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The postbinding activity of scavenger receptor class B type I mediates initiation of hepatitis C virus infection and viral dissemination

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

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2 Hepatitis C virus, scavenger receptor BI, virus entry, viral dissemination, antiviral

3

4 **Abbreviations**

5 CE: cholesteryl ester; CLDN1: claudin-1; HCV: hepatitis C virus; HCVcc: cell culture-derived

6 HCV; HCVpp: HCV pseudoparticle; HDL: high-density lipoprotein; SR-BI: scavenger receptor

7 class B type I

8

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18 **Author contribution**

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20 G., I. F., P. B., J. T., D. B., T. P., M. D., F.-L. C, F. G. and M. B. Z. performed experiments.

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22 G., T. F. B. and M. B. Z. analyzed data. M.B.Z. and T. F. B. wrote the paper. The authors

23 declare no conflict of interest. M. N. Z and M. T. as well as T. F. B. and M. B. Z contributed

24 equally.

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28

1 **Conflict of interest**

2 The authors do not report any conflict of interest. Inserm and the University of Strasbourg
3 have filed a patent application on SR-BI-specific monoclonal antibodies and inhibition of HCV
4 infection.

5

Abstract

1
2
3 Scavenger receptor class B type I (SR-BI) is a high-density lipoprotein (HDL) receptor highly
4 expressed in the liver and modulating HDL metabolism. Hepatitis C virus (HCV) is able to
5 directly interact with SR-BI and requires this receptor to efficiently enter into hepatocytes to
6 establish productive infection. A complex interplay between lipoproteins, SR-BI and HCV
7 envelope glycoproteins has been reported to take place during this process. SR-BI has been
8 demonstrated to act during binding and post-binding steps of HCV entry. While the SR-BI
9 determinants involved in HCV binding have been partially characterized, the post-binding
10 function of SR-BI remains largely unknown. To uncover the mechanistic role of SR-BI in viral
11 initiation and dissemination we generated a novel class of anti-SR-BI monoclonal antibodies
12 that interfere with post-binding steps during the HCV entry process without interfering with
13 HCV particle binding to the target cell surface. Using the novel class of antibodies and cell
14 lines expressing murine and human SR-BI we demonstrate that the post-binding function of
15 SR-BI is of key impact for both initiation of HCV infection and viral dissemination.
16 Interestingly, this post-binding function of SR-BI seems not to be related to HDL interaction
17 but appears to be directly linked to its lipid transfer function. Conclusion: Taken together, our
18 results uncover a crucial role of the SR-BI post-binding function for initiation and
19 maintenance of viral HCV infection which does not require receptor-E2/HDL interactions. The
20 dissection of the molecular mechanisms of SR-BI-mediated HCV entry opens a novel
21 perspective for the design of entry inhibitors interfering specifically with the proviral function
22 of SR-BI.
23

1 Hepatitis C virus (HCV) is a major cause of liver cirrhosis and hepatocellular carcinoma.
2 Preventive modalities are absent and the current antiviral treatment is limited by resistance,
3 toxicity and high costs.¹ Viral entry is required for initiation, spread, and maintenance of
4 infection, and thus is a promising target for antiviral therapy. HCV binding and entry into
5 hepatocytes is a complex process involving the viral envelope glycoproteins E1 and E2, as
6 well as several host factors, among which highly sulfated heparan sulfate, CD81, the low-
7 density lipoprotein receptor, scavenger receptor class BI (SR-BI), claudin-1 (CLDN1),
8 occludin (OCLN), and receptor tyrosine kinases.^{2,3}

9 Human SR-BI is a glycoprotein highly expressed in tissues with a high cholesterol
10 need for steroidogenesis and the liver.⁴ SR-BI is a multifunctional molecule well known to
11 modulate high-density lipoprotein (HDL) metabolism. SR-BI binds a variety of lipoproteins
12 and mediates selective uptake of HDL cholesterol ester (CE) as well as bidirectional free
13 cholesterol transport at the cell membrane. Genetic SR-BI variants have been associated
14 with HDL levels in humans and a recent study uncovered a functional mutation in SR-BI
15 impairing SR-BI function and affecting cholesterol homeostasis.⁵ SR-BI also interacts with
16 different pathogens, including HCV⁶⁻⁸, and mediates their entry/uptake into host cells. SR-BI
17 is relevant for HCV infection *in vivo* and its potential as an antiviral target has recently been
18 reported.⁹

19 SR-BI directly binds HCV E2^{6, 8} but virus-associated lipoproteins also contribute to
20 host cell binding and uptake.^{10,11} Moreover, physiological SR-BI ligands modulate HCV
21 infection.¹²⁻¹⁴ This suggests the existence of a complex interplay between lipoproteins, SR-BI
22 and HCV envelope glycoproteins for HCV entry. SR-BI has also been demonstrated to
23 mediate post-binding events during HCV entry.¹⁵⁻¹⁷ HCV-SR-BI interaction during post-
24 binding steps occurs at similar time-points as the HCV utilization of CD81 and CLDN1,
25 suggesting that HCV entry may be mediated through the formation of co-receptor
26 complex(es).^{15,18,19} These data suggest that SR-BI plays a multifunctional role during HCV
27 entry at both binding and post-binding steps.^{15,20} This is corroborated by the fact that murine
28 SR-BI does not bind E2^{20, 21} although it is capable of promoting HCV entry.^{20,22}

1 To elucidate the mechanistic function of SR-BI in the HCV entry process and to
2 explore its potential as an antiviral target, we generated a novel class of monoclonal
3 antibodies directed against human SR-BI that inhibit HCV entry during post-binding steps
4 without preventing E2 binding to target cells.

5

Material and Methods

For more experimental details please refer to Supplementary material.

