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Neutralizing antibody resistant hepatitis C virus cell-to-cell transmission.

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Abstract. 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 co-culture 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 to escape 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 multi-step 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 virus cell-to-cell transmission, 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.

Introduction. 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 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 viral 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 and yet demonstrate 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 (30)).

We (44) and others (48) previously reported that HCV strain JFH-1 could transmit 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 transfer via cell-cell contacts. HCV readily transmits in the presence of patient derived antibodies that are able to neutralize cell-free virus infectivity. However, HCV cell-to-cell transmission was sensitive to some glycoprotein specific monoclonal antibodies, notably those targeting the first hypervariable region in E2 (HVR-1). A diverse panel of chimeric HCVcc viruses 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 and Occludin (5, 29, 43). Co-expression of human SR-BI, CD81, Claudin-1 and Occludin renders non-liver 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 were required for cell-free and cell-to-cell transmission. However, antibodies 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 and have major implications for the development of targeted anti-glycoprotein immune therapies and highlight the importance of targeting virus receptors, in particular SR-BI, as a method to curtail HCV transmission and immune evasion.

Materials and Methods.

Cells lines and antibodies. Huh-7.5 cells (C. Rice, Rockefeller University, NY), and Huh-7 Lunet cells (T. Pietschmann, TWINCORE, Hanover (4)) were propagated in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% nonessential amino acids. Huh-7.5 cells were transduced to over express SR-BI as previously described (13). Rat anti-E2 mAbs (9/27, 3/11 and 11/20) and control (10/76b) were generated as previously described (16), IgG was isolated from the serum of six chronically infected HCV patients by protein G-conjugated Sepharose beads and pooled (GE Healthcare, United Kingdom). Anti-CD81 mAbs were generated by immunizing mice with full-length purified CD81, 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 complementary DNA expression vector, as previously described (22). Anti-Occludin was purchased from Invitrogen. Lentiviral shRNA vectors (pLK01) specific for Occludin were purchased from Open Biosystems (Alabama, US). The anti-SRBI ligands ITX5061 and ITX7650 were the kind gift of Flossie Wong-Staal (iTherx, San Diego, CA USA).

Infectious co-culture assay. Huh-7.5 cells were electroporated with *in vitro* transcribed full-length HCV RNA 72h prior to their use in the assay (24, 47). Unlabelled naïve target cells were seeded in a collagen coated 12 well plate ($1.25x10^5$ cells/well) and allowed to rest for 1h at 37ºC in the presence of either control or neutralizing anti-glycoprotein specific antibodies. HCV infected producer cells were labeled with CMFDA (Invitrogen, CA USA) by incubating the cells at 37°C with 5μM CMFDA (DMEM/3%FBS) for 30 minutes. Cells were then washed and trypsinized. An equal number of CMFDA-labeled producer cells were seeded into co-culture with the naïve target cells (total of $2.5x10^5$ cells in 1ml DMEM/3% FBS). 'Indirect' co-culture assays were performed by seeding $2.5x10^5$ cells in 6 well plates on either side of a 0.1μm transwell insert (BD Falcon, CA USA). After 48h co-cultured cells were trypsinized, harvested and fixed, and the culture medium collected to allow quantification of infectious cell-free virus. De novo transmission events were determined by staining for HCV non-structural protein NS5A and were quantified by flow cytometry (Supplementary Fig.1). To investigate the role of receptors in HCV co-culture transmission, receptor antagonists were added to co-cultures 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 glass coverslips at a 1:20 ratio at 0.75x standard seeding density (1.75 x 10^5 cells/well).

Cell-free infectivity. To assess the infectivity of cell free particles generated in the co-culture assay the culture supernatant was titrated in a standard infectious assay. Briefly, Huh-7.5 cells were seeded at $0.75x10^4$ cells/well of a 96 well plate and the following day infected with a serially diluted sample under test. After 48h the cells were stained for NS5A, foci counted and infectivity expressed as the number of foci forming units/ml (FFU/ml).

