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Hepatitis C Virus Core Protein Inhibits Human T Lymphocyte Responses by a Complement-Dependent Regulatory Pathway\textsuperscript{1,2}

Zhi Qiang Yao, Duong Tony Nguyen, Apostolos I. Hiotellis, and Young S. Hahn\textsuperscript{3}

Complement proteins are involved in early innate immune responses against pathogens and play a role in clearing circulating viral Ags from the blood of infected hosts. We have previously demonstrated that hepatitis C virus (HCV) core, the first protein to be expressed and circulating in the blood of infected individuals, inhibited human T cell proliferative response through interaction with the complement receptor, globular domain of C1q receptor (gC1qR). To investigate the mechanisms of HCV core/gC1qR-induced inhibition of T cell proliferation, we examined the effect of core protein on the early events in T cell activation. We found that HCV core inhibited phosphorylation of extracellular signal-regulated kinase (ERK) and mitogen-activated ERK kinase (MEK). HCV core-induced impairment of ERK/MEK mitogen-activated protein kinase resulted in the inhibition of IL-2 and IL-2R\textalpha\ gene transcription, which led to the inhibition of IL-2 production and high-affinity IL-2R expression. Importantly, the ability of anti-gC1qR Ab treatment to reverse HCV core-induced inhibition of ERK/MEK phosphorylation reveals that the interaction between HCV core and gC1qR is linked to the interference of ERK/MEK mitogen-activated protein kinase activation. These results imply that HCV core-induced blockage of intracellular events in T cell activation by a complement-dependent regulatory pathway may play a critical role in the establishment of HCV persistence during the acute phase of viral infection. The Journal of Immunology, 2001, 167: 5264–5272.

The

natural ligand C1q is the first component in the classical pathway of complement activation (1) and plays a crucial role as a first defense against foreign agents such as viruses and bacteria circulating in blood (2). In addition, C1q can control the inflammatory and adaptive immune responses through binding to its receptors. Indeed, mice deficient in C1q can control the inflammatory and adaptive immune responses, including both antiviral CTL response and Th1 responses (5). Engagement of C1q with the gC1qR complex delivers an antiproliferative signal to human T lymphocytes (6, 7). However, the molecular mechanism for inhibition of T lymphocyte responses mediated by C1q/gC1qR has yet to be identified.

Hepatitis C virus (HCV) is a serious and growing threat to human health, having infected approximately 170 million people worldwide (8). HCV genome is a positive single-stranded RNA virus related to the Flaviviridae family (8). HCV transmission has been linked to a blood-borne route (e.g., patients receiving organ transplants, blood products, or i.v. drug use) (8). The most remarkable feature of HCV is its ability to efficiently establish persistent infection by evading host immune surveillance (9). HCV persistent infection is highly associated with the development of liver cirrhosis, hepatocellular carcinoma, and autoimmune disease (10, 11). Unfortunately, no vaccine or effective treatment for HCV is currently available, and the mechanism for HCV persistence is not understood. Although T lymphocyte responses to HCV gene products have been demonstrated (12, 13), the role of such responses in controlling HCV infection is not well defined. Strong and broad CTL and Th1 responses have been detected in an individual with acute hepatitis C, who subsequently cleared the virus (14, 15). However, in patients chronically infected with HCV, the frequencies of antiviral CTL are relatively low (16). In addition, the production of Th1-type cytokines (i.e., IFN-\textgamma\ and IL-2) is dramatically suppressed in peripheral T cells of chronic HCV patients (17, 18). The presence of inefficient T lymphocyte responses in chronic HCV patients suggests that HCV gene products might be involved in suppressing host immune response.

Strikingly, free HCV core particles are circulating in the bloodstream of HCV-infected patients, suggesting the immunopathological role of core protein in HCV infection (19). Indeed, several reports demonstrate that HCV core, the first protein produced upon viral infection, is able to inhibit the T lymphocyte responses that are critical in viral clearance (20–22). Our prior studies demonstrate that HCV core is responsible for the suppression of protective immune responses, including both antiviral CTL response and IL-2/IFN-\textgamma\ production in mice infected with recombinant vaccinia virus expressing HCV core (20). In addition, soluble HCV core inhibited the proliferation of human T lymphocytes by its interaction with the complement receptor, gC1qR (21). The immunomodulatory function of HCV core has been further evidenced by a diminished T cell response to hepatitis B virus (HBV) envelope proteins in mice immunized with HCV core-HBV chimeric constructs (22). It is notable that CD4+ T cell response to core protein is well correlated with a benign course of infection, presumably by
maintaining humoral and cellular responses to control HCV infection (23).

