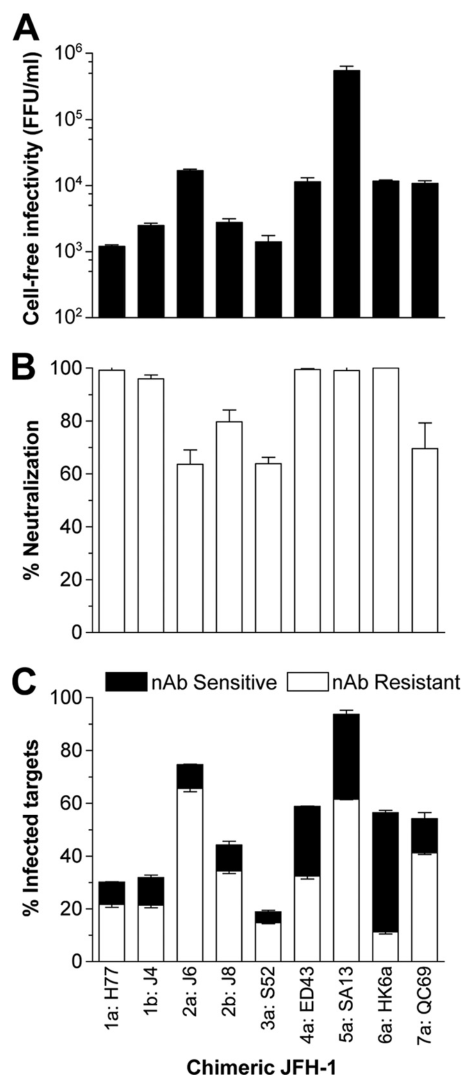

FIG. 3. Diverse HCVcc transmission. Huh-7.5 cells were electroporated with a panel of chimeric JFH-1 HCV RNAs. At 72 h postelectroporation, the cells were labeled with CMFDA and were cocultured with Huh-7.5 target cells in the presence of control or cross-reactive pooled patient HCV$^+$ IgG at 300 μg/ml for 48 h. Extracellular medium was collected. (A) The levels of infectious virus were quantified. (B) The percentage of neutralization by patient HCV$^+$ IgG was determined. (C) The stacked histograms display nAb-resistant cell-to-cell transmission (open portions of bars) and nAb-sensitive transmission (filled portions of bars) for each virus. The infecting genotypes and strain designations are shown at the bottom. All treatments were performed in duplicate, and error bars indicate standard deviations. The data set is representative of 3 experiments.

mission, whereas JFH-1 G451R foci remained unaltered (Fig. 6D) (13). Given this observation, we employed these cells as targets in an infectious coculture assay. HCV strains H77/JFH and JFH-1 demonstrated a significant increase in nAb-resistant cell-to-cell transmission in Huh-7 cells expressing 2-fold-greater levels of SR-BI (Fig. 6E), suggesting that SR-BI levels in Huh-7.5 hepatoma cells limit HCV cell-to-cell transmission. To further investigate the role of SR-BI in HCV cell-to-cell transmission, we studied the efficacies of two recently reported entry inhibitors that bind SR-BI (41). Both compounds, ITX5061 and ITX7650, significantly reduced H77/JFH nAb-resistant cell-to-cell transmission and had a minimal effect on nAb-sensitive cell-free transmission (Fig. 6F).

Receptor dependency of diverse HCV cell-to-cell transmission. We selected the chimeric JFH-1 viruses in which robust (>90%) cell-free neutralization was achieved (Fig. 4B) and examined their sensitivities to receptor antagonists (Table 1). Anti-CD81 MAb 2.s131 inhibited nAb-sensitive and -resistant transmission of all HCV strains tested with comparable efficiencies, with the exception of SA13/JFH. Targeting of SR-BI by a receptor-specific antibody or ITX5061 displayed a broader range of efficiencies, but with excellent agreement between the two treatments. Notably, for all viruses, SR-BI antagonists were more effective at reducing nAb-resistant cell-to-cell transmission. In contrast, anti-CLDN1 demonstrated greater inhibition of nAb-sensitive routes of viral transmission, with only a minimal effect on either route of SA13/JFH or HK6a/JFH transmission (Table 1). Although these findings suggest a spectrum of receptor dependencies among viral strains, SR-BI remains an attractive therapeutic target, with ITX5061 displaying cross-genotype inhibition of cell-to-cell transmission.

