Supplementary material

The post-binding activity of scavenger receptor BI mediates initiation of hepatitis C virus infection and viral dissemination

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Supplementary Material and Methods

Cell culture-derived HCV (HCVcc) and HCV pseudoparticles (HCVpp). Luciferase reporter chimeric HCVcc of genotypes 1-4 (H77/1a/R2a, Con1/1b/R2a, J8/2b/R2a, S52/3a/R2a and ED43/4a/R2a) and HCVpp of genotypes 1-6 (H77, HCV-J, JFH1, UKN3A1.28, UKN4.21.16, UKN5.14.4 and UKN6.5.340) have been described.[1-3] Patient-derived HCVpp (P02VJ) from a patient (P02) undergoing liver transplantation have been as described.[2,4] HCVpp bearing the envelope glycoproteins of strain H77, H77 deleted of hypervariable region 1 (HVR1) within E2 (ΔG384-N411) or H77 containing a point mutation within HVR1 (L399R) have been described.[5] Luc-Jc1 HCVcc lacking HVR1 (ΔHVR1) have been described.[6]

Investigation of the antiviral effects of antibodies and antibody combinations on HCV entry and infection. HCVcc and pseudoparticle infection and kinetic assays have been described.[2,7-10] Briefly, for infection experiments, Huh7.5.1 cells were pre-incubated in the presence or absence of antibodies for 1h at 37°C and infected at 37°C for 4h with HCVcc or pseudoparticles. 72h later infection was analyzed in cell lysates by quantification of luciferase activity using a Promega kit. For kinetic entry experiments, Huh7.5.1 were inoculated with

HCVcc (10^4-10^5 $TCID_{50}$/mL) for 1h at 4°C in the presence or absence of compounds. Subsequently, cells were washed three times with ice cold PBS, supplied with fresh culture fluid pre-warmed to 37°C and supplemented with the respective compounds and shifted to 37°C. The compounds were removed after 4h and cells were supplied with fresh culture fluid without compounds and cultured an additional 48h at 37°C before quantification of luciferase activity in cell lysates. For combination experiments, each antibody was tested individually or in combination with the second antibody. Huh7.5.1 cells were pre-incubated with anti-SR-BI or control antibody for 1 h and then incubated for 4h at 37°C with HCVcc (Luc-Jc1) or HCVpp (P02VJ) (pre-incubated for 1h with or without anti-envelope antibodies).

Assessment of synergy. Synergy was assessed by two independent methods comprising the Combination Index and the method of Prichard and Shipman. The Combination Index (CI) was calculated as described.[11,12] A CI less than, equal to, and more than 1 indicates synergy, additivity, and antagonism, respectively[11]. The method of Prichard and Shipman was applied as described.[13] In brief, the theoretical additive effect is calculated from the dose-response curves of individual compounds by the equation Z=X+Y(1-X) where X and Y represent the inhibition produced by the individual compounds and Z represents the effect produced by the combination of compounds. The theoretical additive surface is subtracted from the actual experimental surface, resulting in a horizontal surface that equals the zero plane when the combination is additive. A surface raising more than 20% above the zero plane indicates a synergistic effect of the combination and a surface dropping lower than 20% below the zero plane indicates antagonism. The antiviral assay was performed as described above except that the compound dilutions were added in a checkerboard format. Combination studies for each pair of compounds were performed in triplicate.

Chimeric human/mouse SR-BI and human SR-BI mutants. Retroviral vectors expressing human SR-BI (Z22555) or human SR-BI point mutants Q402R, E418R, Q402R-E418R and G420H-G424H as well as mouse SR-BI (NM_016741) or human/mouse chimeric SR-BI were