Flow cytometry. For CD81 staining, $2x10^5$ cells were incubated in PBS containing 1% BSA and 0.01% sodium azide (PBA) for 20 minutes at 37°C. The CD81 specific mAb 2.s131 or an irrelevant IgG control was incubated with cells in PBS for 30 minutes (2µg/ml) at RT and unbound antibody removed by washing. Secondary anti-mouse Alexa-488 conjugated antibody (1/1000 dilution, Invitrogen, CA USA) was incubated for a further 30 minutes at RT, the cells washed and fixed in 1% paraformaldehyde. To detect infection, cells were fixed with 1% paraformaldehyde, permeabilized in buffer containing PBS + 1% BSA and 0.5% saponin and an anti-NS5A 9E10 primary antibody (C. Rice, Rockefeller University, NY) or an irrelevant IgG control added for 30 minutes at RT. Unbound antibody was removed by washing and the cells incubated for a further 30 minutes at RT with a secondary anti-mouse IgG2a isotype specific Alexa-fluor RPE conjugated antibody (1/1000 dilution, Invitrogen, CA USA), followed by a buffer wash. Bound antibody was detected by flow cytometry using a FACSCalibur (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 cover slips and fixed with ice-cold methanol (Occludin and Claudin-1) or 3% paraformaldehyde (CD81) 24h post seeding. Primary antibodies were applied for 1h at room temperature. After washing twice with PBS, anti-mouse, rabbit or rat Alexa Fluor 488 (Invitrogen, CA) secondary antibody was applied for 1h at room temperature. For imaging infectious co-culture transmission, cells were fixed with ice-cold methanol and stained for NS5A using 9E10 primary antibody and anti-mouse IgG2a Alexa-fluor 594 secondary antibody. Cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen) for nuclei visualization and mounted with ProLong Gold antifade (Invitrogen). Cells were viewed by laser-scanning confocal microscopy on a Zeiss META head confocal microscope with a 40x (co-culture) or 60x (receptor expression) water-immersion objective.

Results.

Co-culture HCV transmission is resistant to the neutralizing effects of anti-glycoprotein antibodies. We assessed the sensitivity of HCV strain H77/JFH co-culture transmission to a panel of anti-E2 glycoprotein antibodies with diverse specificities: rodent mAb 9/27 is specific for amino acids 396-407 within the first hypervariable region (HVR1); rodent mAbs 3/11 and 11/20 recognize linear amino acid 412-423 and 436-447 epitopes, 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, with >95% of infectious cell-free particles being 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 co-culture transmission, neither treatment was able to completely ablate transmission at the maximum concentration tested, reaching ~80 and ~70% inhibition respectively (Fig.1). Similar results were obtained with the human mAbs (Supplementary Fig.2). These data demonstrate that HCV co-culture 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 co-culture (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 co-culture both cell-free and cell-cell modes of transmission can occur, however upon segregation of producer and target cells HCV can only transmit 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 trans-well insert (Fig.2A). 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 non-encapsidated HCV genome is capable of cell-to-cell spread. For both genomic constructs the number of infected ($NS5A^+$) producer cells was comparable (J6/JFH 20.6% and J6/JFH/(del B) 24.6%) (Fig.2B). J6/JFH co-cultures efficiently transmitted infection to 21% of target cells after 48h, however, no target cells became infected when co-cultured with J6/JFH/(del B) expressing cells, showing that non-encapsidated HCV genomes are not transferred between cells (Fig.2B). These observations suggest that particle assembly is essential for HCV co-culture transmission.