Cells. HEK293T, Chinese hamster ovary (CHO), Buffalo Rat Liver (BRL3A), Huh7, Huh7.5-GFP and Huh7.5.1 cells were cultured as described.^{18,23-25} Primary human hepatocytes were isolated as described.¹⁸ CHO and BRL3A cells expressing SR-BI were produced as described.^{11,15,23}

Antibodies. Polyclonal¹⁵ and monoclonal antibodies (mAbs) directed against the extracellular loop of SR-BI were raised by genetic immunization of Wistar rats and Balb/c mice as described¹⁵ according to proprietary technology (Aldevron GmbH, Freiburg, Germany). Anti-SR-BI mAbs were purified using protein G affinity columns and selected by flow cytometry for their ability to bind to human SR-BI.¹⁵ To determine the affinity of the anti-SR-BI mAbs for human SR-BI, Huh7.5.1 cells were incubated with increasing concentrations of mAbs and binding was assessed using flow cytometry. Kd values were determined as half-saturating concentrations of the mAbs.^{26,27} Antibodies will be provided on request using an MTA. Anti-CD81 (JS-81), anti-SR-BI (CLA-1) and phycoerythrin (PE)-conjugated anti-mouse antibodies were from BD Biosciences. Anti-His and FITC-conjugated anti-His antibodies were from Qiagen, rabbit anti-actin (AA20-30) antibodies from Sigma-Aldrich and mouse anti-NS5A from Virostat. Anti-E1 (IGH520, IGH526, Innogenetics), anti-E2 (IGH461, Innogenetics; AP33, Genentech; CBH23, a kind gift from S.K.H. Fong) and patient-derived anti-HCV IgG have been described.^{16,25,27}

Cell culture-derived HCV (HCVcc) and pseudoparticle production and infection.

Luciferase reporter HCVcc, HCVpp, MLVpp and VSV-Gpp, infection and kinetic experiments have been described.^{15,18,25,27,28} Unless otherwise stated, HCVcc experiments were performed using Luc-Jc1 and infection was analyzed in cell lysates by quantification of

1 luciferase activity.²⁹ For combination experiments, each antibody was tested individually or in
2 combination with a second antibody. Huh7.5.1 cells were pre-incubated with anti-SR-BI or
3 control mAb for 1h and then incubated for 4h at 37°C with HCVcc (Luc-Jc1) or HCVpp
4 (P02VJ) (pre-incubated for 1h with or without anti-envelope antibodies). Synergy was
5 assessed using the combination index and the method of Prichard and Shipman.³⁰⁻³² Cell
6 viability was assessed using a MTT test.²

7
8 **Cellular binding of envelope glycoprotein E2.** Recombinant His-tagged soluble E2 (sE2)
9 was produced as described.²³ Huh7.5.1 cells were pre-incubated with control or anti-SR-BI
10 serum (1:50), anti-SR-BI or control mAbs (20 µg/mL) for 1h at room temperature (RT) and
11 then incubated with sE2 for 1h at RT. Binding of sE2 was revealed using flow cytometry as
12 described.^{18,23}

13
14 **HCVcc binding.** Huh7.5.1 cells were pre-incubated with heparin (100 µg/mL), control or anti-
15 SR-BI serum (1:50), anti-SR-BI or control mAbs (20 µg/mL) for 1h prior to incubation with
16 HCVcc at 4°C as described.¹⁸ Non-bound HCVcc were removed by washing of cells with
17 PBS and cell bound HCV RNA was then quantified by RT-PCR.¹⁸

18
19 **HCV cell-to-cell transmission.** HCV cell-to-cell transmission was assessed as described.^{2,24}
20 Producer Huh7.5.1 cells were electroporated with Jc1 RNA³³ and cultured with naive target
21 Huh7.5-GFP cells in the presence or absence of anti-SR-BI or control mAbs. An HCV E2-
22 neutralizing antibody (AP33, 25 µg/mL) was added to block cell-free transmission.²⁴ After 24h
23 of co-culture, cells were fixed with paraformaldehyde, stained with an NS5A-specific antibody
24 (Virostat) and analyzed by flow cytometry.^{2,24}

25
26 **Immunofluorescence of viral dissemination.** Cell spread was assessed by visualizing Jc1-
27 infected Huh7.5.1 cells by immunofluorescence using anti-NS5A (Virostat) and anti-E2
28 (CBH23) antibodies as described.²

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HDL binding. HDL was labeled using Amersham Cy5 Mono-Reactive Dye Pack (GE Healthcare). Unbound Cy5 was removed by applying labeled HDL on illustra MicroSpin G-25 Columns (GE Healthcare). Blocking of Cy5-HDL binding with indicated reagents was performed for 1h at RT prior to Cy5-HDL binding for 1h at 4°C on 10⁶ target cells.

Lipid transfer assays. Selective HDL-CE uptake and lipid efflux assays were performed as described.^{23,34} HDL-CE uptake was assessed in the presence or absence of anti-SR-BI mAbs (20 µg/mL) and ³H-CE-labelled HDL (60 µg protein) for 5h at 37°C. Selective uptake was calculated from the known specific radioactivity of radiolabelled HDL-CE and is denoted in µg HDL-CE/µg cell protein. For lipid efflux assay, Huh7 cells were labeled with ³H-cholesterol (1 µCi/mL) and incubated at 37°C for 48h as described.^{23,35} Cells were incubated with anti-SR-BI mAbs (20 µg/mL) for 1h prior to incubation with unlabeled HDL for 4h. Fractional cholesterol efflux was calculated as the amount of label obtained in the medium divided by the total in each well (radioactivity in the medium + radioactivity in the cells) regained after lipid extraction from cells.

Statistical analysis. Unless otherwise stated, data are presented as means ± SD of three independent experiments. Statistical analyses were performed using Student's t test and/or Mann-Whitney test with a P value of <0.01 being considered statistically significant.