described previously.[14,15] Briefly, mouse SR-BI (NM_016741) or human/mouse chimeric SR-BI cDNAs were inserted in CNC MLV (murine leukemia virus) vector backbones (kind gift of M. Collins) harboring selectable marker genes for puromycine and G418, respectively. Using SR-BI sequence comparisons as well as structural features predictions, three regions in the SR-BI ectodomain were delineated between amino acid (aa) positions 38-215, 216-398 and 399-432. cDNAs encoding three human/mouse SR-BI chimeras were generated by PCR by swapping these three SR-BI regions. While the HHH and MMM SR-BI constructs refer to the wild-type human (H) and mouse (M) SR-BI molecules, respectively, the human/mouse SR-BI chimeras were denominated according to the origin of either SR-BI region, e.g., HMM bears region 1 from human SR-BI and regions 2 and 3 from murine SR-BI (Supplementary Figure 5).[15] All mutants were sequenced to ensure that the clones possessed only the expected mutation.[15] Retroviral vectors containing these cDNAs were produced from 293T cells as VSVG-pseudoparticles as described previously. Stable expression of either receptor in target cells was obtained by transduction with vector particle-containing supernatants of 293T producer cells, followed by antibiotic selection. CHO and BRL3A cells expressing human, mouse, human/mouse chimeric as well as mutant SR-BI were produced as described.[8,14,15]

Epitope mapping. BRL3A or CHO cells were transduced with retroviral vectors expressing human, mouse or human-mouse chimeric SR-BI or previously described human SR-BI mutants.[14,15]Transduced cells were selected using antibiotics and proper SR-BI expression was studied using flow cytometry and commercial anti-SR-BI antibodies. Anti-SR-BI mAb binding was assessed using flow cytometry.[14]

Immunoblotting. Huh7.5.1 cells were lysed with Glo lysis buffer (Promega) and 50 µg of protein of each sample were separated by 12% SDS-PAGE, transferred to HyBond-P nitrocellulose membranes (GE Healthcare) and then incubated with anti-SR-BI mAbs QQ-4A3-A1, QQ-2A10-A5, QQ-4G9-A6, PS-6A7-C4, NK-8H5-E3, NK-6B10-E6 and NK-6G8-B5 (5 µg/mL) or EP1556Y (Abcam, 1:100) and AP-labelled secondary antibodies.[8]

Supplementary Results

Anti-SR-BI antibodies do not interfere with sE2 binding to target cells. As HCV E2 directly binds hSR-BI, we assessed their ability to interfere with E2-SR-BI binding using recombinant soluble E2 (sE2) as a surrogate model for HCV and Huh7.5.1 cells as target cells. In contrast to a polyclonal anti-SR-BI rat serum[8] and an anti-CD81 mAb, none of the anti-SR-BI mAbs inhibited sE2-SR-BI binding (Supplementary Figure 1B-C, statistically not significant). Given that Huh7.5.1 cells express all known HCV receptors that may also contribute to sE2 binding, we also used rat BRL cells lacking SR-BI[14] to assess sE2 binding to exogenously expressed hSR-BI in the absence of other HCV receptors. Although one antibody (NK-8H5-E3) appeared to have a minor inhibitory effect in some experiments, inhibition of sE2 binding was not statistically significant and not robust compared to the polyclonal anti-SRBI rat serum (Supplementary Figure 1D). Surprisingly, rat anti-SR-BI mAbs increased sE2-SR-BI binding. It is conceivable that binding of the rat mAb to SR-BI results in a different interaction of sE2 with other HCV attachment factors on BRL cells such as heparan sulfate which subsequently enhances HCV attachment. Taken together, these data confirm the findings obtained for cellular attachment of HCVcc (Figure 2) and suggest that interference with E2 binding to target cells does not play a major role for the antiviral action of SR-BI-specific mAbs described in this study.

Functional impact of HCV HVR1 and SR-BI during post-binding steps of the viral entry process. The 27 amino acid long hypervariable region 1 (HVR1) at the N-terminus of HCV E2 has been shown to mediate E2 binding to SR-BI and also to contribute to HDL-mediated enhancement of HCV entry that is dependent on the lipid transfer function of SR-BI but independent of HDL binding.[14-16] Given this complex role of HVR1 in SR-BI-dependent HCV entry steps, we investigated the effect of anti-SR-BI mAbs inhibiting HCV post-binding steps on HCVcc and HCVpp deleted in HVR1 (ΔHVR1). Interestingly, HCVcc and HCVpp lacking HVR1 were less sensitive to inhibition by anti-SR-BI mAbs (Supplementary Figure 4A-B, $p<0.01$) although requiring SR-BI for cell entry as cells lacking SR-BI are not permissive for

ΔHVR1 HCV (data not shown). This was also confirmed using HCVpp L399R containing a point mutation within HVR1 (Supplementary Figure 4C, $p<0.01$). Taken together, these data suggest that HVR1 may play a role during SR-BI-mediated post-binding steps of the HCV entry process. Since anti-SR-BI antibodies did not interfere with cellular binding of sE2, it is conceivable that the functional role of HVR during SR-Bi mediated post-binding steps may be beyond direct E2-HVR1-SR-BI interactions.