nAb resistant cell-to-cell transmission of diverse HCV genotypes. We performed infectious co-culture assays with a panel of chimeric JFH viruses bearing the structural proteins of genotype 1a-7a viruses (12). The different viral strains generated a range of cell-free infectious virus (Fig.3A) that was generally predictive of transmission efficiency (Fig.3C), in agreement with our earlier results demonstrating that co-culture transmission is dependent on infectious particles. To neutralize cell-free particle infectivity, cross-reactive pooled HCV patient IgG (300μg/ml) was added to the co-cultures (Fig.3B). All of the viral strains demonstrated nAb resistant and sensitive co-culture transmission (Fig.3C), however, differences were noted between viral strains in their relative mode of transmission. 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 co-cultures enables us to monitor cell-to-cell transmission. We employed a H77/JFH co-culture 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 co-culture target cells were treated for 1h with anti-CD81 mAbs specific for a range of conformation-dependent epitopes or a control antibody. A ll of the anti-CD81 mAbs inhibited nAb sensitive and nAb resistant transmission by more than 90%, suggesting a similar CD81 dependency for both routes of infection (Fig.4A). Indeed, titration of anti-CD81 mAb 2s131 demonstrated comparable inhibition of nAb sensitive and nAb resistant transmission (Fig.4B). To confirm that HCV co-culture 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 co-cultured with parental Huh-7 Lunet cells or those transduced to express human CD81. Robust target cell infection was only detected 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, that 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.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 the producer cells were CMFDA labeled, consequently multi-cell aggregates of infected producer and naïve target cells can be wrongly registered as positive transmission events, an example of this can be found in Supplementary Fig.3. The current experimental design eliminates these false positives. To corroborate these findings we imaged HCV infected producer cells co-cultured 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 using a using a recently reported rat polyclonal antiserum that can inhibit cell-free virus infectivity (22). Target cells were treated for 1hr with anti-Claudin-1 or control antiserum prior to co-culturing 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 shRNA silenced 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 Occludin in Huh-7.5 target cells reduced both nAb sensitive and resistant routes of transmission (Fig.5C). Thus, both tight junction proteins have a comparable role 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-SRBI 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 anti-SR-BI mAb. Interestingly, anti-SRBI demonstrated significantly greater inhibition of nAb resistant cell-to-cell transmission than 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 (G451R) that is relatively SR-BI-independent (14, 51). Co-culture 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, 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 and yet had minimal effect on JFH G451R transmission (Fig.6C). We previously reported that SR-BI over-expression 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 transmission, whereas JFH-1 G451R foci remain unaltered (Fig.6D) (13). Given this observation we employed these cells as targets in an infectious co-culture assay. HCV strains H77/JFH and JFH-1 demonstrate a significant increase in nAb resistant cell-to-cell transmission to 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 efficacy 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 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 sensitivity to receptor antagonists (Table 1). Anti-CD81 mAb 2s131 inhibited nAb sensitive and resistant transmission of all HCV strains tested with comparable efficiency, with the exception of SA13/JFH. Targeting SR-BI by 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 minimal effect on either route of SA13/JFH or HK6a/JFH transmission (Table 1). Although these findings suggest a spectrum of receptor dependencies between 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) 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 transmit directly between cells, for instance HIV and herpes simplex virus (HSV) spread across specific cellular contacts, in the case of HIV a virally induced structure termed the virological synapse is formed (17). In contrast, HSV exploits pre-existing 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 a 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 be exploiting a contact structure similar to the virological synapse of HIV (17), which has recently been shown to be permeable to antibodies (27).

We find co-culture transmission to be relatively insensitive to neutralization by a panel of diverse anti-glycoprotein antibodies, whereas cell-free infectivity was readily eliminated (Fig.1 & Supplementary Fig.1). 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 non-encapsidated HCV genomes (Fig.2B). Furthermore, we found that HCVcc chimeras representative of seven genotypes 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 CD81 mAbs inhibited both cell-free and cell-to-cell infection, consistent with both routes of transmission being CD81-dependent (Fig.4). Huh-7 Lunet cells were resistant to infection by both H77/JFH and SA13/JFH. Furthermore, the low level SA13/JFH infection of Huh-7 Lunet cells was blocked by

anti-CD81 mAb 2s131, suggesting that infection occurred in the minority of Huh-7 Lunet cells expressing low levels of CD81. This observation is in contrast to previous findings by ourselves and others of CD81 independent co-culture transmission (18, 44, 48). Further investigation into our observation that HCV could transmit to CD81 negative HepG2 cells suggest that our earlier finding was due to cell aggregates between labeled target and producer cells resulting in false positives, as described in Supplementary Fig.3.

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 that disrupts Claudin-1/CD81 co-receptor 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 Occludin expression in Huh-7.5 target cells reduced both nAb sensitive and 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 the greatest potential to reduce 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 transmitted 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, over-expression 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 this receptor may limit nAb resistant modes of HCV transmission. SR-BI specific compounds have been developed to treat atherosclerosis (1, 31) and, 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 re-infection 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 pre-clinical evaluation of HCV glycoprotein-specific antibody and therapeutic B-cell vaccines, as it would appear that HCV has evolved methods to evade such immune surveillance. In contrast, targeting the viral receptors or entry factors, and in particular SR-BI, may provide a means of augmenting the host immune response by inhibiting cell-to-cell transmission.