The molecular basis for the core-induced immune suppression is presumably due to its interaction with the TNFR family (e.g., TNFR1, Fas, lymphotxin β receptor) (24) and the complement receptor, gC1qR (21), both identified by yeast two-hybrid screening. Intracellular expression of core protein has been reported to modulate TNFR family-mediated apoptosis by interaction with the cytoplasmic death domains of TNFR family (24). We have previously demonstrated that, like the natural ligand C1q, the interaction of soluble core protein with the gC1qR on T cell surface inhibits human T lymphocyte proliferation (21). Importantly, there is accumulating evidence that supports the presence of circulating core protein in the blood of HCV-infected patients. Core protein has been shown to be secreted from transfected cell lines expressing the core gene (25), and circulating core protein is detectable in the plasma of HCV-infected patients (26). These results suggest that core-induced immune suppression may play a critical role in establishing and maintaining HCV persistence during early viral infection. It implies that blocking agents of the immunomodulatory function of core protein would be a potential therapeutics for HCV persistent infection.

To determine the mechanisms for core-induced inhibition of human T cell proliferative response through its interaction with the complement receptor, gC1qR (21), we analyzed the effect of HCV core on the early events of T cell activation. We examined the production of cytokines (i.e., IL-2, IFN-γ), the cell-surface expression of IL-2R and CD69, and the phosphorylation of mitogen-activated protein (MAP) kinase in mitogen-activated PBMC in the presence of HCV core or control proteins. We further characterized the role of gC1qR in core-induced immunosuppression by addition of anti-gC1qR Ab to the cultures of PBMC in the presence of core protein. In this report, we describe the ability of HCV core to impair T cell activation in response to Con A or TCR and further investigate the role of core/gC1qR interaction in core-induced immunosuppression, 1/10 diluted rabbit anti-gC1qR (produced in our laboratory, ELISA titer 1/6,400–12,800) was added to PBMC stimulated with Con A in the presence of core protein (1 μg/ml). Prebleeding serum of the same rabbit was used as a negative control. After 24 or 48 h of coincubation, the expression of IL-2Rα (CD25) and IL-2Rβ (CD122) chain was determined by FACS as described above.

Materials and Methods

PBMC isolation and culture

Human PBMC were isolated from peripheral blood obtained from Virginia Blood Service (Richmond, VA) of three healthy donors, free of human pathogens such as HIV, HCV, HBV, and CMV, by Ficoll-density centrifugation using lympholyte-H (Cedarlane Laboratories, Hornby, Ontario, Canada). PBMC were washed twice and incubated with RPMI 1640 (Life Technologies, Gaithersburg, MD) containing 10% (v/v) FBS (HyClone Laboratories, Logan, UT), penicillin/streptomycin (100 U/ml, BD PharMingen, San Diego, CA) and anti-CD3 (2 μg/ml, Sigma-Aldrich, St. Louis, MO), 2) anti-CD3 (UCHT-1, 1 μg/ml, BD PharMingen, San Diego, CA) and anti-CD28 (1 μg/ml, BD PharMingen). Two different recombinant HCV core proteins (genotype 1a) were also assessed in these studies: 1) β-galactosidase (β-gal) core, with β-gal fused with the full-length (amino acid residue 1–191) HCV core protein (ViroGen, Watertown, MA), 2) GST core where GST was fused with truncated HCV core protein spanning amino acid residue 1–124 as described previously (27) β-gal and GST alone were used to serve as negative controls. For measurement of T cell proliferation, varying amounts of β-gal core or β-gal, and GST core or GST were added to PBMC (2 × 10^6 cells/ml). Cells were activated with either Con A (2 μg/ml) or anti-CD3/CD28 (1 μg/ml) and were cultured in the presence of 1 μg/ml β-gal core or GST core. Cells were also treated with the same dose of β-gal or GST to serve as a negative control. Cultures were harvested at different time points and cells were removed by centrifugation at 14,000 rpm for 1 min. Supernatants were stored at ~80°C until analysis. The production of IL-2 and IFN-γ was analyzed using OptEIA Human IL-2 and IFN-γ ELISA kit (BD Pharamingen) under conditions specified by the manufacturer. Each experimental condition was performed in duplicate and repeated at least three times.

