Supplementary Information

Preferential Association of Hepatitis C Virus with CD19+ B Cells Is Mediated by Complement System

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Supporting Methods:

Cell culture

The human hepatoma cell line, Huh7.5.1 was obtained from Dr. Francis V. Chisari (1). Huh7.5.1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% heat inactivated fetal bovine serum, 2 mM L-glutamine, 10 mM HEPES, pH7.2, 100 unit/ml penicillin, 100 µg/ml streptomycin, and 1% non-essential amino acids (Thermo Fisher Scientific, Waltham, MA) in a humidified 37°C, 5% CO_2 incubator. PBMCs and Raji cells were cultured in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum, 2 mM L-glutamine, 100 unit/ml penicillin, and 100 µg/ml streptomycin (complete RPMI medium).

In-vitro RNA synthesis

The plasmid pH77-S, which carries a full length HCV1a sequence with five cell culture-adaptive mutations was kindly provided by Dr. Stanley Lemon (2). The plasmid was linearized with XbaI restriction enzyme and purified by Qiagen PCR purification kit. In

vitro transcribed RNA, using the MEGA-script T7 kit (Thermo Fisher Scientific, Waltham, MA), was purified with Qiagen RNA clean up kit. The RNA pellet was aliquoted and stored at -80°C until use.

HCV production in cell culture

1.4 µg of HCV1a (H77S) full length RNA was transfected into $2x10^5$ Huh7.5.1 cells per well in 6-well plates by using mRNA boost reagent and TransIT-mRNA reagent (Mirus, MIR2250) according to the manufacturer's instructions. Six hours after transfection, the cells were trypsinized, washed once with complete DMEM medium without antibiotics, and collected by centrifugation. Cells were seeded into 25x150mm culture dishes at $1.2x10^6$ cells per dish with 50 ml complete DMEM medium without antibiotics. After incubation for 6 days, the culture supernatant was collected and filtered through 0.45µm sterile filtration units. The filtrates were aliquoted and stored at -80°C before use. The virus producing cells were continuously sub-cultured every 3-4 days for 21 days post transfection by seeding $4x10^6$ cells per 25x150mm culture dish with 50 ml complete DMEM medium and collecting the culture supernatant after 72 h incubation. For the production of HCV genotype 2a virus, $5x10^6$ Huh-7.5.1 cells in each 25x150mm culture dish were infected with JFH1 virus at MOI=0.1. The culture supernatant was collected 96 h post infection and centrifuged at 1,800 rpm for 10 min. The supernatant was filtered through 0.45µm sterile filtration units. The filtrates were aliquoted and stored at -80°C before use. For the production of JFH1/1a chimeric virus, $3x10^6$ Huh-7.5.1 cells in each 25x150mm culture dish were infected with JFH1/1a virus at MOI=0.1. After 96 h infection, the cells were sub-cultured at $7-8x10^6$ cells per 25x150mm culture dish with 50 ml complete DMEM medium. The culture supernatant was collected 24 h later and

centrifuged at 1,800 rpm for 10 min. The supernatant was filtered through 0.45μm sterile filtration units. The filtrates were stored at -80°C before use.

Conversion of plasma samples to serum samples

Some HCV patients' blood samples and heathy blood donor samples were received as plasma samples. To convert plasma samples to serum samples, $CaCl_2$ was added first to the plasma samples at 10 mM final concentration, mixed, and followed by adding thrombin (Sigma-Aldrich, St. Louis, MO) at final 0.1unit/ml. The samples were incubated at 37°C for 0.5 h, followed by incubating at room temperature for 0.5 h, and then stored at -20°C overnight. The samples were thawed in a 37°C water bath, and the clots were removed and squeezed for residual fluid using sterile forceps in a biosafety hood. The serum samples were further incubated in ice for one hour. If no clot was detected, 1 ml aliquots were prepared and stored at -80°C.