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Figure legends

Fig.1: Effect of anti-glycoprotein antibodies on HCV H77/JFH cell-free infectivity and co-culture 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 a HCV strain H77/JFH co-culture assay. Co-culture transmission (white bars) expressed as the percentage of infected target cells (left Y-axis) and the infectivity of cell-free virus (black bars) defined as focus forming units per ml (FFU/ml, right Y-axis) were measured. All titrations were performed in duplicate and the error bars indicate the standard deviation. The data shown is representative of 3 experiments.

Fig.2: nAb resistant HCV transmission requires cell contact and infectious HCV particles. (**A**) The standard H77/JFH co-culture assay was modified either by performing the co-culture at low (0.25x) seeding density or by seeding the target and producer cells on opposing faces of the culture well using a 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 4μg/ml is shown. The experiments were performed in duplicate and error bars indicate the standard deviation. 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 72h the cells were CMFDA labeled and NS5A expression measured immediately (T=0hr, left) or 48h after co-culture with Huh-7.5 target cells (T=48hr, right).

Fig.3: Diverse HCVcc transmission. Huh-7.5 cells were electroporated with a panel of chimeric JFH-1 HCV RNAs, where the infecting genotype is depicted prior to the strain nomenclature. 72h post electroporation the cells were labeled with CMFDA and co-cultured with Huh-7.5 target cells in the presence of control or cross reactive patient pooled HCV+ IgG at 300μg/ml for 48h. Extracellular media was collected and the levels of infectious virus quantified (**A**) and the % neutralization by patient HCV+ IgG determined (**B**). The stacked histogram displays nAb resistant cell-to-cell (white bars) and nAb sensitive transmission (black bars) for each virus (**C**)**.** All treatments were performed in duplicate and the error bars indicate the standard deviation. The data set is representative of 3 experiments.

Fig.4: HCV co-culture transmission is CD81 dependent. (**A**) Anti-CD81 inhibition of H77/JFH co-culture transmission. The stacked histogram displays nAb resistant cell-to-cell (white bars) and nAb sensitive (black bars) transmission in the presence of control or five anti-CD81 mAbs (5μg/ml) specific for distinct epitopes. (**B**) Anti-CD81 mAb 2.s131 was titrated in a standard H77/JFH co-culture assay and nAb sensitive (■) and resistant (Δ) transmission is shown. All treatments were performed in duplicate and error bars indicate the standard deviation. (**C**) H77/JFH and SA13/JFH infected producer cells were co-cultured with Huh-7 Lunet cells or cells

transduced to express human CD81 ± anti-CD81 (10μg/ml mAb 2s131), representative flow cytometry plots are annotated with the percentage of infected target cells. **(D)** To corroborate the infectious co-culture assay cells were fixed, stained for NS5A and imaged by confocal microscopy. HCV infected target cells (red), uninfected producer (green) and infected producer cells (orange) are clearly visible, with cell nuclei shown in grey. White arrows depict 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.

Fig.5: A role for Claudin-1 and Occludin in HCV co-culture transmission. Infectious co-culture 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 (1/100 dilution) prior to co-culture. Inhibition of nAb sensitive (black bars) and nAb resistant (white bars) transmission is shown. (**B**) Huh-7.5 cells were transduced with irrelevant and shRNA-Occludin lentiviruses and 120h later the cells were fixed, permeabilised and stained for Occludin expression. (**C**) Irrelevant control and shRNA-Occludin silenced Huh-7.5 target cells were co-cultured with H77/JFH infected producer cells and the frequency of nAb sensitive (black) and resistant (white) transmission quantified by flow cytometry. All treatments were performed in duplicate, the error bars indicate the standard deviation and this data set is representative of two experiments.