Results

1
2
3 **Production of SR-BI-specific monoclonal antibodies interfering with the post-binding**
4 **steps of viral entry.** To further explore the role of HCV-SR-BI interaction during HCV
5 infection, we generated five rat and three mouse monoclonal antibodies (mAbs) directed
6 against the human SR-BI (hSR-BI) ectodomain (Table 1). These antibodies bound to
7 endogenous SR-BI on human hepatoma Huh7.5.1 cells and primary human hepatocytes
8 (PHH) but did not bind to mouse SR-BI (mSR-BI) expressed on rat BRL cells (Figure 1A-B,
9 Supplementary Figure 1). Three rat (QQ-4A3-A1, QQ-2A10-A5 and QQ-4G9-A6) and one
10 mouse mAb (NK-8H5-E3) significantly ($p < 0.01$) inhibited HCVcc infection in a dose-
11 dependent manner with 50% inhibitory concentrations (IC_{50}) between 0.2 to 8 $\mu\text{g/mL}$ (Figure
12 1C-D, Table 1). The apparent K_d (K_{dapp}) corresponding to the half-saturating concentrations
13 for binding to Huh7.5.1 cells ranged from 0.5 to 7.4 nM demonstrating that these antibodies
14 recognize SR-BI with high affinity (Table 1). It is noteworthy that there seems to be a
15 correlation between the antibody affinity and inhibitory capacity with the low affinity
16 antibodies unable to block HCV infection. We next aimed to characterize the viral entry steps
17 targeted by these anti-SR-BI mAbs. We first assessed their ability to interfere with viral
18 binding. To reflect the complex interaction between HCV and hSR-BI during viral binding, we
19 studied the effect of anti-SR-BI mAbs on HCVcc binding to Huh7.5.1 cells at 4°C. Incubation
20 of Huh7.5.1 cells with anti-SR-BI mAbs prior to and during HCVcc binding did not inhibit virus
21 particle binding (Figure 2A). Similar results were obtained using sE2 as a surrogate model for
22 HCV (Supplementary results, Supplementary Figure 1). These data suggest that, in contrast
23 to previously described anti-SR-BI mAbs²⁰, these novel anti-SR-BI mAbs do not inhibit HCV
24 binding but interfere with HCV entry during post-binding steps. Next, to characterize potential
25 post-binding steps targeted by these anti-SR-BI mAbs, we assessed HCVcc entry kinetics
26 into Huh7.5.1 cells in the presence of anti-SR-BI mAbs inhibiting HCV infection (QQ-4A3-A1,
27 QQ-2A10-A5 and QQ-4G9-A6 and NK-8H5-E3) added at different time-points during or after
28 viral binding (Figure 2B). This assay was performed side-by-side with an anti-CD81 mAb

1 inhibiting HCV post-binding^{15,18,29} and proteinase K³⁶ to remove HCV from the cell surface.
2 HCVcc binding to Huh7.5.1 cells was performed for 1h at 4°C in the presence or absence of
3 compounds. Subsequently, unbound virus was washed away, cells were shifted to 37°C to
4 allow HCVcc entry and compounds were added every 20 min for up to 120 min after viral
5 binding. These kinetic experiments indicate that anti-SR-BI mAbs inhibited HCVcc infection
6 when added immediately after viral binding as well as 20 to 30 min after initiation of viral
7 entry (Figure 2C) demonstrating that QQ-4A3-A1, QQ-2A10-A5 and QQ-4G9-A6 and NK-
8 8H5-E3 indeed target post-binding steps of the HCV entry process. This timeframe is
9 comparable to the kinetics of resistance of internalized virus to proteinase K (Figure 2C)
10 indicating that these post-binding steps precede completion of virus internalization. Taken
11 together, these data indicate that a post-binding function of SR-BI is essential for initiation of
12 HCV infection. In contrast to previous anti-SR-BI mAbs inhibiting HCV binding²⁰ as well as
13 polyclonal anti-SR-BI antibodies and small molecules interfering with both viral binding and
14 post-binding^{15,17,23}, these antibodies are the first molecules exclusively targeting the post-
15 binding function of SR-BI and thus represent a unique tool to more thoroughly assess the
16 relevance of this function for HCV infection.

17
18 **A post-binding function of SR-BI is essential for cell-to-cell transmission and viral**
19 **spread.** HCV disseminates via direct cell-to-cell transmission.^{24,37} To assess the role of SR-
20 BI post-binding function in viral dissemination, we first investigated the ability of the anti-SR-
21 BI mAbs to interfere with neutralizing antibody-resistant viral spread by studying direct HCV
22 cell-to-cell transmission in the presence of anti-SR-BI mAbs QQ-2A10-A5 and QQ-4G9-A6.
23 Viral “producer” cells containing replicating HCV Jc1 (Pi) are co-cultured with GFP-
24 expressing “target” cells (T) in the presence of E2-neutralizing mAb (AP33, 25 µg/mL) to
25 prevent cell-free HCV transmission.²⁴ AP33 reduces cell-free transmission by >90% and
26 infectivity of producer cell supernatants is minimal at the time of co-culture; viral transmission
27 thus occurs predominantly by cell-to-cell transmission in this assay.^{2,24} HCV cell-to-cell
28 transmission is assessed by quantifying HCV-infected, GFP-positive target cells (Ti) by flow

1 cytometry.^{2,24} Both anti-SR-BI mAbs (10 $\mu\text{g}/\text{mL}$) efficiently blocked HCV cell-to-cell
2 transmission (Figure 3A, $p < 0.01$; Supplementary Figure 2 A-B) indicating that these
3 antibodies may prevent viral spread *in vitro*. As these anti-SR-BI mAbs do not block HCV-
4 SR-BI binding (Figure 2A) but inhibit HCV entry during post-binding steps (Figure 2C), these
5 data suggest that a SR-BI post-binding function plays an important role during HCV cell-to-
6 cell transmission. To ascertain the importance of the SR-BI post-binding function in this
7 process, we performed additional cell-to-cell transmission assays using mSR-BI, which in
8 contrast to hSR-BI is unable to bind E2. Cells lacking SR-BI and robustly replicating HCV,
9 that would be an ideal model cell to study cell-to-cell transmission by mSR-BI in the absence
10 of hSR-BI, have not been described. However, hSR-BI has been reported to be a limiting
11 factor for HCV spread in Huh7-derived cells as overexpression of hSR-BI increases cell-to-
12 cell transmission.³⁷ We thus used Huh7.5 cells or Huh7.5 cells overexpressing either mSR-BI
13 or hSR-BI as target cells. Cell-to-cell transmission was enhanced in Huh7.5 cells
14 overexpressing either hSR-BI (2.04 ± 0.03 fold) or mSR-BI (1.92 ± 0.19 fold) as compared to
15 parental cells (Figure 3B, $p < 0.01$). These data indicate that E2-SR-BI binding is not essential
16 for viral dissemination and confirm the crucial role of SR-BI post-binding function in this
17 process. Furthermore, to assess whether anti-SR-BI mAbs prevent viral dissemination in
18 already HCV-infected cell cultures when added post-infection, we performed a long-term
19 analysis of HCVcc infection by culturing Luc-Jc1 infected Huh7.5.1 cells in the presence or
20 absence of control or anti-SR-BI mAbs QQ-4G9-A6 and NK-8H5-E3 as previously
21 described.² When added 48h after infection and maintained in cell culture medium
22 throughout the experiment, these anti-SR-BI mAbs efficiently inhibited HCV spread over 2
23 weeks in a dose-dependent manner without affecting cell viability (Figure 3C-D,
24 Supplementary Figure 2C-D). We also assessed Jc1 spread in Huh7.5.1 cells by
25 immunostaining of infected cells as described.² While $74.5 \pm 2.3\%$ and $70.0 \pm 3.2\%$ of cells
26 incubated with control rat or mouse mAbs stained positive for NS5A and E2, respectively,
27 incubation with QQ-4G9-A6 and NK-8H5-E3 markedly reduced the number of NS5A- ($14.2 \pm$

1 3,4%) and E2-positive ($16.7 \pm 2.6\%$) cells (Figure 3E-F). Taken together, these data indicate
2 that a post-binding function of SR-BI is required for HCV cell-to-cell transmission and spread.