Protein determinants relevant for HCV post-binding steps lie within the N-terminal half of the human SR-BI ectodomain. To map the protein determinants important for SR-BI post-binding function during HCV entry, we first performed cross-competition studies in order to determine whether these antibodies recognize overlapping or distinct epitopes. Labelled anti-SR-BI mAb NK-8H5-E3 was incubated with Huh7.5.1 cells in the presence of increasing concentrations of unlabelled anti-SR-BI mAbs. Cross-competition experiments with labelled versions of QQ-4A3-A1, QQ-2A10-A5 and QQ-4G9-A6 demonstrated that each of these mAbs reduced binding of unlabelled rat mAbs but not mouse mAb (Supplementary Figure 6A-C). Moreover, in contrast to unlabelled mouse NK-8H5-E3, none of the three unlabelled rat mAbs (QQ-4A3-A1, QQ-2A10-A5 and QQ-4G9-A6) reduced binding of NK-8H5-E3 to Huh7.5.1 cells, comparable to control isotype mAb (Supplementary Figure 6D). The mutual cross competition between the three rat mAbs suggests that they recognize overlapping or closely related epitopes on SR-BI while the mouse mAb recognizes a distinct epitope. To further define the epitopes targeted by these antibodies, we investigated their ability to bind to human-mouse SR-BI chimeras, where part of the mouse SR-BI ectodomain was replaced by the corresponding human sequence (Supplementary Figure 5A-C).[15] While the HHH and MMM SR-BI constructs refer to the wild-type human (H) and mouse (M) SR-BI molecules, respectively, the human/mouse SR-BI chimeras were denominated according to the origin of either SR-BI region, e.g., HMM bears region 1 from human SR-BI and regions 2 and 3 from murine SR-BI (Supplementary Figure 5B-C). The overall homology between human and mouse SR-BI is 80% (54 aa difference) (Supplementary Figure 5A). There are a total of 31,

14 and 9 different aa within the first, second and third region of the SR-BI human/mouse chimeras, respectively (Supplementary Figure 5A). The three rat anti-SR-BI mAbs QQ-4A3-A1, QQ-2A10-A5, QQ-4G9-A6 bind to HMM SR-BI, i. e. aa 38-215, with high affinity and also to MHM, i. e. 216-398, to a lesser extent while the mouse mAb NK-8H5-E3 only recognizes HMM SR-BI with high affinity (Supplementary Figure 5D). These data suggest that the epitope targeted by NK-8H5-E3 lies in the N-terminal half of the human SR-BI ectodomain, between aa 38 and aa 215, while the epitope(s) targeted by QQ-4A3-A1, QQ-2A10-A5, QQ-4G9-A6 probably lie more downstream within the SR-BI ectodomain. To further map residues within SR-BI contributing to antibody binding we used previously described SR-BI point mutants.[14] Interestingly, point mutation G420H and double mutations Q402R-E418R and G420H-G424H within human SR-BI markedly reduced binding of the four anti-SR-BI mAbs inhibiting HCV infection (Supplementary Figure 5E). These data suggest that aa 402, 418, 420 and 424 may be part of the epitopes of these antibodies or that these mutations may induce conformational changes within the epitopes. Finally, to further characterize the nature of the epitopes targeted by our panel of anti-SR-BI mAbs, we assessed the ability of the anti-SR-BI mAbs to bind to human SR-BI using SDS-PAGE and Western blot. Immunostaining of SR-BI by anti-SR-BI mAbs PS-6A7-C4, NK-6B10-E6 and NK-6G8-B5 suggest that the epitopes interacting with these antibodies, that do not inhibit HCV infection, probably include linear domains (data not shown). In contrast, none of the antibodies inhibiting HCV infection interacted with linear SR-BI in Western blot experiments suggesting that the antibodies inhibiting HCV infection likely recognize predominantly conformational epitopes (data not shown). Taken together, these data indicate that anti-SR-BI mAbs inhibiting HCVcc infection recognize conformational epitopes within the N-terminal half of the SR-BI ectodomain. Moreover, these data suggest that the N-terminal ectodomain of SR-BI contains protein determinants relevant for the SR-BI post-binding function in HCV entry.