Fig.6: HCV co-culture transmission is SR-BI dependent. (**A**) Anti-SR-BI mAb was titrated in a standard H77/JFH co-culture assay and inhibition of nAb sensitive (■) and nAb resistant (Δ) infection is shown. (**B)** JFH-1 and JFH-1 G451R infected producer cells were co-cultured with Huh-7.5 target cells in the presence or absence of nAb patient IgG (300μg/ml). The stacked histogram displays nAb resistant cell-to-cell transmission (white bars) and nAb sensitive transmission (black bars) for each virus. (**C)** The effect of anti-SR-BI mAb (1μg/ml) on JFH-1 and JFH-1 G451 nAb resistant (white bars) and sensitive (black bars) transmission. **(D)** Parental Huh-7.5 cells (white bars) or those over-expressing SR-BI (black) were infected with JFH-1 or JFH/G451R for 72h in a standard cell-free infectious assay. HCV positive cells were enumerated by immunofluorescent microscopy allowing quantification of infected cell focus size. The histogram displays the percentage of total infection residing in small (1-2 cells), medium (3-10), large (11-30) or very large (31-100) infected cell foci. (**E)** H77/JFH and JFH-1 infected producer cells were co-cultured with parental Huh-7.5 cells or Huh-7.5 cells transduced to over-express SR-BI (>>), in the presence or absence of neutralizing patient IgG (300μg/ml), and infected target cells quantified. H77/JFH and JFH-1 nAb resistant infection of cells over expressing SR-BI was significantly increased ($p=0.0096$ and $p=0.0037$ respectively). (**F)** Effect of the SR-BI specific small molecules ITX5061 and ITX7650 (1μM) on H77/JFH nAb sensitive (black bars) and

resistant (white bars) transmission. Treatments were performed in duplicate and error bars indicate the standard deviation.

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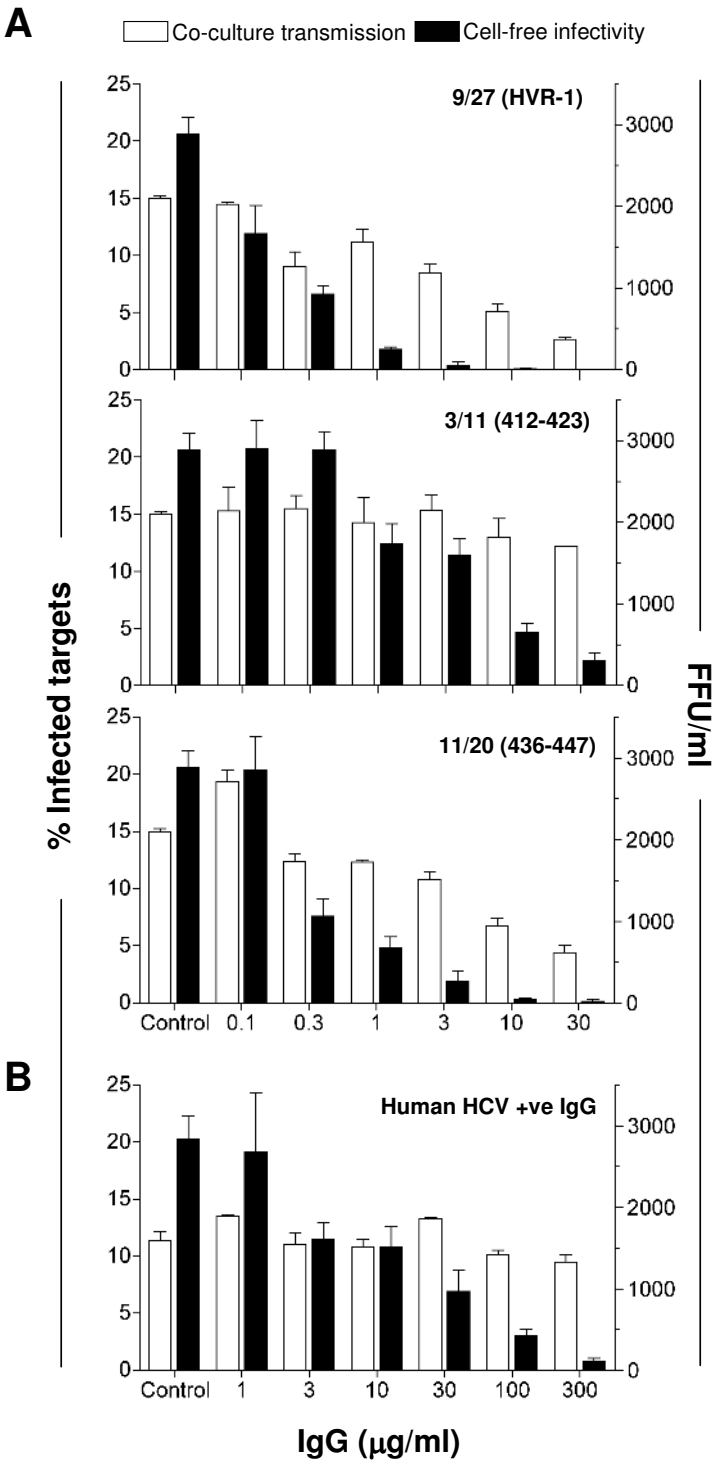