3
4 **SR-BI determinants relevant for HCV post-binding steps may be linked to the lipid**

5 **transfer function of the entry factor.** The SR-BI ectodomain has been demonstrated to be

6 important for both HDL binding and CE uptake but the determinants involved in these

7 processes have not been precisely defined yet. To assess whether anti-SR-BI mAbs

8 inhibiting HCV post-binding steps affect HDL binding to SR-BI, we studied Cy5-labeled HDL

9 binding to hSR-BI in the presence or absence of anti-SR-BI mAbs. In contrast to polyclonal

10 anti-SR-BI serum which inhibited Cy5-labeled HDL binding, none of the anti-SR-BI mAbs

11 markedly interfered with HDL-SR-BI binding at concentrations inhibiting HCV infection by up

12 to 90% (Figure 4A, statistically not significant). Furthermore, we investigated the effect of

13 these mAbs on CE uptake and cholesterol efflux. While PS-6A7-C4, PS-7B11-E3, NK-6B10-

14 E6 and NK-6G8-B5 had no effect on lipid transfer, QQ-4A3-A1, QQ-2A10-A5, QQ-4G9-A6

15 and NK-8H5-E3 partially reduced both CE uptake and cholesterol efflux at concentrations

16 inhibiting HCV infection by up to 90% (Figure 4B-C, $p < 0.01$). These data indicate that the

17 anti-SR-BI mAbs inhibiting HCVcc infection also partially inhibit SR-BI mediated lipid transfer

18 (Table 1). Taken together, these data suggest that SR-BI determinants involved in HCV post-

19 binding events do not mediate HDL binding but may contribute to lipid transfer, in line with

20 the reported link between the SR-BI lipid transfer function and HCV infection.^{11,12,23}

21
22 **Synergy between antibodies targeting SR-BI post-binding function and neutralizing**

23 **antibodies on inhibition of HCV infection.** To assess the clinical relevance of blocking SR-

24 BI post-binding function to inhibit HCV infection, we determined the effect of anti-SR-BI mAbs

25 on entry into Huh7.5.1 cells of HCVcc and HCVpp of major genotypes and highly infectious

26 HCV strains selected during liver transplantation (P02VJ). All anti-SR-BI mAbs inhibiting

27 HCVcc genotype 2a (Jc1) infection (QQ-4A3-A1, QQ-2A10-A5, QQ-4G9-A6 and NK-8H5-E3)

28 also inhibited infection of HCVcc and HCVpp of all major genotypes ($p < 0.01$) whereas VSV-

1 Gpp entry was unaffected (Figure 5, Supplementary Figure 3). Moreover, entry of patient-
2 derived HCVpp P02VJ into both Huh7.5.1 cells and PHH was also efficiently inhibited by
3 these anti-SR-BI mAbs (Supplementary Figure 7 and data not shown). Given that
4 combinations of drugs targeting both viral and host factors represents a promising future
5 approach to prevent and treat HCV infection, we next determined whether the combination of
6 anti-SR-BI mAb NK-8H5-E3 or QQ-2A10-A5 and anti-HCV envelope antibodies results in an
7 additive or synergistic effect on inhibiting HCV infection. Thereto, each antibody was tested
8 individually or in combination with a second antibody in a checkerboard format and synergy
9 was assessed using the Combination Index (CI) and the method of Prichard and Shipman³⁰⁻
10 ³². Combination of anti-SR-BI and anti-HCV envelope antibodies resulted in a synergistic
11 effect on inhibition of HCVpp P02VJ entry and HCVcc infection as reflected by a CI of 0.06 to
12 0.67 (Supplementary Figure 7) and synergy of low doses was confirmed using the method of
13 Prichard and Shipman (Figure 6). These combinations reduced the IC₅₀ of anti-SR-BI mAb by
14 up to 100-fold (Supplementary Figure 7). The marked observed synergy may be explained
15 by the fact the anti-envelope- and SR-BI-specific antibodies target highly complementary
16 steps during HCV entry. Taken together, these data indicate that interfering with SR-BI post-
17 binding function may hold promise for the design of novel antiviral strategies targeting HCV
18 entry factors.
19

Discussion

We generated novel anti-SR-BI mAbs specifically inhibiting HCV entry during post-binding steps that enabled us for the first time, using endogenous SR-BI, to explore and validate the hypothesis that SR-BI has a multifunctional role during HCV entry and to elucidate the functional role of SR-BI post-binding activity for HCV infection. Our data demonstrate that the HCV post-binding function of hSR-BI can indeed be dissociated from its E2-binding function. Moreover, we demonstrate that the post-binding activity of SR-BI is of key relevance for cell-free HCV infection as well as cell-to-cell transmission.

SR-BI mediates uptake of HDL-CE in a two-step process including HDL binding and subsequent transfer of CE into the cell without internalization of HDL. At the same time, SR-BI also participates in HCV binding and entry into target cells. SR-BI is able to directly bind E2 and virus-associated lipoproteins but additional function(s) of SR-BI have been reported to be at play during HCV infection.^{11,12,15,23} The results from this study highlight the importance of a SR-BI post-binding function for HCV entry and further extend the relevance of this function for HCV cell-to-cell transmission.