Flow cytometry analysis

PBMC (1 × 10^6) were activated with anti-CD3/CD28 and treated with C1q (100 μg/ml) or 1 μg/ml β-gal core or β-gal for appropriate times, as described above. The cells were pelleted in 96-well U-bottom plate at 200 × g for 5 min at 4°C, resuspended in 50 μl of RPMI 1640 supplemented with 10% FBS, 0.1% NaCl, and incubated with the appropriate Abs (anti-CD3, −CD69, −CD28, −CD122, −CD132, from BD Pharamingen) on ice for 1 h. After washing three times, the cells were incubated with FITC-anti-mouse Ig (BD Pharamigen) on ice for 1 h in the dark. The cells were washed three times at 4°C and fixed with 1% paraformaldehyde in PBS before analysis of cellular markers by three-color flow cytometry (BD Biosciences, San Jose, CA). The unstained cells and primary isotype controls were used to determine the levels of background fluorescence. Viable cell gates were used to collect at least 20,000 events with the T cell populations. To further investigate the role of core/gC1qR interaction in core-induced immunosuppression, 1/10 diluted rabbit anti-gC1qR (produced in our laboratory, ELISA titer 1/6,400–12,800) was added to PBMC stimulated with Con A in the presence of core protein (1 μg/ml). Prebleeding serum of the same rabbit was used as a negative control. After 24 or 48 h of coincubation, the expression of IL-2Rα (CD25) and IL-2Rβ (CD122) chain was determined by FACS as described above.

Western blot analysis

PBMC (2 × 10^5) were activated with anti-CD3/CD28 and treated with 1 μg/ml β-gal core or β-gal for 12, 24, or 48 h. Aliquots of cells were harvested and resuspended in cell lysis buffer (200 μM Tris, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, and protease inhibitor mixtures) (Roche Molecular Biochemicals). The lysates were incubated at 4°C for 20 min with moderate shaking. Cell debris was pelleted by centrifugation and the supernatants were collected and frozen at ~80°C. For MAP kinase assay, 40 μg of cellular lysates were run on a 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA) using a SemiDry Blotting apparatus (PharMacia, Piscataway, NJ). After blocking in Blotto-Tween 20 (10 mM Tris, 0.9% NaCl, 50% glycerol, 0.1% Tween 20, and 5% nonfat dry milk) at room temperature for 1 h, the membranes were first probed with the specific Ab detecting the phosphorylated protein at 4°C overnight. The blots were then incubated with an HRP-conjugated secondary Ab and developed using the ECL plus kit (Amersham, Arlington Heights, IL). Subsequently, the blots were stripped in 0.1 M glycine (pH 2.9), blocked in Blotto-Tween 20, and reprobed with Abs for detection of total protein levels (according to the manufacturer’s instructions). PhosphoPlus p44/p42 extracellular signal-regulated kinase (ERK)1/2 MAP kinase (Thr202/Tyr 204) Ab and PhosphoPlus mitogen-activated ERK kinase (MEK)1/2 (Ser217/Ser221) Ab kit was purchased from New England Biolabs (Beverly, MA). To determine the effect of the HCV core/gC1qR interaction on the activation status of ERK/MEK MAP kinase, 1/10 diluted rabbit anti-gC1qR polyclonal Ab was added to PBMC stimulated with Con A in the presence of core (1 μg/ml). Prebleeding serum of the same rabbit was used as negative control. After 24 h of coinubation, the cell lysates were prepared and the ERK/MEK MAP kinase proteins were detected by Western blot as described above.
Analysis of IL-2 and IL-2R mRNA expression

To assess mRNA levels for IL-2 and IL-2Rα, IL-2Rβ, and IL-2Rγ chains, total RNA was isolated from 5 × 10^6 PBMC stimulated with anti-CD3/CD28 in the presence of Clq (100 μg/ml), or 1 μg/ml β-gal core or β-gal. Furthermore, 1/10 diluted rabbit anti-gClqR or prebleeding serum was added to PBMC stimulated with anti-CD3/CD28 in the presence of core protein to block the interaction between core and gClqR. After 6 h of incubation, the cells were collected and total cellular RNA was isolated by TRizol reagents (Life Technologies) following the manufacturer’s protocol. RNA (1 μg) was treated by RNase-free DNase (Life Technologies), and cDNA synthesis was performed in 20 μl of reaction mix for 60 min at 37°C using a cDNA synthesis kit (PE Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions. The integrity of RNA extraction and cDNA synthesis was verified by measuring the amount of GAPDH or β-actin cDNA in each sample.