DISCUSSION

HCV persists in the face of a robust nAb response (40). Indeed, the ability to evade the host immune responses is a common feature of many viruses capable of establishing chronic infection. *In vitro* model systems suggest that HCV is a relatively sensitive target for antibody-mediated neutralization (3, 24, 32, 50). We (44) and others (48) have reported efficient HCV transmission in the presence of polyclonal patient IgG and a limited number of anti-E2 MAbs, suggestive of direct cell-to-cell transfer.

There are various mechanisms by which a virus particle may be transmitted directly between cells. For instance, HIV and herpes simplex virus (HSV) spread across specific cellular contacts, and in the case of HIV, a virally induced structure termed the virological synapse is formed (17). In contrast, HSV exploits preexisting cellular contacts (45). Other viruses remain tethered to the infected-cell surface, allowing directed lateral movement to adjacent naïve target cells (8, 36, 38). While these varied modes of cell-to-cell transmission are believed to provide an efficient alternative to cell-free infection, they may also confer resistance to the humoral immune response (27, 30).

The observation that HCV entry is dependent on two tight-junction components (10, 34) offers the tantalizing possibility that HCV may exploit such junctional contacts in transmission. However, the Huh-7.5 cell line used in this study does not form functional tight junctions (28), and further studies are required to investigate the mechanism(s) of HCV transmission in polarized cell systems. Our observation that HCV E2 glycoprotein- and receptor-specific antibodies reduce cell-to-cell transmission suggests that spread is not occurring across a sealed cellular junction, which would exclude such antibodies (27). HCV may exploit a contact structure similar to the virological synapse of HIV (17), which has recently been shown to be permeable to antibodies (27).

We find coculture transmission to be relatively insensitive to neutralization by a panel of diverse anti-glycoprotein antibod-