The molecular mechanisms underlying HCV cell-to-cell transmission are only partially understood. A recent study showed that SR-BI contributes to this process³⁷ and that E2-SR-BI interaction and/or SR-BI-mediated lipid transfer likely takes place during HCV dissemination as antibodies and small molecule inhibitors targeting both SR-BI binding and lipid transfer reduce HCV cell-to-cell transmission.^{9,17} However, which SR-BI functions are relevant for this process remained to be determined. Taking advantage of our novel mAbs uniquely inhibiting SR-BI post-binding activity required for HCV entry, we demonstrated that an E2 binding-independent post-binding function is involved in neutralizing antibody-resistant cell-to-cell transmission. E2-independent SR-BI function in HCV dissemination is in line with the observation that cell-to-cell transmission is largely insensitive to E2-specific antiviral mAbs.³⁷ Given that mSR-BI does not bind sE2 but mediates HCV entry and promotes cell-to-cell transmission, the post-binding function of SR-BI seems to be essential for HCV infection

1 and dissemination while the binding function may be dispensable. Furthermore, since HVR1-
2 deleted HCV is less sensitive to inhibition by anti-SR-BI mAbs (Supplementary results,
3 Supplementary Figure 4), HVR1-SR-BI interaction may play an important role during post-
4 binding steps of HCV entry.

5 Previous studies using small molecule inhibitors indicated a role for SR-BI lipid
6 transfer function in HCV infection and HDL-mediated entry enhancement.^{12,13,23} As inhibition
7 of cell-free HCV entry and cell-to-cell transmission by our anti-SR-BI mAbs was associated
8 with interference with lipid transfer, our data suggest that the SR-BI lipid transfer function
9 may be relevant for both initiation of HCV infection and viral dissemination. Noteworthy, our
10 anti-SR-BI mAbs are the first anti-SR-BI mAbs that do not inhibit HDL binding to SR-BI.
11 These data suggest that HCV entry and dissemination can be inhibited without blocking
12 HDL-SR-BI binding. The further characterization of the SR-BI post-binding function will allow
13 to determine whether the SR-BI-mediated post-binding steps of HCV entry and dissemination
14 are directly linked to its lipid transfer function.

15 Using SR-BI chimeras, we demonstrate that the determinants relevant for HCV post-
16 binding steps lie within N-terminal half of the human SR-BI ectodomain (Supplementary
17 results, Supplementary Figures 5 and 6). Amino acids 70 to 87 and residue E210 of SR-BI
18 are required for E2 binding while distinct protein regions are involved in HDL binding.^{20,38}
19 Although the SR-BI determinants involved in HDL binding and CE uptake have not been
20 precisely defined yet, a recent study reported that amino acid C323 is critical for these
21 processes.³⁸ Given that our anti-SR-BI mAbs do not interfere with E2 and HDL binding,
22 amino acids 70-87 and residues E210 and C323 are most likely not part of the targeted
23 epitope(s). Interestingly, the amino acid associated with cholesterol homeostasis⁵ probably
24 also lies outside these epitope(s). The further characterization of the(se) epitope(s) may
25 allow to more thoroughly determine the regions of SR-BI relevant for its post-binding function
26 during initiation of HCV infection and spread.

27 Finally, our data suggest that the SR-BI post-binding function is a highly relevant
28 target for antivirals. Therapeutic options for a large proportion of HCV-infected patients are

1 still limited by drug resistance and adverse effects.¹ Furthermore, a strategy for prevention of
2 HCV liver graft infection is absent. Antivirals targeting essential host factors required for the
3 HCV life cycle are attractive since they may increase the genetic barrier to antiviral
4 resistance.^{2,3} Indeed, our data demonstrate a marked synergy on the inhibition of HCV entry
5 when combining antibodies directed against the viral envelope and SR-BI. These results
6 suggest that combining molecules directed against viral and host entry factors is a promising
7 strategy for prevention of HCV infection such as liver graft infection. The potent effect on cell-
8 to-cell transmission and viral spread also opens a perspective of SR-BI-based entry inhibitors
9 for treatment of chronic infection.

10 Small molecules and mAbs targeting SR-BI and interfering with HCV infection have
11 previously been described.^{12,17,26} A human anti-SR-BI mAb has been reported to inhibit HDL
12 binding, to interfere with cholesterol efflux and to decrease HCVcc entry during attachment
13 steps without having a relevant impact on SR-BI mediated post-binding steps.^{20,26} A codon-
14 optimized version of this mAb has been demonstrated to prevent HCV spread *in vivo*⁹
15 underscoring the potential of SR-BI as an antiviral target. The mAbs generated in our study
16 are highly novel in their function as they do not interfere with sE2-SR-BI binding but inhibit
17 HCV entry during post-binding steps of cell-free infection and cell-to-cell transmission.
18 Furthermore, in contrast to previously described anti-SR-BI mAbs²⁶, these mAbs do not
19 hinder HDL-SR-BI binding and only partially inhibit lipid transfer at concentrations
20 significantly inhibiting HCV infection. Given their novel mechanism of action and their
21 potential differential toxicity profile, QQ-4A3-A1, QQ-2A10-A5, QQ-4G9-A6 and NK-8H5-E3
22 define a novel class of anti-SR-BI mAbs for prevention and treatment of HCV infection.

23

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References

- 1
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3 1. Pawlotsky JM. Treatment failure and resistance with direct-acting antiviral drugs
4 against hepatitis C virus. *Hepatology* 2011;53:1742-1751.
- 5 2. Lupberger J, Zeisel MB, Xiao F, Thumann C, Fofana I, Zona L, Davis C, et al. EGFR
6 and EphA2 are host factors for hepatitis C virus entry and possible targets for antiviral
7 therapy. *Nature Medicine* 2011;17:589-595.
- 8 3. Zeisel MB, Fofana I, Fafi-Kremer S, Baumert TF. Hepatitis C virus entry into
9 hepatocytes: Molecular mechanisms and targets for antiviral therapies. *J Hepatol*
10 2011;54:566-576.
- 11 4. Krieger M. Scavenger receptor class B type I is a multiligand HDL receptor that
12 influences diverse physiologic systems. *J Clin Invest* 2001;108:793-797.
- 13 5. Vergeer M, Korporaal SJ, Franssen R, Meurs I, Out R, Hovingh GK, Hoekstra M, et
14 al. Genetic variant of the scavenger receptor BI in humans. *N Engl J Med* 2011;364:136-145.
- 15 6. Scarselli E, Ansuini H, Cerino R, Roccasecca RM, Acali S, Filocamo G, Traboni C, et
16 al. The human scavenger receptor class B type I is a novel candidate receptor for the
17 hepatitis C virus. *EMBO J* 2002;21:5017-5025.
- 18 7. Bartosch B, Vitelli A, Granier C, Goujon C, Dubuisson J, Pascale S, Scarselli E, et al.
19 Cell entry of hepatitis C virus requires a set of co-receptors that include the CD81 tetraspanin
20 and the SR-B1 scavenger receptor. *J Biol Chem* 2003;278:41624-41630.
- 21 8. Evans MJ, von Hahn T, Tscherne DM, Syder AJ, Panis M, Wolk B, Hatzioannou T, et
22 al. Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry. *Nature*
23 2007;446:801-805.
- 24 9. Meuleman P, Catanese MT, Verhoye L, Desombere I, Farhoudi A, Jones CT,
25 Sheahan T, et al. A human monoclonal antibody targeting scavenger receptor class B type I
26 precludes hepatitis C virus infection and viral spread in vitro and in vivo. *Hepatology*
27 2012;55:364-372.