PBMCs and PBMC subset separation

PBMCs were isolated by Ficoll-Paque density gradient centrifugation from buffy coat. Each buffy coat (about 18-30 ml) was diluted with 1X PBS, pH7.4 to 120 ml, and divided equally into four sterile 50 ml conical tubes. Each tube was then underlay with 15 ml of Ficoll-Paque Plus solution (GE Healthcare Life Sciences, Marlborough, MA), and centrifuged at 400 x g for 30 min at 25°C. The lymphocytes were collected from the interphase layer and washed twice with 1X PBS, pH7.4. The cells were pelleted by centrifugation at 400 x g and 200 x g for 10 min each at 25°C for the first wash and the second wash, respectively. The cells were further washed once with complete RPMI medium and collected by centrifugation at 200 x g for 10 min at 25°C. The B cell subset

was purified by positive selection using MACS magnetic beads coated with anti-human CD19 antibody (Miltenyi Biotec Inc., Auburn, CA). The efficiency of separation was > 85% for B cells as confirmed by FACS analysis (BD Biosciences, San Diego, CA). For isolation of untouched B cells from PBMCs, human B cell isolation kit II (Miltenyi Biotec Inc., Auburn, CA) was used according to the manufacturer's instructions.

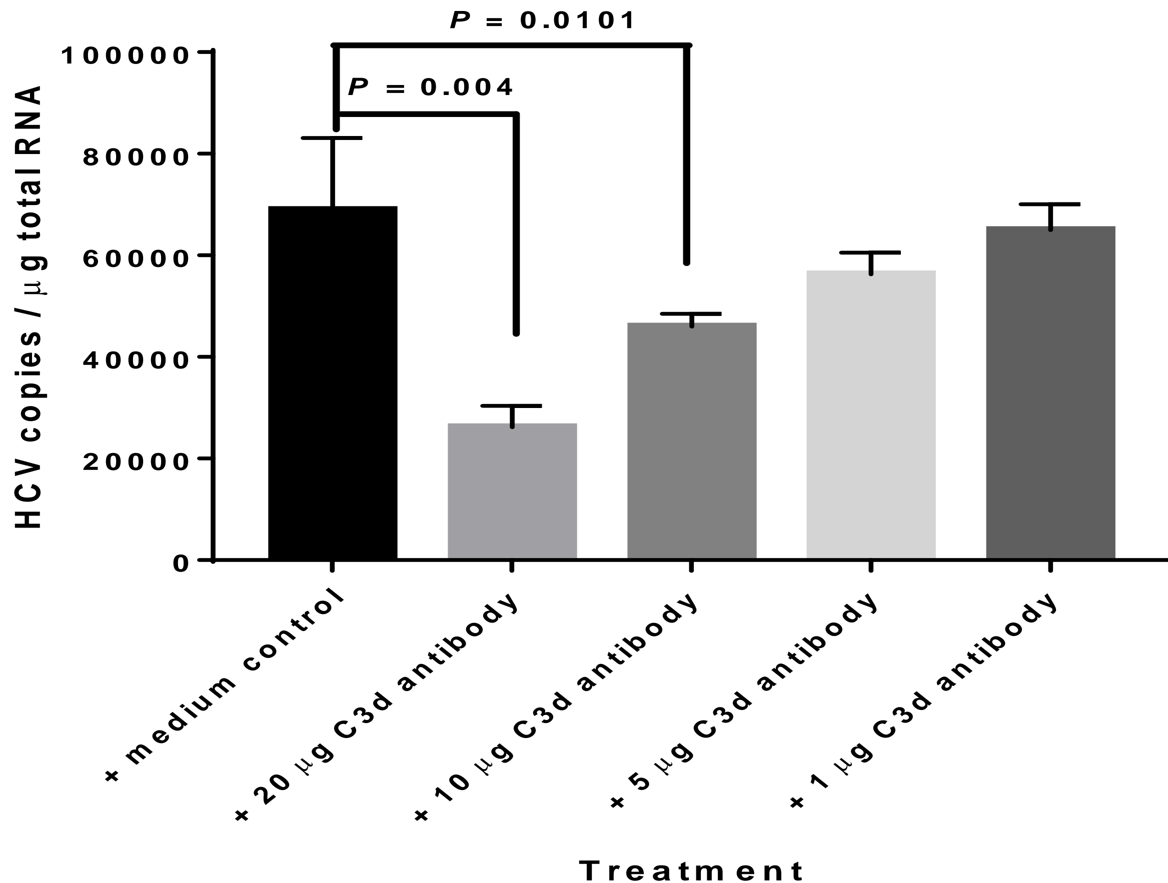
In vitro HCV binding assay

For assessing the effect of anti-complement C3 antibodies on blocking HCV binding to B cells, 25 µl of complement active serum sample was mixed with 10-20 µg each of anti-C3 antibodies as indicated in the figure legend and incubated at 25°C for 30 min before adding 3 ml virus to the reaction mixture. All the anti-C3 antibodies used in this study were from Santa Cruz Biotechnology (Dallas, TX). For conducting HCV binding assays using complement depleted sera reconstituted with purified complement proteins, 100 µl of the indicated complement-depleted serum was used for each reaction. All the complement-depleted sera and the purified complement proteins used in this study were obtained from Complement Technology (Tyler, TX). To measure the effect of antibodies against various cell surface receptors on HCV binding to B cells, 2 ml of PBMCs (4-5x10^7 cells) or 2 ml of untouched B cells (2-4x10^6 cells) were incubated with 10 µg of mouse monoclonal antibodies against the indicated receptors at 25°C for 30 min, followed by adding 3 ml of complement activated virus and incubating for 1 h. All the antibodies for blocking cell surface receptors used in this study were obtained from BD Biosciences (San Diego, CA) except that a few anti-CD19 and anti-CD81 antibodies from Santa Cruz Biotechnology (Dallas, TX) were also tested. The clone numbers for antibodies from BD Biosciences are as follows: CD11b, ICRF44; CD19,