Figure 1

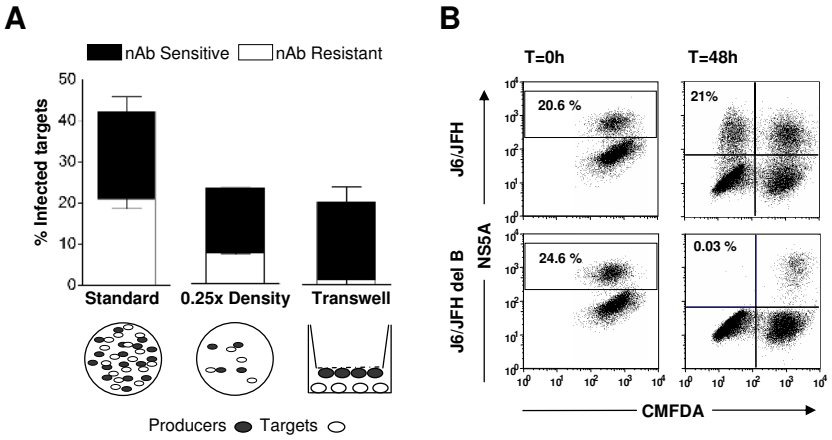
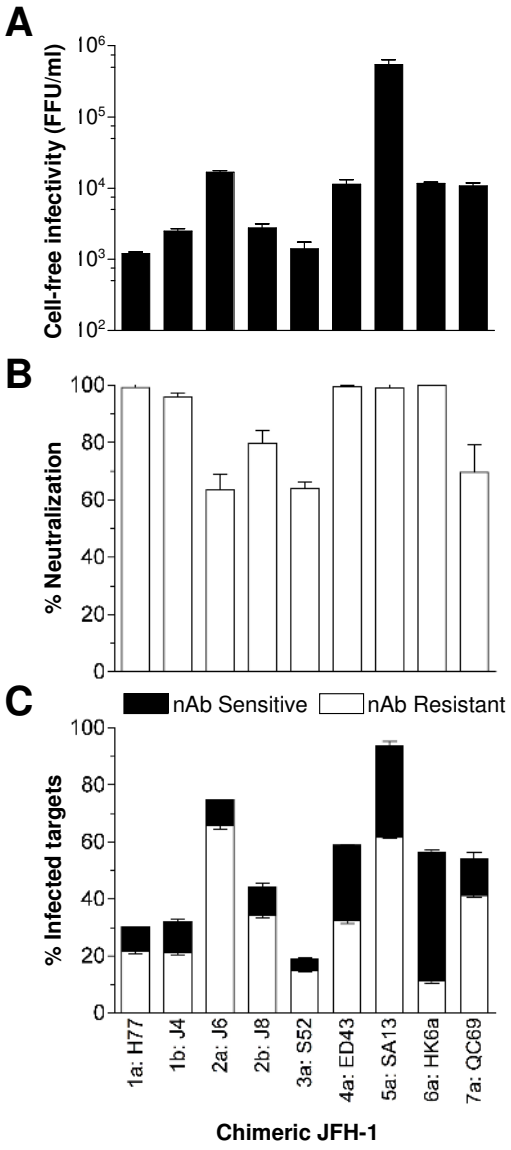