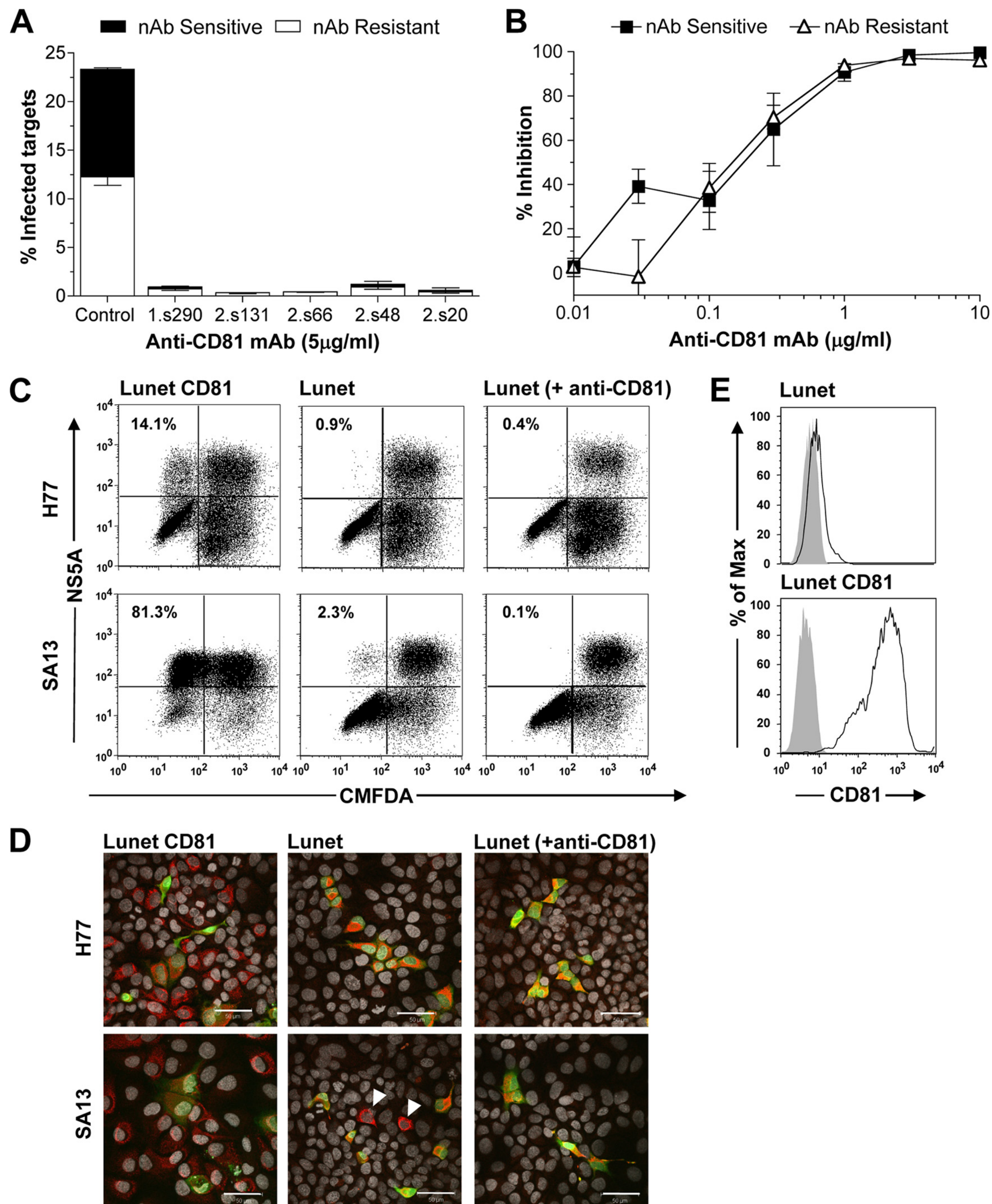

FIG. 4. HCV coculture transmission is CD81 dependent. (A) Anti-CD81 inhibition of H77/JFH coculture transmission. The stacked histogram displays nAb-resistant cell-to-cell (open portion of bar) and nAb-sensitive (filled portion of bar) transmission in the presence of a control MAb or one of five anti-CD81 MAbs (5 μg/ml) specific for distinct epitopes. (B) Anti-CD81 MAb 2.s131 was titrated in a standard H77/JFH coculture assay, and nAb-sensitive (■) and -resistant (△) transmission is shown. All treatments were performed in duplicate, and error bars indicate standard deviations. (C) H77/JFH- and SA13/JFH-infected producer cells were cocultured with Huh-7 Lunet cells or with cells transduced to express human CD81 with or without anti-CD81 (10 μg/ml MAb 2.s131). Representative flow cytometry plots are shown, and the percentages of target cells infected are given in the upper left quadrants. (D) To corroborate the infectious coculture assay, cells were fixed, stained for NS5A, and imaged by confocal microscopy. HCV-infected target cells (red), uninfected producer cells (green), and infected producer cells (orange) are clearly visible, with cell nuclei shown in gray. White arrowheads indicate rare SA13/JFH-infected Lunet target cells. (E) Flow cytometric analysis of CD81 expression in parental Huh-7 Lunet cells and those transduced to express human CD81. Max, maximum.