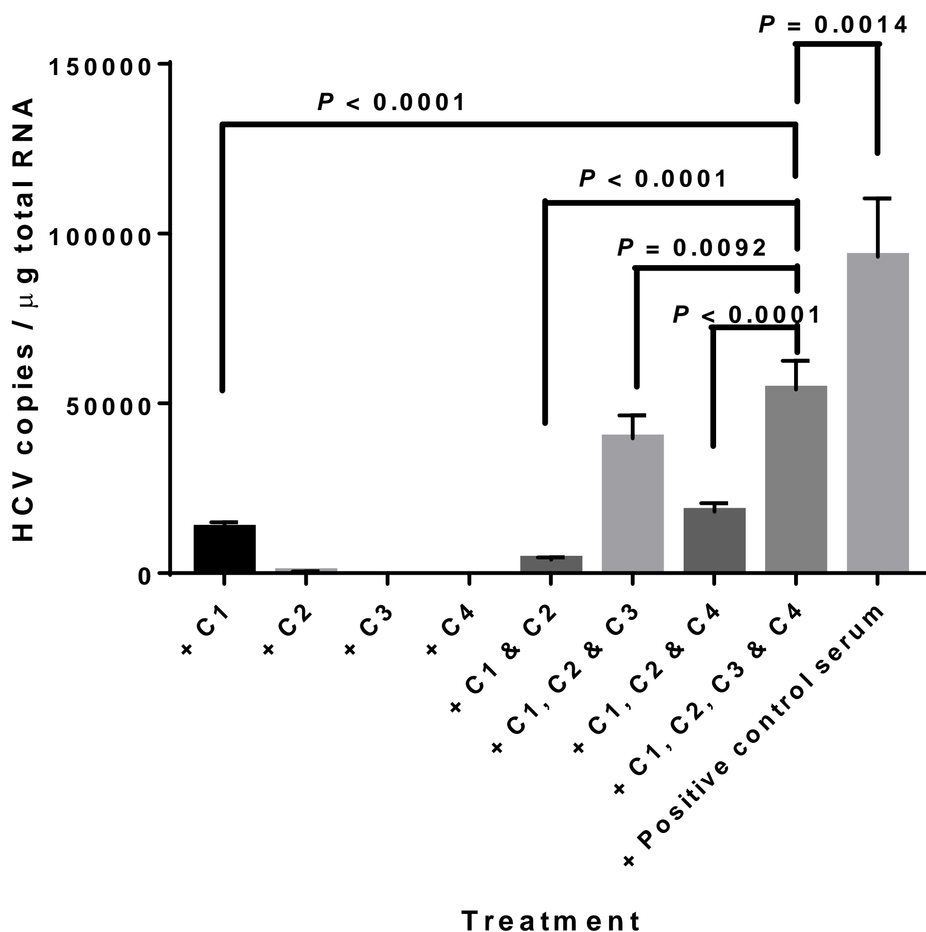
HIB19; CD20, 2H7; CD21, 1048; CD32, 3D3; CD35, E11; CD55, IA10; and CD81, JS-81. The clone numbers for antibodies from Santa Cruz Biotechnology are as follows: C3, C-4, B-9 and 2898; C3d, 003-05; and CD81, 1.3.3.22. C3 (C-4) and C3 (B-9) are raised against amino acids 541-840 of human C3. C3 (2898) is raised against the C-terminus of human C3. C3d (003-05) is raised against full length native C3 of human origin. The epitope mapping for anti-CD21 and anti-CD35 has been previously reported (3, 4). For determination of HCV binding to Raji cells, Raji cells were cultured in complete RPMI medium until the cell density reached to 1-2x10^6 cells per ml. The cells were then collected by centrifugation at 200x g for 10 min at 25°C. After removing the supernatant, the cells were re-suspended in complete RPMI medium and adjusted to 1x10^6 cells per ml before use.