Supplementary Figure legends

Supplementary Figure 1. Monoclonal antibodies specific for human SR-BI do not block HCV E2 binding. (A) BRL3A cells engineered to express mouse (m) or human (h) SR-BI were first incubated with monoclonal anti-SR-BI antibodies (20 µg/mL) for 1h at RT before bound antibodies were detected using PE-labelled secondary antibodies and flow cytometry. Results are expressed as net mean fluorescence intensity (∆MFI). (B-C) Huh7.5.1 cells were pre-incubated with anti-CD81 (5 µg/mL), anti-SR-BI or control serum (1:100), anti-SR-BI (20 µg/mL) or control antibodies for 1h at room temperature (RT) before incubation with sE2 for 1h at RT. (B) sE2 binding was detected using mouse anti-His antibody followed by PE-labelled anti-mouse antibody and flow cytometry. (C) sE2 binding was detected using FITC-labelled mouse anti-His antibody and flow cytometry. Results are expressed as means ± SD % sE2 binding in the absence of antibody of three independent experiments performed in duplicate. (D) BRL cells engineered to express human SR-BI were pre-incubated with polyclonal anti-SR-BI or control (CTRL) serum (1:50), anti-SR-BI (20 µg/mL) or control (CTRL) antibodies for 1h at room temperature (RT) before incubation with sE2 for 1h at RT. sE2 binding was detected using FITC-labelled mouse anti-His antibody and flow cytometry. Results are expressed as means ± SD % sE2 binding in the absence of antibody of four independent experiments. * $P<0.01$

Supplementary Figure 2. Monoclonal anti-SR-BI antibodies block HCV cell-to-cell transmission and spread. (A-B) Quantification of HCV–infected target cells (Ti) after co–cultivation with HCV producer cells (Pi) during incubation with (A) control or anti-SR-BI mAb QQ-4G9-A6 (10 µg/mL) or (B) control or anti-SR-BI mAb QQ-2A10-A5 (10 µg/mL) in the presence of E2-neutralizing antibody AP33 (25 µg/mL) by flow cytometry. (C) Cell viability after long-term exposure to anti-SR-BI mAbs QQ-4G9-A6 and NK-8H5-E3. Cell viability was assessed using MTT assay after incubation of Huh7.5.1 cells for 14 days in the presence or absence of control or anti-SR-BI mAbs at 1, 10, or 100 µg/mL. Control medium and medium containing antibodies were replenished every 4 days. Data are expressed as % cell viability

relative to cells incubated in the absence of mAb and represent means ± SD from one experiment.

Supplementary Figure 3. Genotype-independent inhibition of HCVpp infection by monoclonal anti-SR-BI antibodies. Inhibition of entry into Huh7.5.1 cells of HCVpp bearing envelope glycoproteins from genotypes 5 and 6. Huh7.5.1 cells were pre-incubated with control or anti-SR-BI mAbs (50 µg/mL) for 1h at 37°C before infection with HCVpp bearing envelope glycoproteins of strains UKN5.14.4 (5) or UKN6.5.340 (6) and VSV-Gpp. HCVpp entry was analyzed by luciferase reporter gene expression. Results are expressed as % HCVpp entry and represent means ± SD from 3 independent experiments performed in triplicate. * P<0.01

Supplementary Figure 4. Inhibition of HVR1-deleted HCVcc and HCVpp by monoclonal anti-SR-BI antibodies. Huh7.5.1 cells were pre-incubated with control or anti-SR-BI mAbs (20 µg/mL) for 1h at 37°C before infection with (A) Luc-Jc1 HCVcc deleted of HVR1 (ΔHVR1) or (B-C) HCVpp bearing the envelope glycoproteins of strain H77, (B) H77 deleted of HVR1 (ΔG384-N411) or (C) H77 containing a point mutation within HVR1 (L399R). HCVpp and HCVcc infection was analyzed by luciferase reporter gene expression. Results are expressed as % HCVpp entry or HCVcc infection and represent means ± SD from (A) one experiment performed in triplicate and (B-C) 3 independent experiments performed in triplicate. * P<0.01

