Interaction between complement receptor gC1qR and hepatitis C virus core protein inhibits T-lymphocyte proliferation

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Hepatitis C virus (HCV) is an important human pathogen that is remarkably efficient at establishing persistent infection. The HCV core protein is the first protein expressed during the early phase of HCV infection. Our previous work demonstrated that the HCV core protein suppresses host immune responses, including anti-viral cytotoxic T-lymphocyte responses in a murine model. To investigate the mechanism of HCV core-mediated immunosuppression, we searched for host proteins capable of associating with the core protein using a yeast two-hybrid system. Using the core protein as bait, we screened a human T cell–enriched expression library and identified a gene encoding the gC1q receptor (gC1qR). C1q is a ligand of gC1qR and is involved in the early host defense against infection. Like C1q, HCV core can inhibit T-cell proliferative responses in vitro. This core-induced anti–T-cell proliferation is reversed by addition of anti-gC1qR Ab in a T-cell proliferation assay. Furthermore, biochemical analysis of the interaction between core and gC1qR indicates that HCV core binds the region spanning amino acids 188 to 259 of gC1qR, a site distinct from the binding region of C1q. The inhibition of T-cell responsiveness by HCV core may have important implications for HCV persistence in humans.

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Introduction

Hepatitis C virus (HCV) is a serious and growing worldwide threat to human health, having already infected approximately 4 million people in the United States alone (1). Unlike hepatitis B, HCV evades effective immune recognition and shows an extremely high rate of viral persistence in the infected individual (>90% versus approximately 10% for HBV) (2, 3). After a subclinical phase lasting 10 to 20 years, HCV infection often progresses to end-stage liver disease and is highly correlated with the development of hepatocellular carcinoma (4, 5) and autoimmune disease (6, 7). While HCV transmission has been linked to a blood-borne route, e.g., patients receiving organ transplants, blood products, or intravenous drug use, almost half of all HCV cases show no such factors and are considered sporadic (8). Unfortunately, no vaccine or effective treatment is available currently.

Structural studies of the HCV genome have shown the virus to be a positive-strand RNA virus related to the Flaviviridae family (9, 10). The viral genome encodes a single polyprotein of approximately 3,000 amino acids, which is subsequently processed by host and viral proteases to form additional nonstructural and structural proteins including a nucleocapsid (core) and two envelope proteins, E1 and E2 (11). At least six genotypes and 30 subtypes have been identified, differing primarily in the region of core, E1, and NS5 (12, 13). The intrinsically high rate of mutation in the RNA genome of this virus and the variability of the envelope protein likely contributes significantly to the ineffective Ab response against the virus (14, 15). While T-lymphocyte responses to HCV gene products have been demonstrated (16, 17), the role of such responses in controlling HCV infection are not well-defined. Furthermore, HCV replication persists in the hepatocytes of these patients although massive lymphocytic infiltration was detectable in the livers of HCV-infected patients (18–20) and specific response of these infiltrating lymphocytes to HCV antigens has been demonstrated (21).

The major difficulty in addressing the mechanism of viral persistence, the role of virus replication, and host immune responses to HCV in the development of liver injury is the lack of a small-animal model of HCV infection. Recently, we have developed a murine model to examine the influence of specific HCV proteins on viral-antigen recognition and virus clearance. These studies have demonstrated that expression of other HCV structural or nonstructural proteins using recombinant vaccinia or Sindbis virus resulted in vigorous T-lymphocyte responses and nonlethal infections with rapid virus clearance. In contrast, expression of the HCV core pro-

tein is sufficient to suppress protective immune responses such as anti-viral cytotoxic T-lymphocyte (CTL) responses (22). The immunomodulatory role of core protein may play a critical role in HCV pathogenesis in conjunction with identification of circulating naked core protein in the plasma of HCV-infected patients (23) and secreted core protein from transfected cell lines (24). Indeed, $CD4^+$ T-cell responses to core protein are well-correlated with a benign course of infection by presumably maintaining humoral and cellular responses that protect against the disease (25).

Based on these data indicating the role of HCV core in suppressing the host immune response, including anti-viral T-cell responses, we searched for possible targets of core protein on immune cells. To this end, we have screened a human leukocyte cDNA library using the yeast two-hybrid system to identify host proteins with which core can interact (26). One fourth of the clones identified encoded the p33 gC1q receptor (gC1qR) specific for the globular heads of the complement C1q protein (27). This finding was particularly noteworthy since the binding of C1q, the natural ligand for the gC1qR, to T lymphocytes can block proliferation of T lymphocytes (28, 29). In this report, we describe the identification of the core binding to gC1qR and have undertaken a comprehensive analysis of the gC1qR/core interaction as well as the effects of core on T-cell responsiveness.

Methods

Plasmid constructions. To construct the plasmid used in the yeast two-hybrid screening, pAS2.1 was purchased from CLONTECH (Emeryville, California, USA). The cDNA encoding the HCV core-protein region (amino acids 1–124) of the HCV H strain (genotype 1a) or gC1qR was subcloned into the pAS2.1 vector downstream of and in-frame with the GAL4 DNA-binding domain. This vector contains the TRP1 nutritional gene allowing for selection of transformants in yeast auxotrophic for this marker. The human phytohemagglutinin-stimulated (PHA-stimulated) leukocyte library was purchased from CLONTECH. This library was inserted into the pGAD10 vector downstream of the GAL4 transcriptional activation domain. The activation domain vector, pGAD10, contains a nutritional marker (LEU2) allowing for selection of transformants. In addition, cDNA-encoding core protein was inserted in-frame with the GAL4 transcriptional activation domain of pACT vector (CLONTECH).

The plasmids used to express the core protein–glutathione S-transferase (GST) fusion proteins in *Escherichia coli* were constructed by inserting HCV cDNA fragments corresponding to the regions of core protein (amino acids 1–192, 1–152, 1–124, 26–124, 125–192) into the pGEX4T.3 vector purchased from Pharmacia Biotech Inc. (Piscataway, New Jersey, USA). The resulting constructs were designated as pGEX:C1-192, pGEX:C1-152, pGEX:C1-124, pGEX:C26-124, and pGEX:C125-192, respectively.

The plasmid used for in vitro transcription of gC1qR protein was constructed by inserting cDNA encoding gC1qR into the multiple cloning region of the pCI:*neo* vector (Promega Corp., Madison, Wisconsin, USA). Deletion constructs for gC1qR were generated by PCR-based amplification of appropriate fragments encoding the amino acid regions 45–115, 45–187, 45–259, and 187–259, respectively. Oligonucleotides with sense strands, 5′ ACCCGGGAATTCGGGATGCTCAGC 3′ (nt 118–141), 5′ GTGTTGAATTCTATGTATCCAGAG 3′ (nt 547–570) were used for PCR amplification with antisense strand oligonucleotides, 5′ CACTAATTTGTCGACTTACCCA-TT 3′ (nt 340–363), 5′ TCCAACCTCGTCGACTGGCTAATG 3′ (nt 559–582), and 5′ GTGCTCCAGGTCGACTCAGAGCTC 3′ (nt 772–795). All PCR-generated products were confirmed by DNA sequencing. Resulting PCR products representing the deletion constructs of gC1qR were inserted into plasmid pCI:*neo* to produce radiolabeled protein by in vitro transcription and translation.