HCV detection

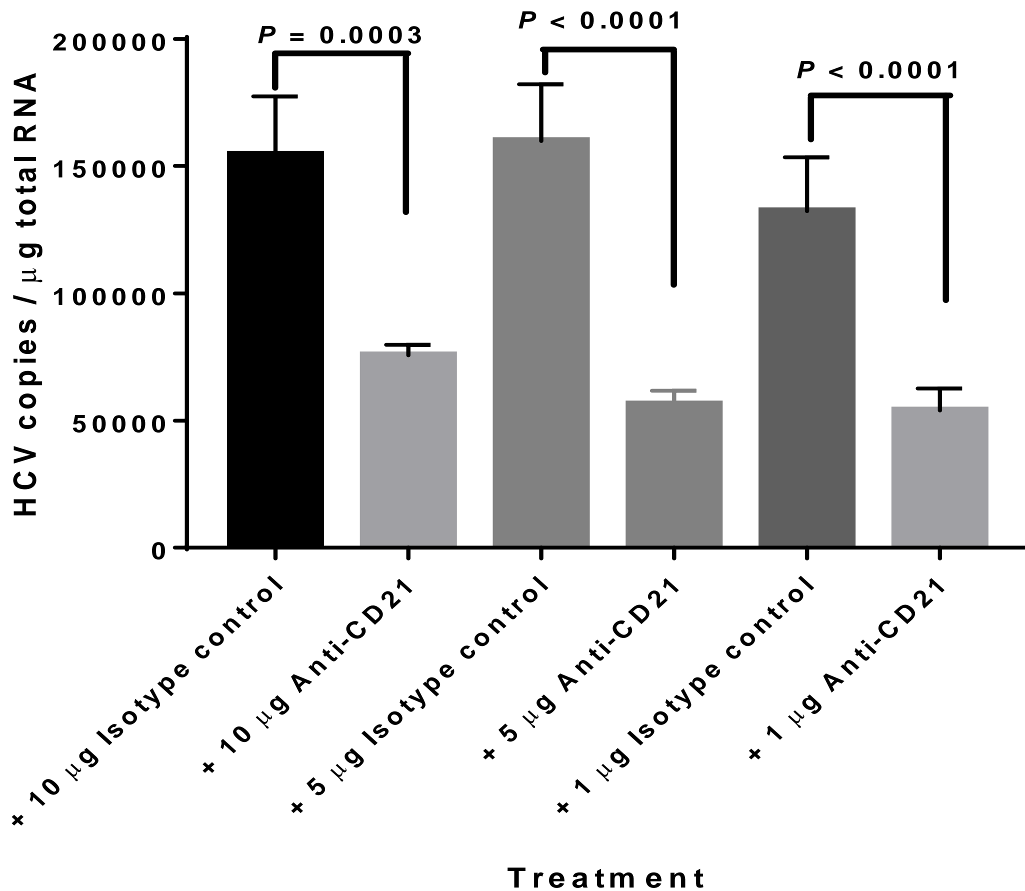
For quantifying HCV genomic copy number in purified total cellular RNA samples, we used the primers and probes as previously described (5). Each sample was quantified in triplicate using a TaqMan Fast Virus 1-Step Master Mix (Thermo Fisher Scientific, Waltham, MA) with 10 µl RNA input per reaction in a 50 µl mixture. The reactions were carried out on ABI 7900HT system with a program of 50°C for 2 min, 60°C for 30 min, 95°C for 3 min, and then 50 cycles at 95°C for 20 sec and 60°C for 1 min. The copies of HCV RNA were determined by in vitro transcribed HCV1a RNA standards (6) with the Sequence Detector Software (version 2.2; Applied Biosystems) and normalized to 1 µg total RNA input.