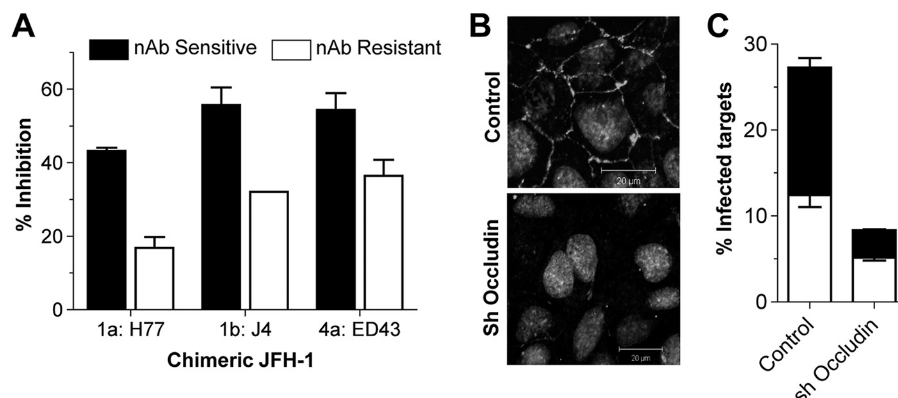

FIG. 5. Role for claudin-1 and occludin in HCV coculture transmission. Infectious coculture assays were performed with chimeric HCV-infected producer cells bearing the structural proteins of genotypes 1a (H77), 1b (J4), and 4a (ED43). (A) Target cells were incubated with polyclonal rat anti-claudin-1 serum (dilution, 1/100) prior to coculture. Inhibition of nAb-sensitive (filled bars) and nAb-resistant (open bars) transmission is shown. (B) Huh-7.5 cells were transduced with irrelevant lentiviruses or lentiviruses expressing occludin shRNA (sh occludin). One hundred twenty hours later, the cells were fixed, permeabilized, and stained for occludin expression. (C) Huh-7.5 target cells either transduced with irrelevant control shRNA or silenced with occludin shRNA were cocultured with H77/JFH-infected producer cells, and the frequency of nAb-sensitive (filled portions of bars) and nAb-resistant (open portions of bars) transmission was quantified by flow cytometry. All treatments were performed in duplicate; error bars indicate standard deviations; and the data set is representative of two experiments.

ies, whereas cell-free infectivity was readily eliminated (Fig. 1; see also Fig. S1 in the supplemental material). nAb-resistant transmission was prevented by separation of the target and producer cells (Fig. 2A), providing compelling evidence that this transmission occurs via direct cell-cell contacts. Cell-to-cell transmission required particle assembly and did not occur via the transfer of nonencapsidated HCV genomes (Fig. 2B). Furthermore, we found that HCVcc chimeras representative of seven genotypes were transmitted via cell-to-cell transmission (Fig. 3), implying that this phenotype is common to all viral strains.

We investigated the receptor dependency of HCV transmission and found that anti-CD81 MAbs inhibited both cell-free and cell-to-cell infection, consistent with the idea that both routes of transmission are CD81 dependent (Fig. 4). Huh-7 Lunet cells were resistant to infection both by H77/JFH and by SA13/JFH. Furthermore, the low-level SA13/JFH infection of Huh-7 Lunet cells was blocked by anti-CD81 MAb 2.s131, suggesting that infection occurred in the minority of Huh-7 Lunet cells expressing low levels of CD81. This observation is in contrast to our previous findings, and those of others, of CD81-independent coculture transmission (18, 44, 48). Further investigation into our observation that HCV could be transmitted to CD81-negative HepG2 cells suggests that our earlier finding was due to cell aggregates between labeled target and producer cells yielding false-positive results, as shown in Fig. S3 in the supplemental material.

The tight-junction components claudin-1 and occludin were recently identified as entry factors for HCV (10, 34). Their elevated expression at points of cellular contact make them interesting candidates in directing cell-to-cell transmission. We used an antiserum directed against the claudin-1 extracellular domain, which disrupts the claudin-1/CD81 coreceptor association(s) (22), to inhibit cell-free and cell-to-cell transmission (Fig. 5). The antiserum exhibited moderate activity against HCVcc chimeric viruses bearing the structural proteins of genotypes 1a, 1b, and 4a and demonstrated greater efficacy against nAb-sensitive viral transmission. shRNA silencing of occludin expression in Huh-7.5 target cells reduced both nAb-sensitive and nAb-resistant routes of HCV transmission (Fig. 5). Thus, both tight-junction proteins have a role in cell-to-cell HCV transmission.

HCV particles are known to associate with host lipoproteins (2, 7), and we previously reported that this association confers nAb resistance (14). This may have implications for localized spread by allowing HCV to remain associated with cells via lipoprotein moieties (26). Interestingly, of all the anti-glycoprotein MAbs tested, the anti-HVR1 MAb 9/27 demonstrated

TABLE 1. Sensitivities of chimeric JFH-1 viruses to receptor antagonists

Genotype	JFH chimera	% Inhibition of virus transmission							
		Anti-CD81		Anti-CLDN1		Anti-SR-BI		ITX5061	
		Sensitive	Resistant	Sensitive	Resistant	Sensitive	Resistant	Sensitive	Resistant
1a	H77	88 (±1)	92 (±2)	43 (±1)	17 (±3)	7 (±6)	87 (±1)	7 (±12)	81 (±1)
1b	J4	82 (±1)	88 (±1)	55 (±5)	32 (±1)	7 (±14)	79 (±1)	8 (±2)	73 (±1)
4a	ED43	97 (±1)	94 (±2)	54 (±5)	36 (±4)	7 (±2)	75 (±2)	4 (±5)	69 (±1)
5a	SA13	46 (±4)	92 (±1)	8 (±5)	22 (±2)	0 (±4)	44 (±2)	0 (±0)	41 (±2)
6a	HK6a	94 (±1)	93 (±1)	10 (±1)	1 (±1)	10 (±2)	34 (±7)	15 (±1)	40 (±3)