Yeast two-hybrid system. The screening procedure was performed as described previously (26). Briefly, *Saccharomyces cerevisiae* strain Y-190 (auxotrophic for leucine, tryptophan, and histidine) was grown in YPD (1% yeast extract, 2% peptone, 2% dextrose). Yeast was cotransformed with pAS2.1/core 1–124 and pGAD10/human leukocyte library by the lithium-acetate method. Cotransformants containing interacting hybrids were selected on synthetic medium lacking leucine, tryptophan, and histidine. Interaction of the two hybrids was further confirmed by β-galactosidase (β-gal) staining. β-gal activity was assayed on nitrocellulose filter replicas of yeast transformants. Filters were placed in liquid nitrogen for 30 seconds and incubated for 8 hours in buffer containing 4 mM 6-bromo-4-choloro-3-indolyl-β-D-galactosidase (X-gal). Potential positive interaction was determined by the appearance of blue colonies, which were isolated, replated, and retested for β-gal activity.

DNA sequencing. The cDNA sequences were obtained by the dideoxynucleotide chain termination–sequencing method. Resulting sequences were compared against the database of the National Center for Biotechnology Information (Bethesda, Maryland, USA) using the basic local alignment search tool (BLAST) program.

GST fusion protein binding assay. GST and GST-core fusion protein were expressed in *E. coli* DH5α after transforming *E. coli* with plasmids pGEX4T, pGEX:C1-192, pGEX:C1-152, and pGEX:C1-124, pGEX:C26-124, and pGEX:C125-192. GST and GST-core fusion proteins were purified using glutathione-agarose beads (Sigma Chemical Co., St. Louis, Missouri, USA) in accordance with the supplier's recommendation. Full-length gC1qR labeled with ^{35}S-methionine was generated using a coupled transcription/translation system (TNT) from Promega Corp. with pCI:gC1qR as template. GST-binding assay was performed as previously described (30). GST (200 ng)

or GST-core fusion proteins coupled to 20 μl of glutathione-agarose beads (50% slurry) was incubated at 4°C with ^{35}S-methionine–labeled gC1qR in 500 μl of a buffer solution containing 40 mM HEPES-KOH, pH7.5, 150 mM KCl, 0.5 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 0.1% Nonidet P-40 for 1 hour. To minimize potential bead loss during subsequent washes, the buffer was mixed with glutathione-agarose beads to adjust to a total bead volume of 20 μl per reaction. After the incubation, the beads were washed five times with the same buffer. The bound proteins were eluted with SDS-PAGE loading buffer and analyzed by SDS-PAGE and autoradiography. To define the binding region of gC1qR to core protein, the GST-binding assay was performed using GST-core fusion proteins expressing the HCV core and ^{35}S-methionine–labeled deletion constructs of gC1qR, as described above.

Purification of human lymphocytes. Peripheral blood lymphocytes (PBLs) were isolated from the blood of healthy donors by Ficoll-Paque gradient (Pharmacia Biotech Inc.). PBLs were then washed three times in cold tissue media, RPMI (Life Technologies, Burlington, Ontario, Canada) containing penicillin (100 μg/ml), streptomycin (100 μg/ml), amphotericin (2.5 μg/ml), and 2-ME (5.5×10^5 M), supplemented with 10% (vol/vol) newborn calf serum (Sigma Chemical Co.).

T-cell proliferation assay. Purified T cells (1×10^6 cells/ml) were grown in RPMI, 10% FCS, and stimulated with allogeneic lymphocytes or conA (2 μg/ml) for 5 days in the presence of varying amounts of C1q (50 μg/ml; Sigma Chemical Co.), HCV core (13, 1.3, 0.13, 0.013 nM) or HCV NS3 (13, 1.3, 0.13, 0.013 nM). Recombinant HCV core and NS3 proteins were purified from bacteria purchased from ViroGen (Watertown, Massachusetts, USA). The cultures were incubated at 37°C with 5% CO_2 in a humidified atmosphere for 72 hours. At the 60-hour time point, 1 μCi/well of ^{3}(H)-thymidine was added (specific activity of 6.7 Ci/mmol; Amersham International, Oakville, Ontario, Canada). Cells were subsequently harvested using a semiautomated Skatron cell harvester (Skatron Instruments Inc., Sterling, Virginia, USA), and the amount of ^{3}(H)-thymidine incorporated into DNA was measured using a liquid scintillation counter. Data are expressed as the mean counts per minute plus or minus the SE of quadruplicate cultures.

To examine whether anti-gC1qR Ab can reverse the effect of core protein on anti–T-cell proliferation, anti-gC1qR mAb's (60.11, 74.5.2) were coincubated in the T-cell proliferation assays as described above. Anti-gC1qR mAb's (60.11, 74.5.2) were generated by injection of BALB/c mice with highly purified recombinant gC1qR (31). These Ab's were generously provided by Berhane Ghebrehiwet at State University of New York (Stony Brook, New York, USA). Furthermore, anti-core mAb (1 μg/ml, 0.2 μg/ml) or isotype-matched Ab (anti-mouse IgG) as a control was added into conA-stimulated lymphocyte culture to examine the effect of anti-core Ab on reversing the core-induced anti–T-cell proliferation.

Flow-cytometric analysis of cell-surface expression of gC1qR. MOLT-4 T-cell lines or human PBMCs were incubated with anti-gC1qR Ab (74.5.2) in PBS containing 1% FCS and 0.04% azide, washed and stained with Alexa 488–conjugated goat anti-mouse Fab fragments (Molecular Probes, Eugene, Oregon, USA). MOLT-4 cells were preincubated with 50 μg/ml of core protein at 4°C for 30 minutes followed by the flow cytometry as described above. Analysis was performed on a FACScan (Becton Dickinson, Mountain View, California, USA) with CellQuest software.

Cell-surface biotinylation of human PBMCs. Biotinylation of cell-surface proteins from human PBMCs (10^7 cells) was performed using NHS-LC-biotin, as previously described (32). The viability of cells after biotinylation was measured by trypan blue–exclusion method and was greater than 95%. After washing, the membrane pellet was resuspended in 1 ml of lysis buffer containing a mixture of protease inhibitors and incubated on ice for 30 minutes. The insoluble cellular debris was removed by centrifugation (850 *g*, 4°C, 10 minutes), and the supernatant containing the labeled proteins was subjected to further centrifugation (45,000 *g*, 4°C, 1 hour). The supernatant was collected and 1/20 of supernatant was used for immunoprecipitation using anti-gC1qR mAbs (60.11, 74.5.2), followed by separating Ab-bound proteins in 10% SDS-PAGE. Gel was transferred to PVDF membrane and the membrane-bound biotinylated proteins were then detected by alkaline phosphatase–conjugated streptavidin followed by a 30-minute incubation with 5-bromo-4-chloro-3-indolyl/phosphate/nitro blue tetrazolium (BCIP/NBT) substrate.