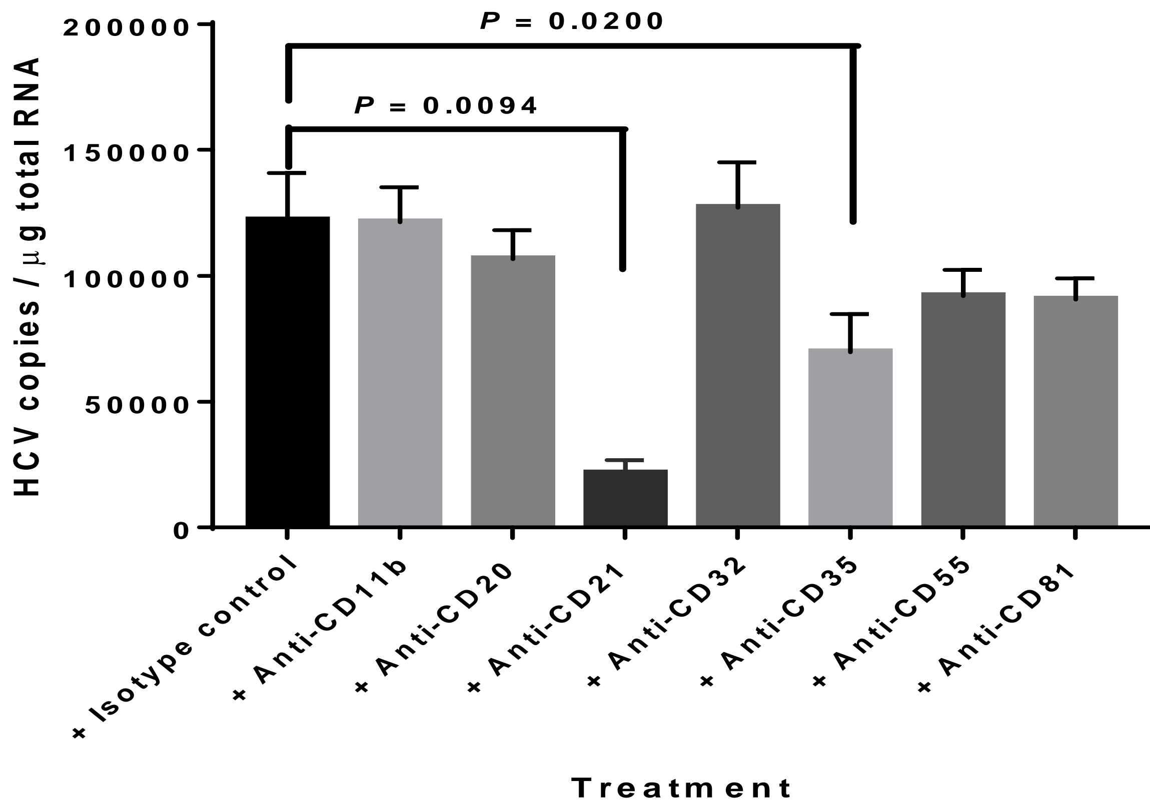
Supporting Fig. S1. Titrating the amount of C3d antibody needed to block HCV binding to B cells. Twenty-five μl serum sample was incubated with the indicated amount of mouse monoclonal antibody C3d (clone 003-05) at 25°C for 30 min, followed by mixing with virus and incubating at 25°C for 1 h. After mixing with PBMC, the mixture was incubated at 25°C for one more hour. The cells then were processed for HCV quantification as described in Methods section. Each value represents the mean ± SD of 6 determinations.