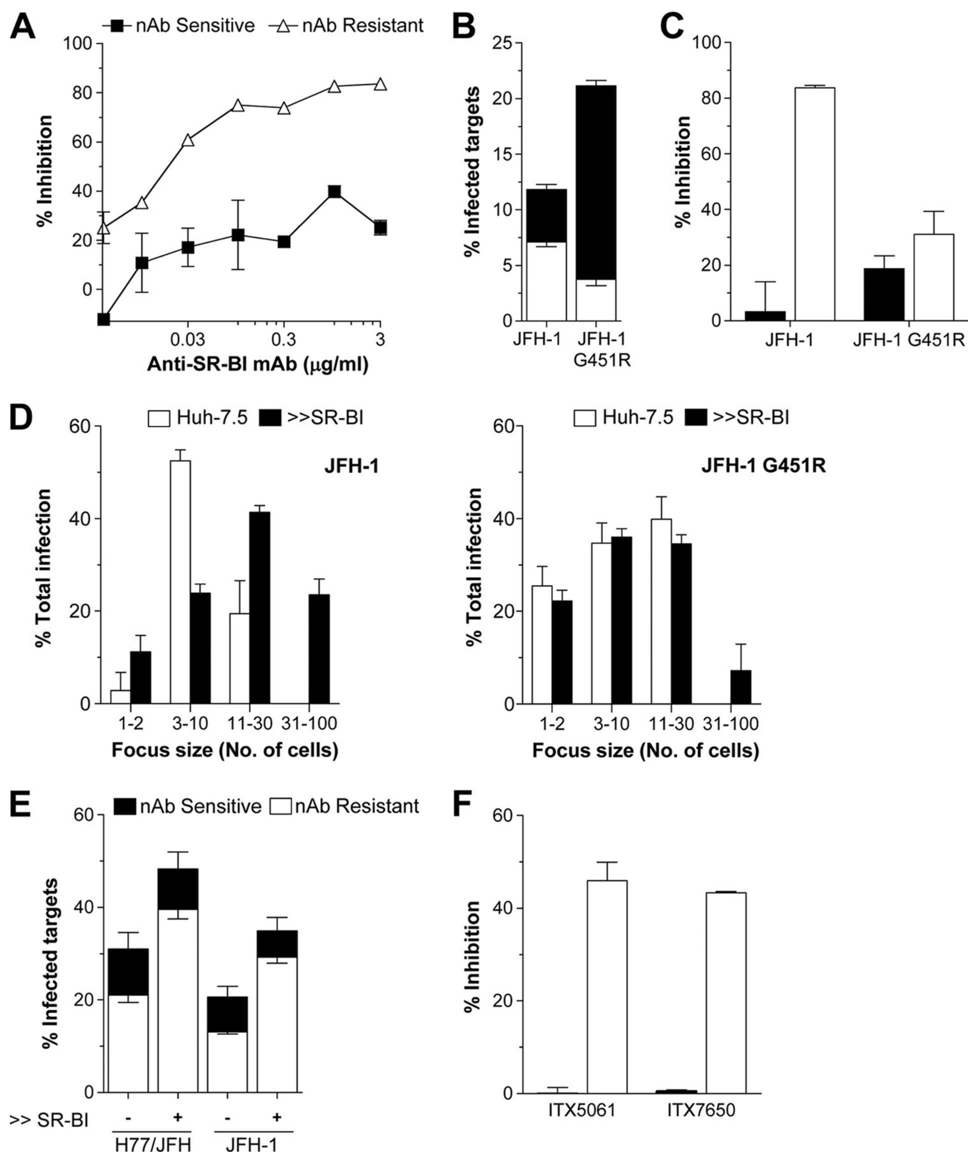

FIG. 6. HCV coculture transmission is SR-BI dependent. (A) An anti-SR-BI MAb was titrated in a standard H77/JFH coculture assay. Inhibition of nAb-sensitive (■) and nAb-resistant (△) infection is shown. (B) JFH-1- and JFH-1 G451R-infected producer cells were cocultured with Huh-7.5 target cells in the presence or absence of neutralizing patient IgG (300 μg/ml). The stacked histogram displays nAb-resistant cell-to-cell transmission (open portions of bars) and nAb-sensitive transmission (filled portions of bars) for each virus. (C) Effect of an anti-SR-BI MAb (1 μg/ml) on JFH-1 and JFH-1 G451 nAb-resistant (open bars) and -sensitive (filled bars) transmission. (D) Parental Huh-7.5 cells (open bars) or Huh-7.5 cells overexpressing SR-BI (filled bars) were infected with JFH-1 or JFH-1 G451R for 72 h in a standard cell-free infectious assay. HCV-positive cells were enumerated by immunofluorescent microscopy, allowing quantification of infected-cell focus size. The histograms display the percentages of total infection residing in small (1 to 2 cells), medium (3 to 10 cells), large (11 to 30 cells), or very large (31 to 100 cells) infected-cell foci. (E) H77/JFH- and JFH-1-infected producer cells were cocultured with parental Huh-7.5 cells or with Huh-7.5 cells transduced to overexpress SR-BI (≫), in the presence or absence of neutralizing patient IgG (300 μg/ml), and infected target cells were quantified. H77/JFH and JFH-1 nAb-resistant infection of cells overexpressing SR-BI was significantly increased (*P*, 0.0096 and 0.0037, respectively). (F) Effects of the SR-BI-specific small molecules ITX5061 and ITX7650 (1 μM) on H77/JFH nAb-sensitive (filled bars) and -resistant (open bars) transmission. Treatments were performed in duplicate, and error bars indicate standard deviations.

the greatest potential for reducing cell-to-cell transmission, suggesting that antibodies targeting HVR1 may limit cell-cell transfer of infection. However, *in vivo*, HCV can escape anti-HVR1 responses by conventional genetic escape (25, 46).