Figure 2



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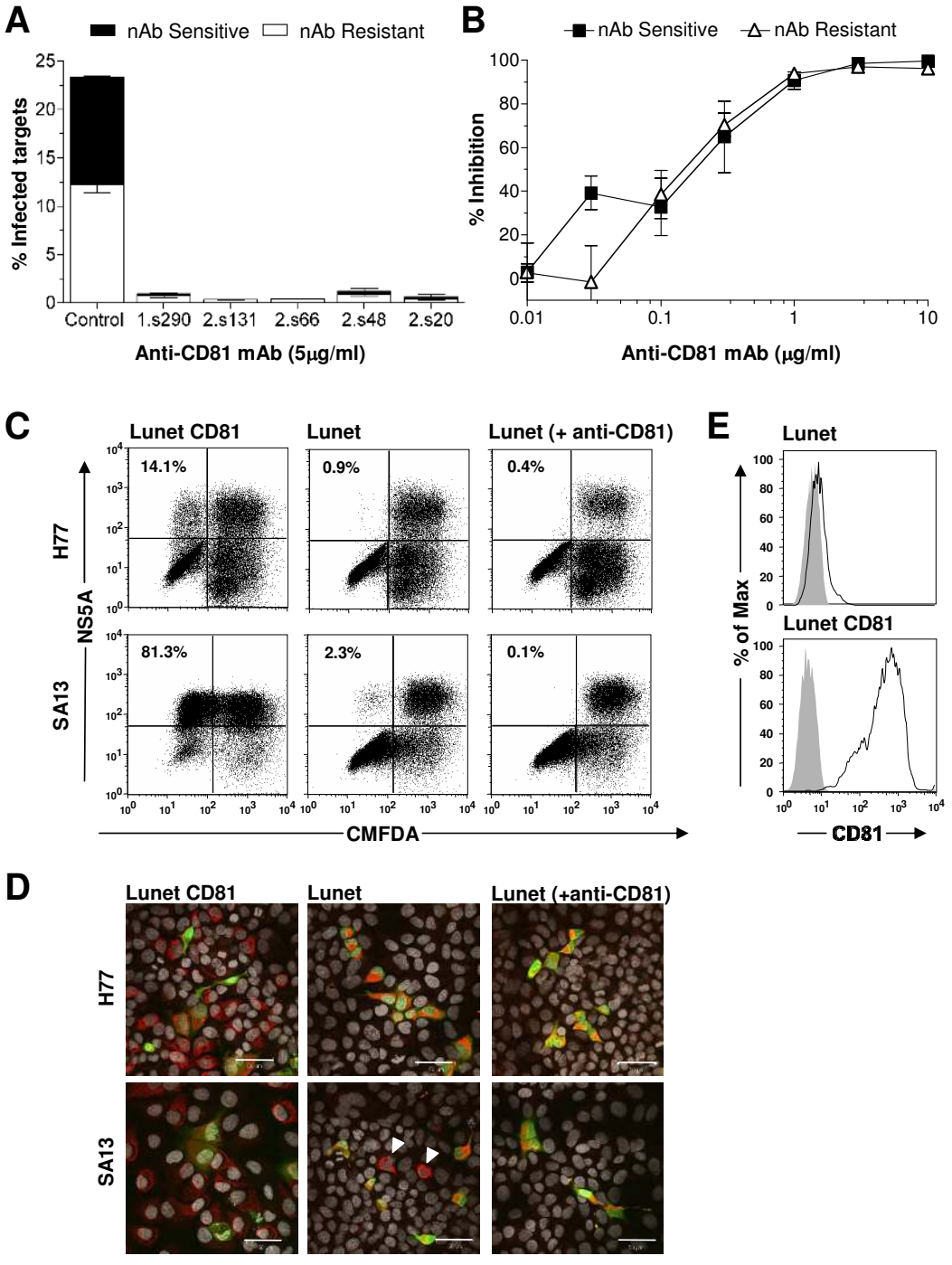