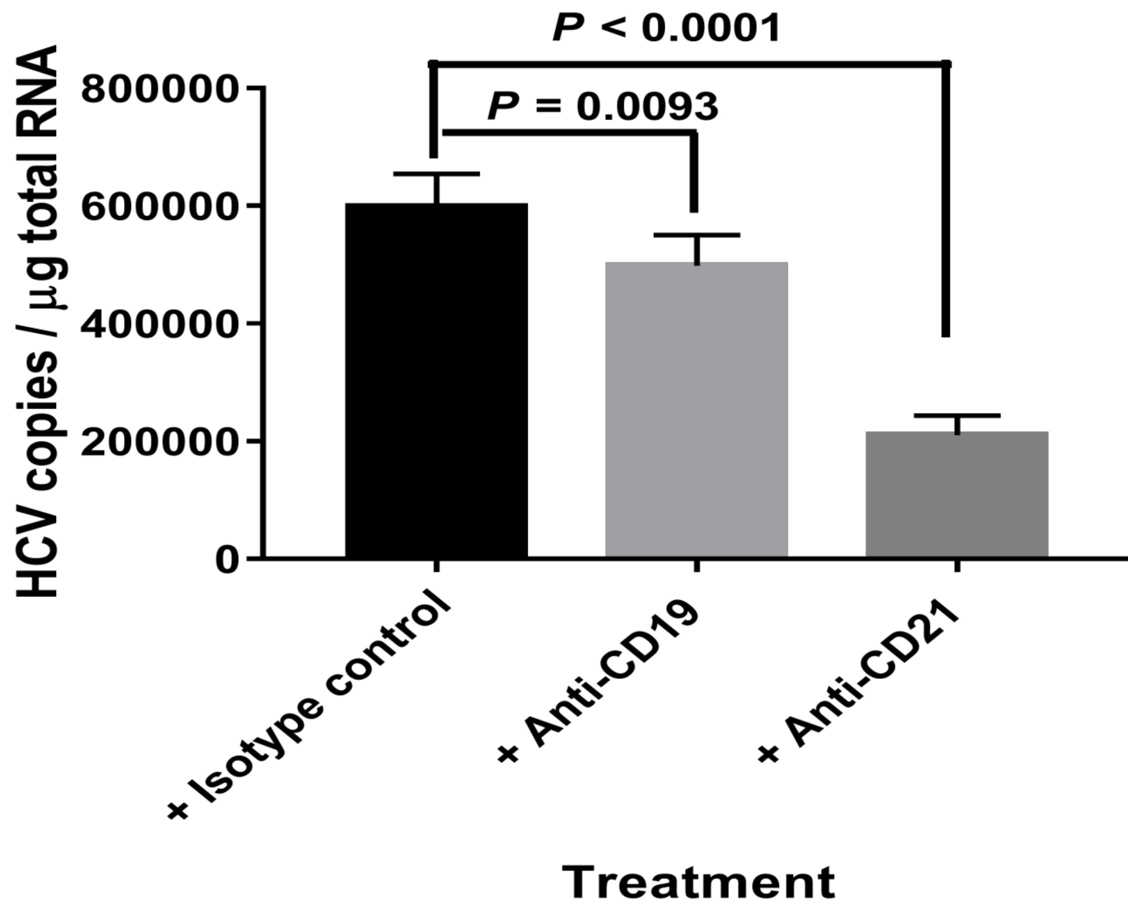
Supporting Fig. S2. A combination of purified complement proteins C1, C2, C3, and C4 can restore HCV binding activity to B cells. Ten million genomic copies of HCV 1a (H77s) in 3 ml medium were incubated with the indicated purified complement protein(s). The amount of each protein used was as follows: C1, 25 µg; C2, 5 µg; C3, 60 µg; and C4, 25 µg. For positive control serum, 100 µl was used in each reaction. After 0.5 h incubation at 25°C, 2 ml PBMCs ($2.5x10^7$ cells per ml) in complete RPMI medium was added. The reaction was carried out at room temperature (25°C) for 1 h. The cells then were processed for HCV quantification as described in Methods section. The values for C1, C2, C3, or C4 only reaction represent the mean ± SD of 3 determinations. The values for a combination of two or more of C1, C2, C3, or C4 reaction represent the mean ± SD of 6 determinations.