SR-BI is thought to play an important role in HCV attachment and entry via its interaction with the E2 glycoprotein (35). SR-BI antagonists display preferential inhibition of cell-to-cell transmission of HCV (Fig. 6 and Table 1). A mutant JFH-1 G451R virus with limited SR-BI dependence (14) demonstrated minimal cell-to-cell transmission (Fig. 6B). Furthermore, overexpression of SR-BI in Huh-7.5 cells promoted cell-to-cell transmission (Fig. 6D and E). Taken together, these data demonstrate that SR-BI plays an essential role in cell-to-cell transmission, and targeting of this receptor may limit nAb-

resistant modes of HCV transmission. SR-BI-specific compounds have been developed to treat atherosclerosis (1, 31), and SR-BI, unlike the other HCV receptors, is predominantly expressed in the liver (39), reducing the possibility for off-target effects.

It is clear that cell-to-cell transmission of pathogens facilitates immune evasion and persistence. The localized spread of HCV may be an adaptation to exploit the compact and highly ordered environment of the liver. This conclusion is supported by the recent observation of HCV-infected foci in liver biopsy samples from HCV-infected patients (23). It is important to remember that extracellular forms of HCV are most likely responsible for spread between hosts and for liver allograft reinfection following transplantation. However, cell-to-cell transmission may represent the dominant route of virus dissemination within chronically infected individuals. Our findings raise a number of issues that will require further consideration for the design and preclinical evaluation of HCV glycoprotein-specific antibody and therapeutic B-cell vaccines, since it appears that HCV has evolved methods to evade such immune surveillance. In contrast, targeting of the viral receptors or entry factors, in particular SR-BI, may provide a means of augmenting the host immune response by inhibiting cell-to-cell transmission.

ACKNOWLEDGMENTS

We thank Michelle J. Farquhar for critical reading of our manuscript; David Mutimer for access to patient sera; Steven Foung for anti-glycoprotein MAbs; Thomas Pietschmann for Lunet Huh-7 cells; and Takaji Wakita, Brett Lindenbach, Charles Rice, Jin Zhong, Frank V. Chisari, and Jens Bukh for infectious HCVcc constructs.

This work was supported by MRC grant G0400802, Pfizer Ltd., the Wellcome Trust, and PHS grants R01 DA024565, AI50798, and AI40034.

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