Supplementary Figure 5. Binding of monoclonal anti-SR-BI antibodies to human, mouse or chimeric mouse and human SR-BI as well as human SR-BI lipid transfer mutants. (A) Alignment of amino acid sequences of mouse and human SR-BI. (B-C) Schematic representations of three human/murine SR-BI chimeras that were generated through PCR by swapping three SR-BI domains between amino-acid positions 38-215 (region 1), 216-398 (region 2) and 399-432 (region 3), respectively. While the HHH and

MMM SR-BI constructs refer to the wild-type human (H) and murine (M) SR-BI molecules, respectively, the human/mouse SR-BI chimeras were denominated according to the origin of either SR-BI domain, e.g., HMM bears the region 1 from human SR-BI and the regions 2 and 3 from murine SR-BI.[15] (D) BRL3A cells engineered to express human (HHH), mouse (MMM) or chimeric mouse and human (HMM, MHM, MMH) SR-BI were first incubated with monoclonal anti-SR-BI antibodies (20 µg/mL) for 1h at room temperature before bound antibodies were detected using PE-labelled secondary antibodies. Results are expressed as means ± SD net mean fluorescence intensity (ΔMFI). (E) BRL3A cells engineered to express wild-type human SR-BI (SR-BI wt) or human SR-BI point mutants (G420H, Q402R, E418R, Q402R-E418R and G420H-G424H) were first incubated with monoclonal anti-SR-BI antibodies (20 µg/mL) for 1h at RT before bound antibodies were detected using PE-labelled secondary antibodies. Results are expressed as % binding of antibodies as compared to SR-BI wt and represent means ± SD from one out of 2 independent experiments.

Supplementary Figure 6. Competition of monoclonal anti-SR-BI antibodies for cellular binding. Huh7.5.1 cells were incubated with 0.1 µg/mL of biotinylated anti-SR-BI mAb (A) QQ-4A3-A1, (B) QQ-2A10-A5, (C) QQ-4G9-A6 or (D) NK-8H5-E3, together with increasing concentrations of unlabeled control or anti-SR-BI mAb (QQ-4A3-A1, QQ-2A10-A5, QQ-4G9-A6, NK-8H5-E3) as competitors. Following washing of cells with PBS, binding of labelled mAbs was determined by flow cytometry and is shown % binding relative to biotinylated mAb incubated in the absence of antibody.

Supplementary Figure 7. Combination of anti-SR-BI and neutralizing antibodies results in a synergistic activity in inhibiting HCV infection. Patient derived HCVpp P02VJ (A, C, E) or HCVcc (Luc-Jc1) (B, D, F) were pre-incubated with (A-B) anti-E1 (IGH526) or (C-D) anti-E2 (IGH461) mAbs or (E-F) purified heterologous anti-HCV IgG obtained from an unrelated chronically infected subject or isotype control IgG at the indicated concentrations for 1h at 37°C and added to Huh7.5.1 cells pre-incubated with increasing concentrations of

control or anti-SR-BI mAbs (NK-8H5-E3). HCVpp and HCVcc infection was analyzed by luciferase reporter gene expression. Results are expressed as mean % HCVpp entry or HCVcc infection from a representative experiment. Synergy was assessed by the Combination Index calculated as described.[11, 12] Combination of anti-E1 or anti-E2 or patient-derived anti-HCV IgG with a sub-IC_{50} concentration of anti-SR-BI mAb - which exerts only minimal inhibitory effect on HCV infection - resulted in a synergistic activity in inhibition of HCVcc infection (CIs of 0.06 to 0.67). These combinations reduced the IC_{50} of anti-SR-BI mAb by up to 100-fold. (A, C, E) CI NK-8H5-E3 + anti-E1 (1 µg/mL): 0.30; CI NK-8H5-E3 + anti-E2 (1 µg/mL): 0.51; CI NK-8H5-E3 + anti-HCV IgG (1 µg/mL): 0.67 (B, D, F) CI NK-8H5-E3 + anti-E1 (0.01 µg/mL): 0.06; CI NK-8H5-E3 + anti-E2 (0.01 µg/mL): 0.25; CI NK-8H5-E3 + anti-HCV IgG (0.1 µg/mL): 0.14.

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