Supporting Fig. S3. Titration of antibody concentration of anti-CD21 (complement receptor 2) in complement-mediated HCV binding to B cells. Two ml of PBMCs ($2.5x10^7$ cells/ml) were incubated with the indicated amount of anti-human CD21 antibody (clone 1048) or isotype control antibody at 25°C for 0.5 h; followed by mixing with 3 ml of activated virus (pre-incubation with serum for 0.5 h at 25°C, $1x10^7$ genomic copies total), and incubating at 25°C for 1 h. The cells then were processed for RNA isolation and HCV quantification as described in Methods section. Each value represents the mean ± SD of 6 determinations.



Supporting Fig. S4. Lowering the concentration of antibodies to receptors involved in complement-mediated HCV binding to B cells. Two ml of PBMCs (2.5×10^7 cells/ml) were incubated with 1 µg of the indicated antibody at 25°C for 0.5 h; followed by mixing with 3 ml of activated virus (pre-incubation with serum for 0.5 h at 25°C, 1×10^7 genomic copies total), and incubating at 25°C for 1 h. The cells then were processed for RNA isolation and HCV quantification as described in Methods section. Each value represents the mean ± SD of 6 determinations.



Supporting Fig. S5. Receptors involved in complement-mediated HCV binding to B cells. Two ml of untouched B cells ($1.0x10^6$ cells/ml) were incubated with 10 μg of the indicated antibody at 25°C for 0.5 h; followed by mixing with 3 ml of activated virus (pre-incubation with serum for 1 h at 25°C, $1.5x10^7$ genomic copies total), and incubating at 25°C for 1 h. The cells then were processed for RNA isolation and HCV quantification as described in Methods section. Each value represents the mean ± SD of 6 determinations. The experiments were repeated three times with similar results using untouched B cells from three different donors.

References

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