IL-2 mRNA was determined by competitive PCR (Cytosurex quantitative PCR kit for human IL-2; BioSource International, Camarillo, CA) according to the manufacturer’s protocol. Briefly, 5 μl of the sample cDNA was mixed with a known copy number (2000 copies) of an exogenous synthesized DNA designate as internal calibration standard (ICS). The ICS has been constructed to contain PCR primer binding sites identical to the IL-2 cDNA and also a unique internal sequence which is used to capture the resulting ICS amplicon (412 bp) and thus be distinguished from the IL-2 amplicon (362 bp). PCR was performed in a total volume of 50 μl using 10 μM dNTPs, 40 μM primers, and 2.5 U of AmpliTaq polymerase (PE Applied Biosystems). After an initial step of 95°C for 5 min, 30 cycles of amplification were performed with a denaturation step at 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min, followed by a common step at 72°C for 10 min. During amplification, the biotinylated IL-2 primers are incorporated into both ICS and IL-2 products. Following PCR, the products are denatured with equal volume of alkaline solution at room temperature (RT) for 10 min, and then hybridized to either ICS or IL-2 sequence-specific capture oligonucleotides, which are prebound to microplate wells at 37°C for 2 h.

The captured sequences are detected and quantified by addition of 100 μl of HRP-streptavidin conjugate at RT for 30 min, followed by 100 μl of tetramethylbenzidine substrate. The signal generated in the reaction is proportional to the amount of ampiclon present. Because the ICS is amplified at an efficiency identical to the IL-2 cDNA, it can serve as a standard for IL-2 cDNA quantitation. The following formula is used to calculate the starting copies of IL-2 cDNA in the PCR: (total IL-2 OD / total ICS OD) × 2 × input copy number of ICS.

Semiquantitative mRNA analysis for IL-2 gene expression was performed by using end-point dilution PCR. This method is used when no internal competitor or external standard is available. After an initial denaturing step of cDNA product at 95°C for 5 min, amplification reaction for competition of the IL-2 was conducted with specific conditions for each amplification product: for the α-chain and γ-chain, 95°C for 60 s, 60°C for 45 s, 72°C for 60 s; for the β-chain and β-actin, 95°C for 60 s, 68°C for 45 s, 72°C for 60 s. After 35 cycles of amplification and a common step at 72°C for 10 min, the resulting cDNA products were separated on a 2% BioGel (Bio 101, Carlsbad, CA). As a control for genomic DNA contamination, equal amounts of RNA extraction products were used for each sample assessed and PCR amplification was performed without the RT step. The following sets of primers were used for the amplification of the IL-2 components: α-chain: sense 5'-CAAATCCATGATATGTAGG-3' and antisense 5'-TCACCTGTGACATATGAGCTG-3' yielding a PCR product of 232 bp; β-chain: sense 5'-GCGTGGCTTCGACCCAC-3' and antisense 5'-GACGGATGAGGGAAGGCCAGAAG-3' yielding a PCR product of 211 bp; and γ-chain: sense 5'-GTCGACCTAACCACACT-3' and antisense 5'-GATCTCTTAGTCTTCAGG-3' yielding a PCR product of 409 bp. As control, the β-actin mRNA was amplified in the same samples: sense 5'-CTGGAGCCGGAAATCTGGTGTAGG-3' and antisense 5'-CTGACACCTCTGCTGATCCACATCT-3' yielding a PCR product of 478 bp.

Statistical analysis

Statistical analysis was performed using Student’s t test (two-tailed). Values of p < 0.05 were considered significant.