Figure 4

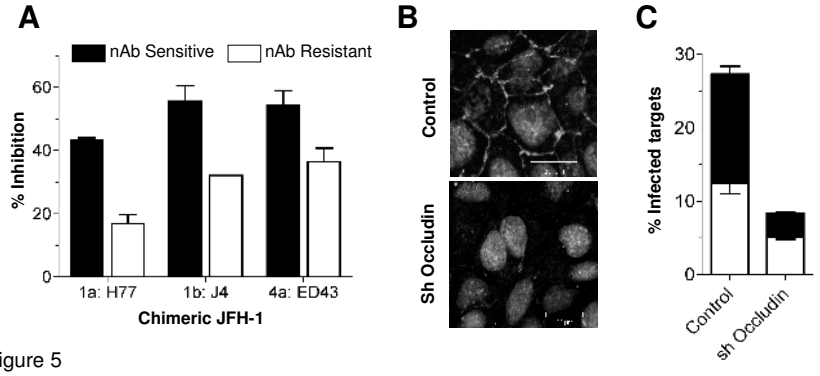

Figure 5

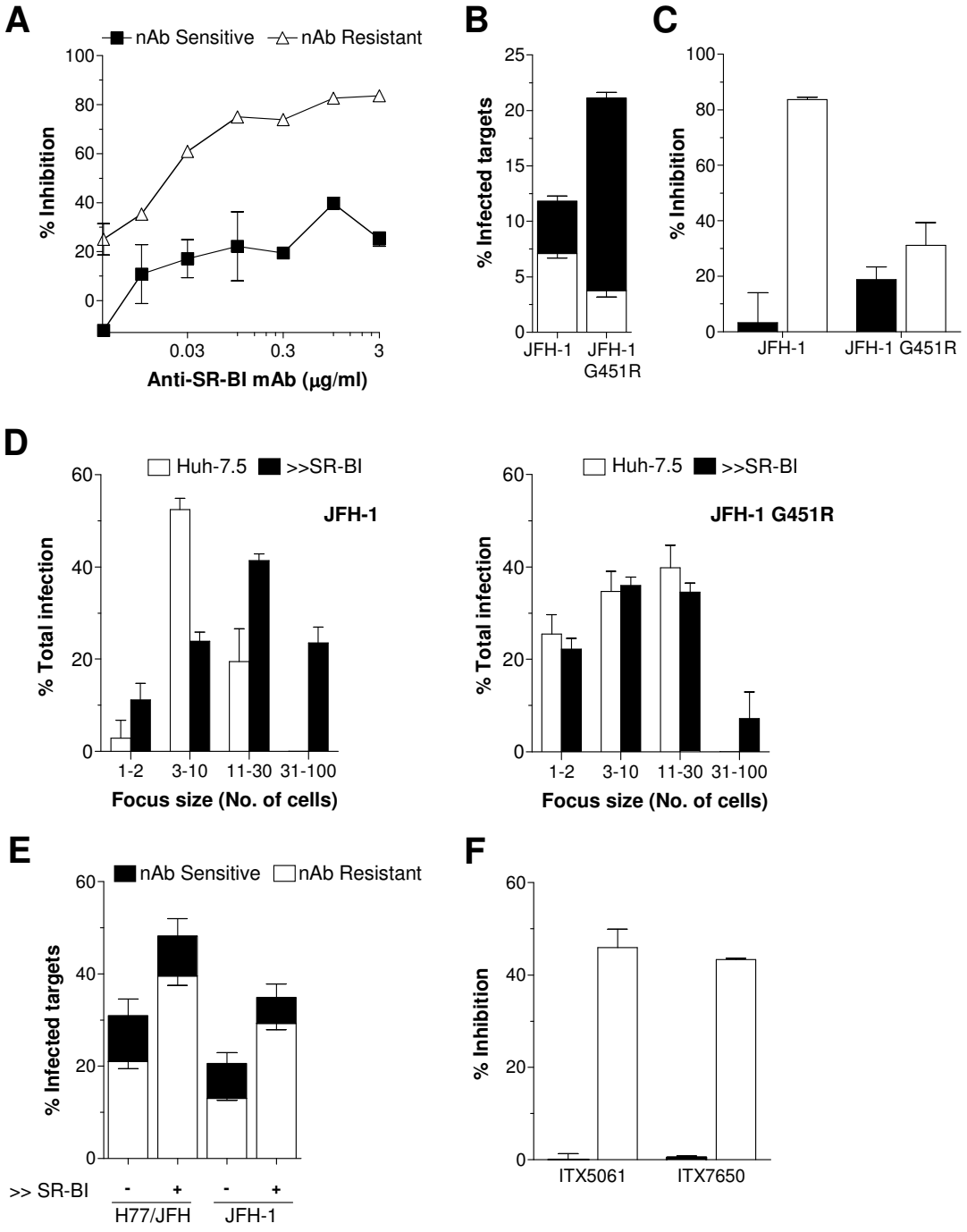

Figure 6

Table 1

		% INHIBITION							
		Anti-CD81		Anti-CLDN1		Anti-SR-BI		ITX5061	
Genotype	JFH chimera	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)