Results

Identification of gC1qR as an HCV core-binding protein. Because our previous data (22) suggested that the HCV core protein might directly inhibit T-lymphocyte function, we used the yeast two-hybrid system to screen a library constructed from PHA-stimulated human leukocytes to identify potential HCV core-binding protein(s). A truncated form of core protein (amino acids 1–124) was used as bait in the screening analyses in order to eliminate the COOH-terminal hydrophobic part of the HCV core protein that may interfere with nuclear transportation of the bait protein in yeast. Among approximately 14×10^6 yeast cotransformants screened, twenty-four clones were identified that consistently demonstrated HCV core-dependent activation of the yeast transcriptional markers (leucine, tryptophan, histidine). These clones were sequenced and compared with nucleotide sequences deposited in the NIH Genbank database (Bethesda, Maryland, USA).

The sequence most frequently selected by the HCV core bait (six of the 24 clones) was the gene encoding the gC1qR (27) (Figure 1a). The gC1qR is reported to associate with cC1qR to form a heterodimer (33). The

gC1qR (p33) binds to the globular head regions of C1q, whereas the cC1q receptor (p66) binds to the collagen-like stalk regions of the C1q molecule (27). Each of the six clones was confirmed to be present in the yeast two-hybrid vector in the correct orientation and reading frame relative to the GAL4 DNA-binding domain gene and therefore produced a proper fusion-protein product. No association was detected in the yeast two-hybrid system between the GAL4 activation domain gC1qR fusion protein and the GAL4-binding domain protein alone or fusion proteins consisting of the GAL4-binding domain and irrelevant bait constructs (Figure 1b). This suggests that the association of core protein with the gC1qR is highly specific. The other 18 clones identified by yeast two-hybrid screening represented genes encoding various members of tumor necrosis receptor family, as reported previously (30). To rule out the possibility of nonspecific binding of proteins, we reversed the screening procedure by using the gC1qR molecule as a bait and core protein as the trap construct. Consistent with the result described above, the gC1qR can interact with core protein (Figure 1c).

In vitro association between gC1qR and HCV core protein. To establish an interaction between HCV core and the gC1qR protein, independent of their association in the yeast two-hybrid screen, we examined the core and gC1qR interaction using the GST fusion protein–binding system. This GST-binding assay allows us to examine specific in vitro interaction between two different proteins. For this analysis, the gC1qR was subcloned from the yeast two-hybrid vector pGAD10 into the pCI:*neo* expression vector. A methionine start codon and Kozak consensus sequence were incorporated into the vector using standard cloning methods. Full-length and truncated (amino acids 1–124) versions of the HCV core gene were subcloned into the GST fusion vector, pGEX4T.3. The potential interaction between core protein and gC1qR was examined in vitro by GST-binding assay using a GST fusion protein of HCV core protein (GST-core) and in vitro–translated and radiolabeled gC1qR. The resulting proteins were separated under denaturing conditions where the interaction of core with gC1qR was dissociated. As shown in Figure 2, in vitro transcribed, translated, and ^{35}S-methionine–labeled gC1qR of the expected molecular weight (39 kDa) was readily detected (lane 1). In addition, the gC1qR was found to associate with both the truncated and the full-length GST-core fusion proteins (lanes 3 and 4). No association between gC1qR and GST alone was observed (lane 2).

Mapping of the core region binding to gC1qR. To define the site within HCV core protein responsible for binding to the gC1qR, we performed a structural analysis examining the binding ability of core deletion constructs to the gC1qR. Deletion constructs encoding four separate regions of core protein (amino acid 1–152, 1–124, 26–124, 125–192) were inserted into the GST fusion vector pGEX4T.3 (Figure 3a). Using the GST fusion protein–binding assay to evaluate the association of the core deletion constructs with the gC1qR, we demonstrated that the region spanning amino acids 26–124 of the core protein was responsible for binding to the gC1qR (Figure 3b). In contrast, a GST fusion protein expressing the amino acids 125–192 failed to bind the gC1qR, although that protein (33.8 kDa) is present as seen by using Coomassie blue staining (Figure 3c, lane 2).

Identification of the region of gC1qR, which binds to the HCV core protein. The gC1qR gene encodes a 39-kDa product consisting of 282 amino acids. Maturation of this protein is accompanied by the cleavage of the NH_2-terminal 73 residues to form a 33-kDa product (residues 74–282) (33). C1q has been reported to bind the region of residues 74–95 of gC1qR (33). Other gC1qR ligands have been reported to interact with either NH_2- or