- 1 10. Maillard P, Huby T, Andreo U, Moreau M, Chapman J, Budkowska A. The interaction
2 of natural hepatitis C virus with human scavenger receptor SR-BI/Cla1 is mediated by ApoB-
3 containing lipoproteins. *Faseb J* 2006;20:735-737.
- 4 11. Dao Thi VL, Granier C, Zeisel MB, Guerin M, Mancip J, Granio O, Penin F, et al.
5 Characterization of hepatitis C virus particle sub-populations reveals multiple usage of the
6 scavenger receptor BI for entry steps. *J Biol Chem* 2012. Jul 6 [Epub ahead of print]
- 7 12. Bartosch B, Verney G, Dreux M, Donot P, Morice Y, Penin F, Pawlotsky JM, et al. An
8 interplay between hypervariable region 1 of the hepatitis C virus E2 glycoprotein, the
9 scavenger receptor BI, and high-density lipoprotein promotes both enhancement of infection
10 and protection against neutralizing antibodies. *J Virol* 2005;79:8217-8229.
- 11 13. Voisset C, Callens N, Blanchard E, Op De Beeck A, Dubuisson J, Vu-Dac N. High
12 density lipoproteins facilitate hepatitis C virus entry through the scavenger receptor class B
13 type I. *J Biol Chem* 2005;280:7793-7799.
- 14 14. von Hahn T, Lindenbach BD, Boullier A, Quehenberger O, Paulson M, Rice CM,
15 McKeating JA. Oxidized low-density lipoprotein inhibits hepatitis C virus cell entry in human
16 hepatoma cells. *Hepatology* 2006;43:932-942.
- 17 15. Zeisel MB, Koutsoudakis G, Schnober EK, Haberstroh A, Blum HE, Cosset F-L,
18 Wakita T, et al. Scavenger receptor BI is a key host factor for Hepatitis C virus infection
19 required for an entry step closely linked to CD81. *Hepatology* 2007; 46:1722-1731.
- 20 16. Haberstroh A, Schnober EK, Zeisel MB, Carolla P, Barth H, Blum HE, Cosset FL, et
21 al. Neutralizing host responses in hepatitis C virus infection target viral entry at postbinding
22 steps and membrane fusion. *Gastroenterology* 2008;135:1719-1728 e1711.
- 23 17. Syder AJ, Lee H, Zeisel MB, Grove J, Soulier E, Macdonald J, Chow S, et al. Small
24 molecule scavenger receptor BI antagonists are potent HCV entry inhibitors. *J Hepatol*
25 2011;54:48-55.
- 26 18. Krieger SE, Zeisel MB, Davis C, Thumann C, Harris HJ, Schnober EK, Mee C, et al.
27 Inhibition of hepatitis C virus infection by anti-claudin-1 antibodies is mediated by
28 neutralization of E2-CD81-claudin-1 associations. *Hepatology* 2010;51:1144-1157.

- 1 19. Harris HJ, Farquhar MJ, Mee CJ, Davis C, Reynolds GM, Jennings A, Hu K, et al.
2 CD81 and claudin 1 coreceptor association: role in hepatitis C virus entry. *J Virol*
3 2008;82:5007-5020.
- 4 20. Catanese MT, Ansuini H, Graziani R, Huby T, Moreau M, Ball JK, Paonessa G, et al.
5 Role of scavenger receptor class B type I in hepatitis C virus entry: kinetics and molecular
6 determinants. *J Virol* 2010;84:34-43.
- 7 21. Barth H, Cerino R, Arcuri M, Hoffmann M, Schurmann P, Adah MI, Gissler B, et al.
8 Scavenger receptor class B type I and hepatitis C virus infection of primary tupaia
9 hepatocytes. *J Virol* 2005;79:5774-5785.
- 10 22. Ploss A, Evans MJ, Gaysinskaya VA, Panis M, You H, de Jong YP, Rice CM. Human
11 occludin is a hepatitis C virus entry factor required for infection of mouse cells. *Nature*
12 2009;457:882-886.
- 13 23. Dreux M, Dao Thi VL, Fresquet J, Guerin M, Julia Z, Verney G, Durantel D, et al.
14 Receptor complementation and mutagenesis reveal SR-BI as an essential HCV entry factor
15 and functionally imply its intra- and extra-cellular domains. *PLoS Pathog* 2009;5:e1000310.
- 16 24. Witteveldt J, Evans MJ, Bitzegeio J, Koutsoudakis G, Owsianka AM, Angus AG, Keck
17 ZY, et al. CD81 is dispensable for hepatitis C virus cell-to-cell transmission in hepatoma
18 cells. *J Gen Virol* 2009;90:48-58.
- 19 25. Fofana I, Fafi-Kremer S, Carolla P, Fauvelle C, Zahid MN, Turek M, Heydmann L, et
20 al. Mutations that alter use of hepatitis C virus cell entry factors mediate escape from
21 neutralizing antibodies. *Gastroenterology* 2012;143:223-233.e229.
- 22 26. Catanese MT, Graziani R, von Hahn T, Moreau M, Huby T, Paonessa G, Santini C, et
23 al. High-avidity monoclonal antibodies against the human scavenger class B type I receptor
24 efficiently block hepatitis C virus infection in the presence of high-density lipoprotein. *J Virol*
25 2007;81:8063-8071.
- 26 27. Fofana I, Krieger SE, Grunert F, Glauben S, Xiao F, Fafi-Kremer S, Soulier E, et al.
27 Monoclonal anti-claudin 1 antibodies prevent hepatitis C virus infection of primary human
28 hepatocytes. *Gastroenterology* 2010;39:953-964.