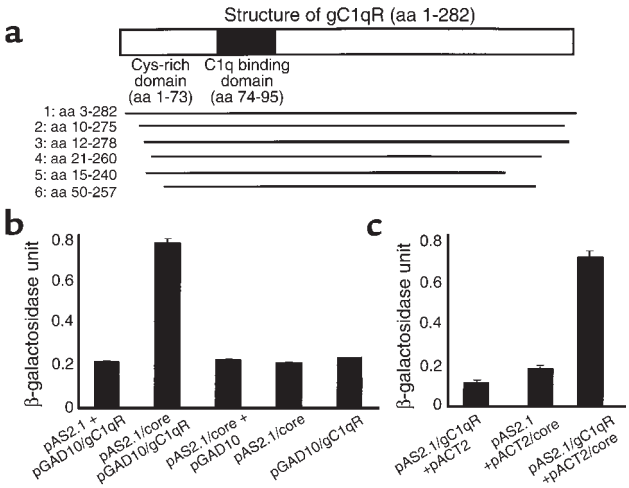

Figure 1

Physical association between HCV core protein and the gC1qR. (**a**) Identification of interaction between core and gC1qR. Structure of gC1qR is drawn as a box, and the C1q-binding site is indicated as a filled box. Six independent clones interacting with core are represented as a line with a location within the gC1qR. (**b**) Quantification of interaction between HCV core and the gC1qR. Yeast cells of strain Y187 were cotransformed with plasmids encoding the DNA-binding and -activation domains of the GAL4 transcriptional complex as fusion proteins with the following bait-prey protein combinations: (first bar) pAS2.1 parental vector (GAL4 DNA-binding domain without any carboxy-terminal fusion construct) + pGAD10/gC1qR (GAL4 transcriptional activation domain as the amino-terminal end of a fusion protein with the human gC1q receptor); (second bar) pAS2.1/core 1–124 (GAL4 DNA-binding domain as the amino-terminal end of a fusion protein with the HCV core protein amino acids 1 to 124) + pGAD10/gC1qR; (third bar) pAS2.1/core 1–124 + pGAD10; (fourth bar) pAS2.1/core 1–124 + pGAD10/CRAT1 (a parental GAL4 activation domain vector encoding an irrelevant prey protein as a carboxy-terminal fusion construct); and (fifth bar) pAS2.1/HE4Z (GAL4 DNA-binding domain as amino-terminal end of fusion protein construct with an irrelevant bait protein) + pGAD10/gC1qR. Yeast cells were grown in media maintaining selection for each of the plasmid pairs. Cells were harvested and tested for GAL4 gene activity, indicating association between bait and prey fusion constructs, using a standard method (26). (**c**) Binding assay for core and gC1qR interaction. Yeast two-hybrid was performed as described above using gC1qR (bait) and core (prey) constructs: (first bar) pAS2.1/gC1qR + pACT2; (second bar) pAS2.1 + pACT2/core; and (third bar) pAS2.1/gC1qR + pACT2/core. aa, amino acid.

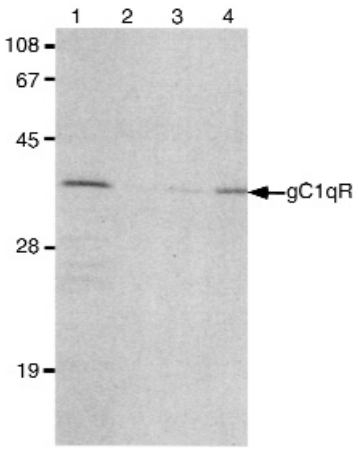

Figure 2
In vitro association between HCV core protein and the gC1qR. GST alone (lane 2), GST/core 1–124 (lane 3), or GST/core 1–194 (lane 4) were incubated with in vitro translated and ^{35}S-methionine–labeled gC1qR (lane 1) protein. After binding to glutathione resin and subsequent washing, samples were separated on SDS-PAGE and detected using autoradiography. The arrow indicates the location of gC1qR (39 kDa).

COOH-terminal domains of this receptor (33). To determine the region in gC1qR that is necessary for core binding, we carried out PCR-based mutagenesis of the gC1qR gene and expressed the molecules either as radiolabeled in vitro translation products or as GST fusion proteins. In Figure 4a the gC1qR constructs employed for this analysis can be seen.

As Figure 4b demonstrates, ^{35}S-methionine–labeled full-length gC1qR in vitro translation product (Figure 4b, lane 4) is efficiently bound by GST core in a GST pull-down analysis (Figure 4b, lane 8). The labeled gC1qR 46–259 deletion construct (Figure 4b, lane 3), which includes the NH_2- and COOH-terminal residues of the receptor necessary for binding of C1q and other ligands, also binds core protein in the GST pull-down, but with an apparently lower efficiency (Figure 4b, lane 7). Additional COOH-terminal truncations of gC1qR, i.e., gC1qR 46–187 (Figure 4b, lane 2) and 46-115 (Figure 4b, lane 1) were not bound by full-length core at detectable levels in the pull-down assay (Figure 4b, lanes 5 and 6, respectively). We also examined the interaction of core with a gC1qR internal deletion construct lacking residues 46–259, but this 68–amino acid product was not bound by HCV core protein (data not shown). These data suggest that the region of gC1qR-spanning residues 46–259 was essential for HCV core binding. Because of the apparent higher efficiency of binding of core to full-length gC1qR, residues in the COOH terminus and possibly the NH_2 terminus, e.g., residues 74–95 of the mature receptor, may also contribute to the gC1qR/core interaction.

To verify the difference in the efficiency of binding of core to full-length gC1qR and 46–259 deletion construct, we carried the GST pull-down experiment by examining the binding ability of in vitro–translated and radiolabeled core and GST-gC1qR fusion proteins. As seen in Figure 4c, labeled core is bound more efficiently by full-length gC1qR than by 46–259 receptor-deletion construct. The other two gC1qR-deletion constructs, 46–187 and 46–115, again failed to bind. This implicates a region of gC1qR (residues 188–259) near the COOH terminus of the mature gC1qR product as a potential association site with the core protein. Indeed, radiolabeled protein expressing the region of gC1qR spanning amino acid 188 to 259 can bind to HCV core protein with a lower efficiency (Figure 4d). However, as noted above, the NH_2-terminal residue in gC1qR may be required for optimal core binding to the mature receptor.

HCV core protein can inhibit human T-cell proliferation induced by allogeneic stimulation. Our previous studies have demonstrated that expression of the HCV core protein in vivo blocks normal viral clearance by suppressing the host immune response, including the induction of viral-specific CTL activity (22). Recently, others have shown that binding of C1q to its receptor on T lymphocytes inhibits the response of these cells to mitogenic stimuli (28, 29). To examine whether the core protein mimics the suppressive effect of C1q and inhibits the T-cell responsiveness, a standard one-way mixed lymphocyte reactions (MLR) was performed in the absence or presence of various doses of purified recombinant HCV core protein.

As shown in Figure 5a, exposure of the T lymphocytes to core protein specifically inhibited T-cell proliferation in a dose-dependent manner. It is notable that the minimal concentration of core protein that inhibits the T-cell proliferative responses is in the nanomolar range (1.3 nM) and may be comparable to the concentration of core protein detectable in the plasma of HCV-infected patients (34). In contrast, neither the addition of recombinant HCV NS3, one of HCV nonstructural proteins prepared in an identical manner as recombinant core, nor the addition of β-gal protein to MLR culture affected T-cell proliferation (Figure 5b). These data are consistent with our earlier in vivo data in the acute infection model that core protein exhibited an immunosuppressive effect on the host immune response to viral infection, but NS3 protein did not (22). In addition to the suppression of MLR by core protein, core protein could also inhibit the mitogen-stimulated (i.e., conA-stimulated) proliferation of human peripheral blood T lymphocytes (Figure 5c).

Anti-gC1qR and anti-core Ab's reverse the HCV core-mediated inhibition of T-cell responses. To establish a direct link between core-induced inhibition of T-cell proliferation and interaction of core protein with gC1qR, we examined the ability of anti-gC1qR Ab to block core-induced inhibition of T-cell proliferation. Two gC1qR-specific mAb's (60.11, 74.5.2) have been used for this study: mAb 60.11 binds to an NH_2-terminal site spanning amino acid residues 76–93 of gC1qR, and mAb 74.5.2 recognizes region bounded by residues 204–218 (31).

Since the gC1qR has been reported to be localized both on the plasma membrane in T lymphocytes (28)

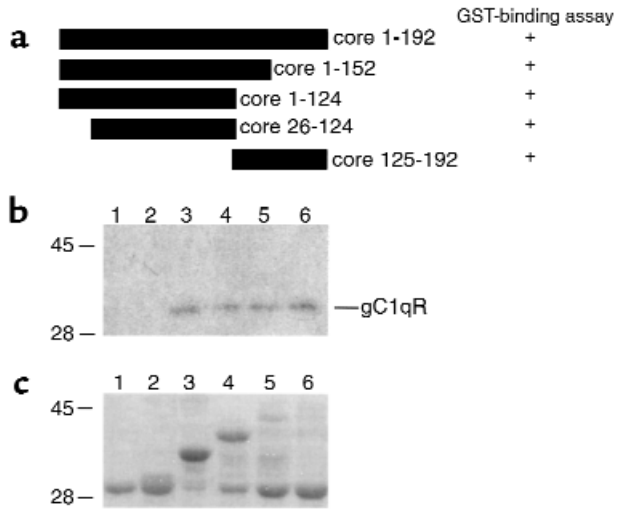

Figure 3
Identification of core-binding region to the gC1qR. (**a**) Diagram for deletion constructs. The COOH-terminal deletion constructs of HCV core protein (amino acids 1–152, 1–124, 26–124, 125–192) were generated by site-directed mutagenesis and inserted into the GST fusion vector pGEX4T.3. (**b**) GST-binding assay. The truncated forms of core protein as shown in the diagram were partially purified from bacteria transformed with the GST fusion plasmids. Purified GST fusion proteins expressing truncated forms of core protein were then examined for their binding ability to gC1qR, as described above. Lane 1: GST alone (29 kDa); lane 2: GST/core 125–192 (33.8 kDa); lane 3: GST/core 26–124 (37.7 kDa); lane 4: GST/core 1–124 (40.7 kDa); lane 5: GST/core 1–152 (43.4 kDa); lane 6: GST/core 1–192 (47.8 kDa). (**c**) Coomassie blue staining of the purified GST-core fusion proteins. Samples for each lane are same as in **b**.

and intracellularly in several cell types (35, 36), we first confirmed the cell-surface localization of the gC1qR on T lymphocytes using flow cytometry with anti-gC1qR Ab. As Figure 6a shows, the anti-gC1qR Ab binds to the MOLT-4 T-cell line as seen by flow-cytometric analysis. Anti-gC1qR Ab (74.5.2) binding to the MOLT-4 T-cell line was inhibited by coincubation of cells with anti-gC1qR Ab and core protein (Figure 6a). To further substantiate that gC1qR is also displayed on the cell surface of primary resting PBMCs, we examined the interaction of the anti-gC1qR mAb and the PBMC populations used as the responder cells in the MLR. As Figure 6b illustrates, approximately 60% of unstimulated PBMCs expressed gC1qR. We also find that, as reported previously (28), 100% of both $CD4^+$ and $CD8^+$ T cells in this population express gC1qR (data not shown). At present we do not know if gC1qR is present on potential antigen-presenting cells (APCs) or accessory cells (i.e., macrophages, circulating dendritic cell precursors) present in MLR-responder and -stimulator populations. This analysis is in progress, although published results indicate that the gC1qR is displayed on the cells of the monocyte/macrophage lineage (27, 28).

Although anti-gC1qR mAb's (60.11, 74.5.2) had been shown previously to precipitate the p33 gC1qR from the surface of cultured lymphoid cells (32), it was important to demonstrate that this Ab could likewise detect this molecule on the surface of primary resting mononuclear cells (Figure 6b). To examine this, we carried out surface biotinylation of primary PBMCs followed by immunoprecipitation of solubilized cells with the two anti-gC1qR mAb's (60.11, 74.5.2) and Western blot analysis with alkaline phosphatase–conjugated streptavidin. Figure 6c shows that a biotinylated protein of the expected molecular mass, i.e., 33 kDa (lane 2), was precipitated by the anti-gC1qR Ab's. The nature of the other band present in the blot is uncertain, but may represent a coreceptor associated with cell-surface gC1qR (see discussion).

We next determined whether the observed core-induced inhibition of T-cell proliferation depends on core protein binding to gC1qR by evaluating the ability of anti-gC1qR Ab's to reverse the core-induced inhibition of T-cell proliferation. As Figure 7a demonstrates, the addition of HCV core protein in one-way MLR inhibited T-cell proliferation by 95%. This inhibition was selectively reversed by addition of either of two independent mAb's specific for the gC1qR in the T-cell proliferation assay. In contrast, Ab to β2 microglobulin had no effect on reversing the core-induced inhibition of T-cell proliferation. Furthermore, the addition of anti-gC1qR Ab alone to the MLR did not enhance T-cell proliferation (Figure 7b), indicating that anti-gC1qR Ab alone does not override the core-mediated suppression by directly activating T cells and does not contain the mitogenic effect on T-cell proliferation. These results suggest that anti-gC1qR Ab reverses core inhibition of the MLR by blocking core binding to the receptor and reinforces the concept that the core protein inhibits in the MLR by binding to gC1qR displayed on the surface of one or more of the cell populations responding in the MLR.

To determine whether depletion of core protein in lymphocyte culture can elicit the normal T-cell proliferation, we examined whether treatment of lymphocyte culture with anti-core Ab can reverse the core-induced suppression of T-cell proliferation. As shown in Figure 7c, anti-core Ab was not capable of increasing conA-stimulated T-cell proliferation but could reverse the core-induced suppression of T-cell proliferation in a dose-dependent manner. In contrast, an isotype-matched control Ab did not reverse the suppression of T-cell proliferation induced by core protein. These results confirm the ability of core protein to suppress the proliferation of T lymphocytes.

Discussion

Here we demonstrate the interaction between the core protein of HCV and the complement p33 gC1qR and describe the impact of this interaction on a human cell–mediated immune response. Using a yeast two-hybrid screen with residues 1–124 of the core protein as bait, we found initial evidence for an interaction between core protein and the p33 gC1qR. The physical association between the HCV core and the gC1qR pro-

teins and the sites of interaction were confirmed in GST pull-down experiments. The interaction site on core protein encompassed residues 26–124 while core binding was primarily localized to the COOH terminus of gC1qR, i.e., residues 188–259. We then demonstrated that the addition of HCV core to alloreactive MLR markedly suppressed T-lymphocyte proliferation in a fashion analogous to the anti-proliferative effect of the C1q protein on T-lymphocyte activation described previously (28). Strikingly, this inhibitory effect of core on lymphocyte proliferation was observed at a concentration of core protein as low as 1.3 nM. The role of the core/gC1qR interaction in mediating the core-induced inhibition of the T-cell proliferation was substantiated by the finding that addition of anti-gC1qR Ab in the MLR-proliferation assay reversed the core-induced inhibition of T-cell proliferation. These findings suggest that the HCV core protein may contribute to HCV pathogenesis through a novel immunosuppressive mechanism involving the interaction of HCV core with the gC1qR on cells of the immune system.