- 1 28. Bartosch B, Dubuisson J, Cosset FL. Infectious hepatitis C virus pseudo-particles
2 containing functional E1-E2 envelope protein complexes. *J. Exp. Med.* 2003;197:633-642.
- 3 29. Koutsoudakis G, Kaul A, Steinmann E, Kallis S, Lohmann V, Pietschmann T,
4 Bartenschlager R. Characterization of the early steps of hepatitis C virus infection by using
5 luciferase reporter viruses. *J Virol* 2006;80:5308-5320.
- 6 30. Zhao L, Wientjes MG, Au JL. Evaluation of combination chemotherapy: integration of
7 nonlinear regression, curve shift, isobologram, and combination index analyses. *Clin Cancer*
8 *Res* 2004;10:7994-8004.
- 9 31. Zhu H, Wong-Staal F, Lee H, Syder A, McKelvy J, Schooley RT, Wyles DL.
10 Evaluation of ITX 5061, a scavenger receptor B1 antagonist: resistance selection and activity
11 in combination with other hepatitis C virus antivirals. *J Infect Dis* 2012;205:656-662.
- 12 32. Prichard MN, Shipman C, Jr. A three-dimensional model to analyze drug-drug
13 interactions. *Antiviral Res* 1990;14:181-205.
- 14 33. Pietschmann T, Kaul A, Koutsoudakis G, Shavinskaya A, Kallis S, Steinmann E, Abid
15 K, et al. Construction and characterization of infectious intragenotypic and intergenotypic
16 hepatitis C virus chimeras. *Proc Natl Acad Sci U S A* 2006;103:7408-7413.
- 17 34. Le Goff W, Settle M, Greene DJ, Morton RE, Smith JD. Reevaluation of the role of the
18 multidrug-resistant P-glycoprotein in cellular cholesterol homeostasis. *J Lipid Res*
19 2006;47:51-58.
- 20 35. de la Llera Moya M, Atger V, Paul JL, Fournier N, Moatti N, Giral P, Friday KE, et al.
21 A cell culture system for screening human serum for ability to promote cellular cholesterol
22 efflux. Relations between serum components and efflux, esterification, and transfer.
23 *Arterioscler Thromb* 1994;14:1056-1065.
- 24 36. Schwarz AK, Grove J, Hu K, Mee CJ, Balfe P, McKeating JA. Hepatoma cell density
25 promotes claudin-1 and scavenger receptor BI expression and hepatitis C virus
26 internalization. *J Virol* 2009;83:12407-12414.

- 1 37. Brimacombe CL, Grove J, Meredith LW, Hu K, Syder AJ, Flores MV, Timpe JM, et al.
2 Neutralizing antibody-resistant hepatitis C virus cell-to-cell transmission. *J Virol* 2011;85:596-
3 605.
- 4 38. Guo L, Chen M, Song Z, Daugherty A, Li XA. C323 of SR-BI is required for SR-BI-
5 mediated HDL binding and cholesteryl ester uptake. *J Lipid Res* 2011;52:2272-2278.
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Figure legends

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Figure 1. Binding of monoclonal anti-SR-BI antibodies to human hepatocytes and inhibition of HCV infection. (A) Huh7.5.1 cells and (B) primary human hepatocytes (PHH) were incubated with anti-SR-BI mAbs and antibody binding was assessed using flow cytometry. Results are expressed as net mean fluorescence intensity (Δ MFI) of a representative experiment. (C) Inhibition of HCVcc infection by anti-SR-BI mAbs. Huh7.5.1 cells were pre-incubated for 1h at 37°C with anti-SR-BI or control mAbs (100 μ g/mL) before infection with HCVcc (Luc-Jc1) for 4h at 37°C. HCV infection was assessed by luciferase activity in lysates of infected Huh7.5.1 cells 72h post-infection. Results are expressed as means \pm SD % HCVcc infectivity in the absence of antibody of three independent experiments. (D) Dose-dependent inhibition of HCVcc infection by anti-SR-BI mAbs. Huh7.5.1 cells were pre-incubated for 1h at 37°C with anti-SR-BI or control mAbs at the indicated concentrations before infection with HCVcc (Luc-Jc1) for 4h at 37°C. HCV infection was assessed by luciferase activity in lysates of infected Huh7.5.1 cells 72h post-infection. Results are expressed as means \pm SD % HCVcc infectivity in the absence of antibody of three independent experiments performed in triplicate. *, P<0.01

Figure 2. Monoclonal anti-SR-BI antibodies do not interfere with HCV binding to SR-BI but inhibit HCV entry at post-binding steps. (A) To assess the effect of anti-SR-BI mAbs on viral binding, Huh7.5.1 cells were pre-incubated with heparin (100 μ g/mL), anti-SR-BI or control (CTRL) serum (1:50) or anti-SR-BI or control (CTRL IgG) mAbs (20 μ g/mL) for 1h prior to incubation with HCVcc (Jc1) at 4°C in the presence of compounds. Non-bound HCVcc were removed by washing of cells with PBS and HCVcc binding was then quantified by RT-PCR of cell bound HCV RNA. Results are expressed as means \pm SD of one representative experiment performed in quintuplicate. (B) Schematic drawing of the experimental setup. To discriminate between virus binding and post-binding events, HCVcc (Luc-Jc1) binding to Huh7.5.1 cells was performed in the presence or absence of anti-CD81

1 (5 µg/mL), anti-SR-BI (20 µg/mL) or control mAbs (20 µg/mL) or proteinase K (50 µg/mL) for
2 1h at 4°C, before cells were washed and incubated for 4h at 37°C with compounds added at
3 different time-points during infection. Compounds were then removed and cells were cultured
4 for an additional 48h. Dashed lines indicate the time intervals where compounds were
5 present. (C) HCV entry kinetics. Time-course of HCVcc infection of Huh7.5.1 cells following
6 addition of the indicated antibodies at different time-points during infection is shown. HCV
7 infection was assessed by luciferase activity in lysates of infected Huh7.5.1 cells 48h post-
8 infection. Results are expressed as mean % HCVcc infectivity in the absence of antibody of
9 three independent experiments performed in triplicate. *, P<0.01