Several groups have identified cellular proteins that interact with HCV core using the yeast two-hybrid screening technique (24, 30). These investigators demonstrated an association of HCV core protein with the lymphotoxin β-receptor, a member of the TNFR family (30), or with apoAII (24). In these studies, human liver-expression libraries were screened with the core protein as bait. Because of our previous evidence indicating core-induced suppression of the host cellular immune response during experimental virus infection (22), we chose to screen a human PBL-expression library for core-binding molecules. The fact that we repeatedly identify the gC1qR as a core protein target by yeast two-hybrid screening might be explained by the use of human lymphocyte-expression library in our screening. The gC1qR has been reported to be expressed at high levels in mononuclear or lymphoid cells but only at moderate levels in hepatocytes (27).

The C1q complement protein is one of natural ligands for gC1qR. Along with its well-defined role in the complement cascade, C1q exhibits immunoregulatory activity since mice deficient in C1q develop symptoms of autoimmune disease, e.g., glomerulonephritis (37, 38). Like HCV core protein, the C1q hexamer is highly positively charged (39, 40), and the globular head of C1q complex binds to the NH_2-terminal portion of the p33 gC1qR (41). By contrast, we report here that the primary interaction site of core protein is within the COOH-terminal portion of the gC1qR, although residues within the NH_2 terminus of the mature form of this receptor may also contribute to the binding of core protein to the receptor (Figure 4). The COOH-terminus of gC1qR is also the site of interaction with inflammation-related proteins including the clotting factor XII and kininogen (33). The recent crystal structure of the p33 gC1qR (42) provides potential insight into the structural basis of the core-gC1qR interaction. The p33 unit structure exists as

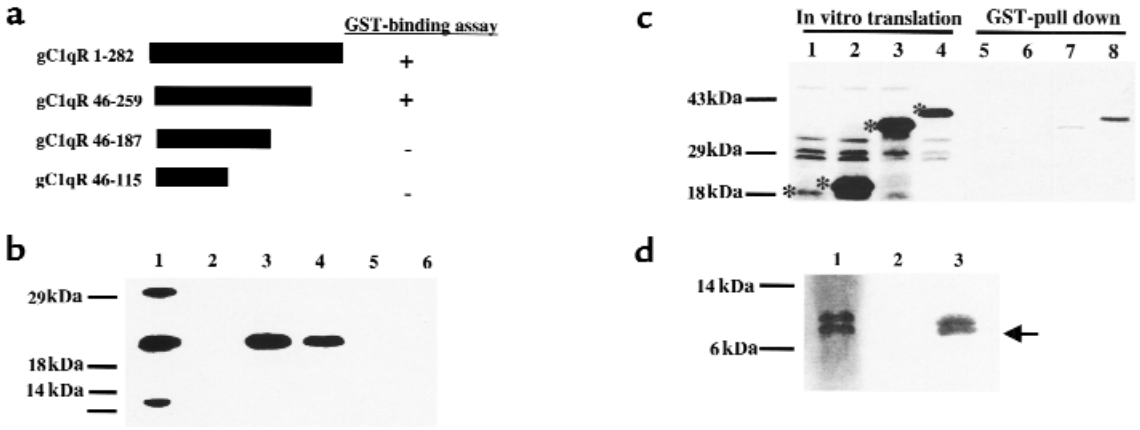

Figure 4

Identification of gC1qR-binding region to HCV core protein. (**a**) Diagram for deletion constructs. The COOH-terminal deletion constructs of gC1qR (amino acids 46–259, 46–187, 46–115) were generated by site-directed mutagenesis. The resulting plasmids were inserted into the pCI:*neo* vector to allow the in vitro transcription and translation. (**b**) GST-binding assay. The truncated forms of gC1qR as shown in the diagram were labeled with ^{35}S-methionine by in vitro transcription and translation reaction. Asterisks indicate the correct size of truncated form of gC1qR. Minor bands present in the left panel represent the preterminated protein during in vitro transcription and translation. ^{35}S-methionine–labeled truncation forms of gC1qR were examined for their binding ability to the GST-core fusion protein. Lane 1: radiolabeled gC1qR 46–115 (17.5 kDa); lane 2: radiolabeled gC1qR 46–187 (25.5 kDa); lane 3: radiolabeled gC1qR 46–259 (33.8 kDa); lane 4: radiolabeled gC1qR 1–282 (39 kDa); lane 5: GST-core/gC1qR 46–115; lane 6: GST-core/gC1qR 46–187; lane 7: GST-core/gC1qR 46–259; lane 8: GST-core/gC1qR 1–282. (**c**) GST-binding assay with GST-truncated forms of gC1qR fusion protein and ^{35}S-methionine–labeled core protein. Lane 1: radiolabeled core (21 kDa); lane 2: GST alone; lane 3: GST-gC1qR 1–282/core; lane 4: GST-gC1qR 46–259/core; lane 5: GST-gC1qR 46–187/core; lane 6: GST-gC1qR 46–115/core. (**d**) GST binding assay with GST-core and ^{35}S-methionine–labeled gC1qR 188–259. Lane 1: radiolabeled gC1qR 188–259 (8.4 kDa); lane 2: GST alone; lane 3: GST-core/gC1qR 188–259.

an asymmetric homotrimer with a negatively charged face, including exposed acidic residues present in the COOH-terminal portion (residues 210–264) of the molecule. In addition, acidic residues clustered in the NH_2-terminal α-helical domain (residues 77–96) of p33 may also contribute to the overall negatively charged face of this molecule. The negatively charged residues in the COOH-terminal core-binding domain defined in this report, along with the contribution of the NH_2-terminal acidic residues displaced on the face of p33, likely represent the binding site for positively charged core protein.

In addition to its role as a cell-surface complement receptor, the gC1qR has also been localized to several compartments within the cell, including in the mitochondria (35), in the nucleus (43), and on the surface of lymphoid cells during apoptotic death (42). In this report we provide direct evidence that gC1qR is constitutively expressed on the surface of primary PBMCs (Figure 6), including $CD4^+$ and $CD8^+$ T cells (Y.S. Hahn, unpublished observation). Our findings support earlier results demonstrating the cell-surface expression of gC1qR on lymphoid cells (28). The mechanism of anchoring of gC1qR on the lymphocyte surface is yet to be defined. Based on crystallographic structure of gC1qR (42), it seems unlikely that this molecule is displayed on the cell surface as an integral membrane protein or a lipid-anchored receptor. We favor the view that the gC1qR is displayed on the cell surface in association with one or more associated proteins, e.g., cC1qR, which could serve both to anchor it to the plasma membrane and possibly function upon engagement with gC1qR.

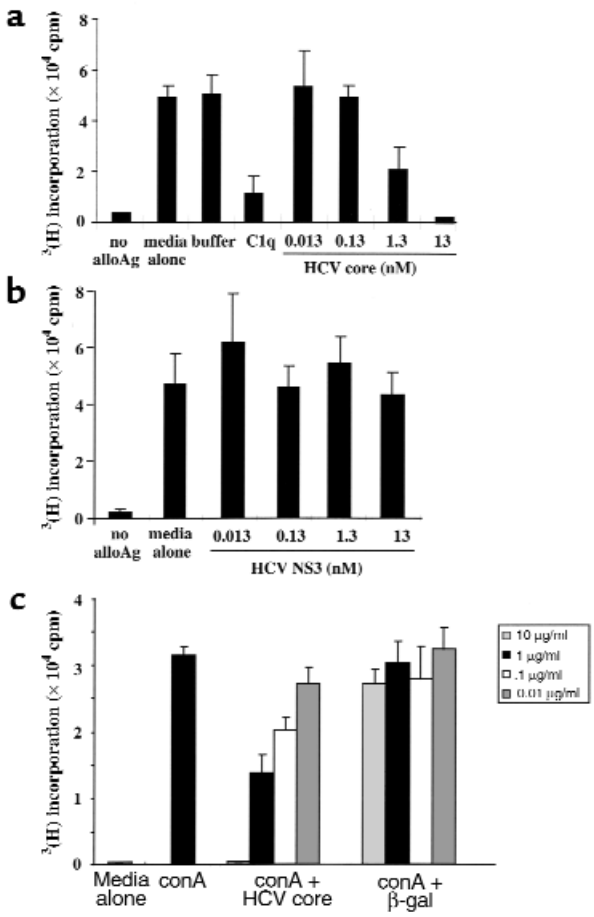

Figure 5
Inhibition of T-lymphocyte proliferation by HCV core protein. (**a**) Effect of HCV core protein on T-cell proliferation. Standard one-way mixed lymphocyte reactions were performed in the presence of C1q (50 μg/ml), the indicated concentration of purified HCV core protein, or core buffer (control response). After 6 days of coculture, proliferation was measured as 18-hour ^{3}(H)-thymidine incorporation. (**b**) Effect of HCV NS3 protein on T-cell proliferation. HCV NS3 protein was tested for its ability to modulate the proliferation of T lymphocytes as described above. (**c**) Effect of HCV core protein on conA-stimulated T-cell proliferation. MLR was performed in the presence of conA (2 μg/ml) and the indicated amount of HCV core protein or control protein, β-gal.

An unexpected outcome of this analysis was our finding that both anti-gC1qR Ab's, 60.11 and 74.5.2, reversed the core-induced inhibition of T-cell proliferation. Ab's 60.11 and 74.5.2 bind to the NH_2 terminus and COOH terminus of gC1qR, respectively. Since our analysis of the sites of core-gC1qR interaction implicated the COOH terminus of the gC1qR as the major site of core binding, it might be anticipated that only the 74.5.2 Ab would reverse the core effect. Again, three-dimensional structure of the gC1qR (42) provides some insight into the impact of these regions of the receptor on the interaction of core with gC1qR. The NH_2-terminal αA-helix (residues 76–93) of the receptor recognized by 60.11 lies in close proximity to the anti-parallel αB-helix (residues 233–244) of the adjoining gC1qR subunit. The αB-helix represents part of the negatively charged face of the receptor and, in turn, abuts on β-strand 6 (residues 205–213). β-strand 6 is within the region (residues 204–218) of gC1qR recognized by the COOH terminal–specific mAb 74.5.2. Thus in the tertiary structure of the gC1qR trimer, the binding sites on these two mAb are in close proximity. The most likely explanation for the ability of both Ab's to reverse core-induced inhibition of T-cell proliferation is that these Ab's may act sterically to block core binding to a site on the negatively charged acidic face of the receptor in close proximity to the sites of Ab binding. Our finding that core protein can inhibit the binding of the NH_2 terminus–specific 60.11 Ab to gC1qR on MOLT-4 cells by flow-cytometry analysis is in keeping with this hypothesis.

The particularly noteworthy observation in this report was our finding that core protein, like C1q, could suppress the proliferative response to alloantigen of human PBMCs in the MLR. That core protein mediates this suppression by binding to gC1qR was strongly suggested by the fact that the suppressive effect of recombinant core protein in the MLR could be blocked by the addition of two different anti-gC1qR Ab's. This observation, along with the finding that recombinant HCV NS3 of bacterial origin prepared in identical fashion did not inhibit the MLR, argues strongly against a nonspecific trace bacterial contaminant present in the

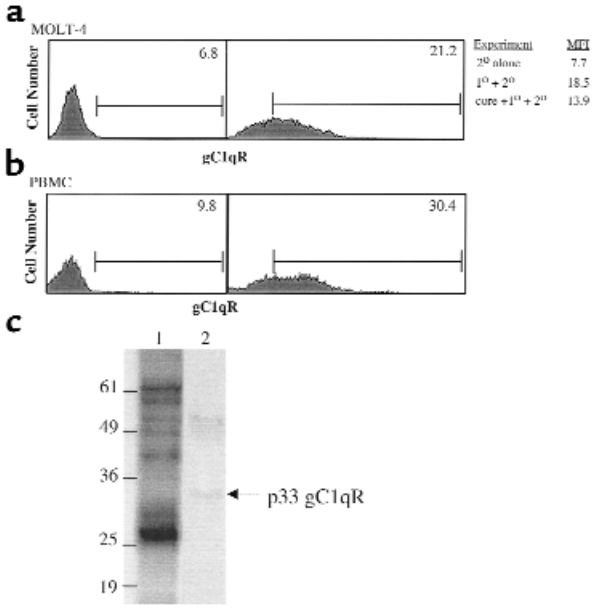

Figure 6
Cell-surface expression of gC1qR on human PBMCs and MOLT-4 cell lines. Cell-surface expression of gC1qR in MOLT-4 T-cell line (**a**), and human PBMCs (**b**). MOLT-4 T-cell line or human PBMCs were incubated with media alone (left panel) or anti-gC1qR Ab, 74.5.2 (right panel), and stained cells were examined for FACS analysis. Value (%) represents the cell population gated with gC1qR-positive cells. (**c**) Analysis of surface-biotinylated gC1qR. Cell-surface proteins of human PBMCs were biotinylated. Solubilized membrane proteins were subjected to SDS-PAGE before (lane 1) or after (lane 2) immunoprecipitation with anti-gC1qR mAb 60.11 and 74.5.2. The separated proteins were transferred to PVDF membranes and developed using streptavidin. MFI, mean fluorescence intensity; 1°, primary; 2°, secondary.

core protein preparation as the mediator of the suppressive effect. The mechanism by which core suppresses T-cell proliferation through its interaction with gC1qR is currently unknown. Because gC1qR is displayed on PBMCs (Figure 6b), including the $CD4^+$ and $CD8^+$ T lymphocytes (28), T lymphocytes are a likely target of core protein through a direct suppressive effect of core on T-lymphocyte activation/proliferation. Consistent with this concept we find that the core protein inhibits IL-2 and IFN-γ production by human T lymphocytes (Y.S. Hahn, unpublished observation). Since gC1qR is expressed on a variety of cell types, including cells of the monocyte/macrophage lineage (27), it is perhaps likely that core protein may suppress early events in lymphocyte activation at the level of antigen presentation and costimulation during HCV infection. In case of core-mediated MLR-inhibition results reported here, it is unlikely that core blocks antigen presentation because the alloantigen complexes recognized by responding T cells would likely be preformed on the stimulator APCs. However, core-mediated suppression of APC activation necessary for MLR stimulation as a result of core/gC1qR engagement could account for our findings, as well. More detailed studies on the effects of core on the response of the individual immune cell types to alloantigens and foreign antigens, such as HCV proteins, will be required to define the cellular site(s) of core-induced suppression.