10

11 **Figure 3. The SR-BI post-binding function is relevant for HCV cell-to-cell transmission**
12 **and viral spread.** (A) Quantification of HCV-infected target cells (Ti) after co-cultivation with
13 HCV (Jc1) producer cells (Pi) during incubation with control or anti-SR-BI mAbs (10 µg/mL)
14 in the presence of E2-neutralizing antibody AP33 (25 µg/mL) by flow cytometry. Data are
15 expressed as % infected target cells and represent means ± SD of three independent
16 experiments. (B) Quantification of HCV cell-to-cell transmission in parental target cells
17 compared to target cells overexpressing mouse (m) or human (h) SR-BI. Data are expressed
18 as means ± SD from three different experiments. (C-D) Long-term analysis of HCVcc (Luc-
19 Jc1) infection in the presence or absence of control (10 µg/mL) or anti-SR-BI mAb (C) QQ-
20 4G9-A6 or (D) NK-8H5-E3 at the indicated concentrations. Antibodies were added 48h after
21 HCVcc infection and control medium or medium containing antibodies were replenished
22 every 4 days. Luciferase activity was determined in cell lysates every 2 days. Data are
23 expressed as Log₁₀ RLU and represent means ± SD of one representative out of three
24 different experiments performed in duplicate. (E-F) Cell spread in the presence or absence of
25 anti-SR-BI mAbs. Antibodies were added 48h after HCVcc (Jc1) infection and control
26 medium or medium containing antibodies were replenished every 4 days. HCV-infected cells
27 were visualized 7 days post-infection by immunofluorescence using (E) anti-NS5A or (F) anti-
28 E2 (CBH23) antibodies. The percentage of infected cells was calculated as the number of

1 infected cells relative to the total number of cells as assessed by DAPI staining of the nuclei.

2 *, P<0.01

3

4 **Figure 4. Anti-SR-BI mAbs do not interfere with HDL binding but partially inhibit lipid**
5 **transfer.** (A) HDL binding to BRL3-hSR-BI cells. BRL3-hSR-BI cells were incubated in the
6 presence or absence of anti-SR-BI mAbs (20 µg/mL) or polyclonal serum (1:50) or respective
7 controls, prior to Cy5-HDL binding for 1h at 4°C. Bound Cy5-HDL was quantified using flow
8 cytometry. Results represent means ± SD of two different experiments performed in
9 duplicate. (B) Lipid uptake by Huh7 cells. Huh7 cells were incubated with a mixture of anti-
10 SR-BI mAbs (20 µg/mL) and ³H-CE-labeled HDL for 5h before incubation with unlabelled
11 HDL for 30 min. Selective uptake was calculated from the known specific radioactivity of
12 radiolabelled HDL-CE and is denoted in µg HDL-CE/µg cell protein. Results represent mean
13 ± SD of three different experiments performed in sixtuplicate. (C) Cholesterol efflux from
14 Huh7 cells. Huh7 cells were first incubated with 3H-cholesterol for 48h and then with BSA
15 (0.5%) for 24h. Subsequently, cells were first incubated with anti-SR-BI mAbs (20 µg/mL) for
16 1h and then with unlabeled HDL for 4h. Fractional cholesterol efflux was calculated as the
17 amount of the label obtained in the medium divided by the total label in each well regained
18 after lipid extraction from cells. Results represent means ± SD of three different experiments
19 performed in triplicate. *, P<0.01

20

21 **Figure 5. Genotype-independent inhibition of HCVpp and HCVcc infection by**
22 **monoclonal anti-SR-BI antibodies.** (A-E) Inhibition of entry into Huh7.5.1 cells of HCVpp
23 bearing envelope glycoproteins from genotypes 1-4. Huh7.5.1 cells were pre-incubated with
24 control (CTRL IgG) or anti-SR-BI mAbs (50 µg/mL) for 1h at 37°C before infection with
25 HCVpp bearing envelope glycoproteins of strains H77 (1a), HCV-J (1b), JFH1 (2a),
26 UKN3A1.28 (3a) or UKN4.21.16 (4) and VSV-Gpp. Means ± SD from 3 experiments
27 performed in triplicate are shown. (F) Inhibition of infection of Huh7.5.1 cells with HCVcc
28 bearing envelope glycoproteins from genotypes 1-4. Huh7.5.1 cells were pre-incubated with

1 anti-SR-BI mAb (NK-8H5-E3, 50 µg/mL) for 1h at 37°C before infection with HCVcc. HCVpp
2 and HCVcc infection was analyzed by luciferase reporter gene expression. Results are
3 expressed as % HCVpp entry or HCVcc infection and represent (A-E) means ± SD from 3
4 independent experiments performed in triplicate and (F) means ± SEM from 3 independent
5 experiments performed at least in triplicate. *, P<0.01

6

7 **Figure 6. Synergy between anti-SR-BI and neutralizing antibodies in inhibiting HCV**

8 **infection.** HCVcc (Luc-Jc1) were pre-incubated with increasing concentrations of (A-B) anti-

9 E1 (IGH520) or (C-D) anti-E2 (AP33) mAbs or (E-F) purified heterologous anti-HCV IgG

10 obtained from an unrelated chronically infected subject or isotype control IgGs for 1h at 37°C

11 and added to Huh7.5.1 cells pre-incubated with increasing concentrations of control or anti-

12 SR-BI mAbs (A, C, E) NK-8H5-E3 or (B, D, F) QQ-2A10-A5 in a checkerboard format.

13 HCVcc infection was analyzed by luciferase reporter gene expression. Effects of antibody

14 combinations on HCVcc infection were evaluated using the method of Prichard and

15 Shipman.³² Combination studies for each pair of compounds were performed in triplicate.

16 The theoretical additive effect is calculated from the dose-response curves of individual

17 compounds by the equation $Z=X+Y(1-X)$ where X and Y represent the inhibition produced by

18 the individual compounds and Z represents the effect produced by the combination of

19 compounds. The theoretical additive surface is subtracted from the actual experimental

20 surface, resulting in a horizontal surface that equals the zero plane when the combination is

21 additive. A surface raising more than 20% above the zero plane indicates a synergistic effect

22 of the combination and a surface dropping lower than 20% below the zero plane indicates

23 antagonism.

24