A hallmark of HCV infection is the establishment and maintenance of persistent infection in the majority of individuals exposed to this virus (44). The results reported here suggest a novel mechanism for the establishment and/or maintenance of HCV persistence by suppression of the host immune response through the interaction of the HCV core protein with the complement gC1q receptor. As demonstrated here (Figure 5) and reported previously (28), the complement protein complex C1q can inhibit the development of specific cellular immune responses. This effect of C1q presumably reflects one of the compensatory inhibitory mechanisms to downregulate the host response during an evolving immune response to pathogenic microorganisms.

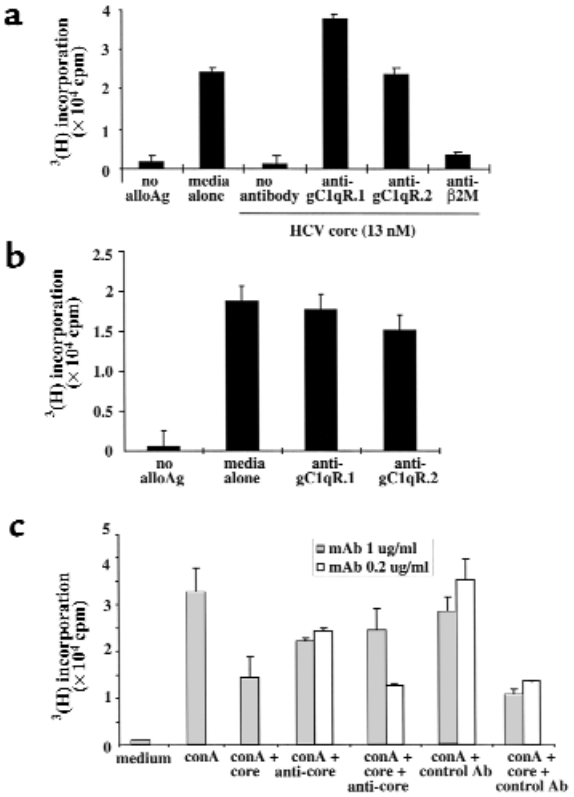

Figure 7
Reversal of core-induced inhibition of T-cell proliferation by anti-gC1qR and anti-core Ab's. (**a**) Inhibition of core-induced anti–T-cell proliferation by the addition of anti-gC1qR Ab's in cultures. To examine the effect of anti-gC1qR Ab on reversing the core-induced anti–T-cell proliferation, anti-gC1qR Ab's (60.11, 74.5.2) were cocultured in the T-cell proliferation assay in the presence of core protein (13 nM) as described. (**b**) The effect of anti-gC1qR Ab's on recovery of core-induced anti–T-cell proliferation. The T-cell proliferation assay was performed in the presence of anti-gC1qR Ab's. (**c**) The reversed effect of anti-core Ab on core-induced anti–T-cell proliferation. The indicated amount of anti-core Ab or an isotype-matched control Ab was added to the conA-stimulated lymphocyte culture.

Similar activity has been reported for other complement components as a result of the interaction of these proteins with their receptors on immunocytes (45). Our results indicate that the HCV core protein can, in a like manner, suppress the host immune response by engaging a complement receptor on the surface of immune cells. There is ample precedent for the subversion of the host response through the interaction of a viral protein with a complement receptor, e.g., the measles glycoprotein interaction with the CD46 receptor on monocytes (46).

It should be noted that purified core protein inhibited the proliferative response in the MLR at a nanomolar concentration range. This suggests that extremely low concentrations of free core protein present in the circulation or tissues of HCV-infected patients could be suppressive in T-lymphocyte responses. While core protein is presumed to be normally present in the circulation of infected patients as the nucleocapsid component of intact HCV virions, the exposure of extra virion-free core protein in the blood and tissues is less clear. Core protein has been reported to be secreted from cells expressing the core gene (24) and free (nonvirion-associated) core protein has been detected in the plasma of HCV-infected patients (34). We have also readily detected core proteins in the plasma of several HCV-infected patients and HCV core transgenic mice without previous detergent treatment of the plasma (Y.S. Hahn, unpublished observation). While these results support the view that core protein (either as free monomer, multimers, or nucleocapsid complexes) may be present in the circulation of infected patients, rigorous biochemical analysis of the nature of core protein in patient material needs to be performed to verify these observations. In this connection, it is noteworthy that the core protein preparation used in the MLR studies reported here exists both as monomers and aggregates in keeping with the ability of the core protein to self-assemble to multimeric complexes (47, 48). Our estimates of the concentration of core protein that inhibited T-lymphocyte proliferation was based on the molecular mass, 21 kDa, of the core monomers. If the inhibitory activity of core requires core multimerization, then concentration of multimeric core protein in the subnanomolar range may be inhibitory. Studies are now in progress to evaluate the requirement for core multimerization in the delivery of the core inhibitory signal through the gC1qR.

Most patients persistently infected with HCV mount a humoral and cellular immune response to the core protein (49, 50). This raises the paradox of how core protein could down-modulate the host response in the presence of an ongoing immune response to HCV constituents. It is tempting to speculate that the suppressive effect of low concentration of free core protein in the circulation could account for the diminished cellular immune response observed in some HCV-infected patients (51, 52). This could, in turn, result in inefficient elimination of virus-infected cells and the maintenance of persistent HCV infection. However, a more likely possibility, which we currently favor, is that HCV core protein released during the acute phase of HCV infection suppresses the early host immune response and promotes the establishment of persistent infection. This concept is supported by our earlier findings of rapid core-induced immune suppression in an animal model of acute viral infection (22). Whether core protein acts in the establishment or maintenance of persistent HCV infection, our results suggest a novel mechanism of immune subversion during HCV infection based on the interaction of core with the gC1qR. At the least, our results provide potential insight into HCV pathogenesis and may offer an opportunity to develop new and specific therapeutic targets for HCV infection.

Acknowledgments

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