Results

HCV core inhibits human T cell proliferation in response to Con A or anti-CD3/CD28 Abs

Our previous studies demonstrated that HCV core protein inhibits the proliferation of human T lymphocytes in a standard one-way mixed lymphocyte reaction through its interaction with the complement receptor, gClqR (21). To determine whether HCV core is also able to inhibit the proliferation of T cells in response to other stimuli, we examined the effect of HCV core protein on T cell proliferation in response to Con A mitogen or TCR and CD28 costimulatory receptor engagement. Human PBMC were isolated from healthy donors and stimulated with either Con A or plate-bound anti-CD3/CD28 Abs in the presence of HCV core or control protein. To ensure that the inhibitory effect of HCV core on T cell proliferation is core-specific, we examined two different sources of recombinant HCV core protein (β-gal core and GST core) on PBMC from three healthy donors.

As shown in Fig. 1A, PBMC treated with HCV core (i.e., β-gal core or GST core) specifically inhibited Con A-stimulated T cell proliferation in a dose-dependent manner. In contrast, β-gal or GST control protein prepared in an identical manner as recombinant core had no inhibitory effect on T cell proliferation. These results are consistent with our previous results, which demonstrated that HCV core inhibited human T cell proliferation in a standard one-way mixed lymphocyte reaction through its interaction with the gClqR (21). We next tested whether HCV core could inhibit T cell proliferation in response to TCR and CD28 costimulatory receptor engagement by examining the effect of HCV core on T cell proliferation while stimulating naive PBMC with plate-bound anti-CD3/CD28 Abs. Consistent with the inhibitory effect...
of core protein on the proliferation of T cells in response to Con A, the addition of HCV core to T lymphocyte cultures stimulated with anti-CD3/CD28 Abs also inhibited T cell proliferation in a dose-dependent manner, as compared with cells treated with a control protein. At 1 \( \mu \)g/ml HCV core, the \( \beta \)-gal core exhibited 60% inhibition, while the GST core exhibited 50% inhibition, as compared with the control anti-CD3/CD28-stimulated cells (\( p < 0.05 \)) (Fig. 1B). In contrast, we did not observe any inhibition in cells treated with any concentration of \( \beta \)-gal or GST alone (\( p > 0.05 \)).

**HCV core inhibits the production of IL-2 and IFN-\( \gamma \) in PBMC stimulated by Con A or anti-CD3/CD28**

Because cytokines including IL-2 and IFN-\( \gamma \) are crucial for supporting T cell proliferation and differentiation (28), we examined whether HCV core could suppress T cell proliferation by inhibiting the production of these cytokines by activated T cells. Purified human PBMC were stimulated with Con A or plate-bound anti-CD3/CD28 Abs in the presence of HCV core or control proteins, and the production of IL-2 and IFN-\( \gamma \) was measured by ELISA. As shown in Fig. 2, HCV core markedly inhibited the production of IL-2 and IFN-\( \gamma \) in T cells stimulated with Con A or anti-CD3/CD28 Abs at various time-points (12, 24, or 48 h). In contrast, the addition of \( \beta \)-gal or GST did not inhibit the production of IL-2 and IFN-\( \gamma \). This suggests that HCV core may interfere with T cell activation in response to stimulation with Con A or anti-CD3/CD28 Abs and inhibit IL-2 and IFN-\( \gamma \) production by activated T cells.

**High dose of exogenous rIL-2 partially restores HCV core-induced inhibition of T cell proliferation**

To assess whether the effect of HCV core on the inhibition of T cell proliferation might be due to insufficient supply of IL-2 or a functional defect of IL-2R, we examined the ability of exogenous rIL-2 to reverse the core-induced inhibition of T cell proliferation by adding various concentrations of rIL-2 (12.5, 25, 50, or 100 U/ml) to core-treated T cells and examining T cell proliferation. As shown in Fig. 3, HCV core (1 \( \mu \)g/ml) inhibited Con A-stimulated T cell proliferation by 60%, as compared with control cells. In the absence of core protein, addition of rIL-2 (12.5 U/ml) in Con A-stimulated cultures increased T cell proliferation by 2-fold, as compared with cells stimulated with Con A alone. In contrast, addition of the same dose of rIL-2 (12.5 U/ml) in core-treated T cells did not reverse the core-induced inhibition of T cell proliferation, i.e., there was a 66% inhibition of T cell proliferation in core-treated cells, compared with Con A and rIL-2 (12.5 U/ml) stimulated cells in the absence of core protein. When the dose of rIL-2 was increased 2-fold (25 U/ml), the core-induced inhibition of T cell proliferation was still observed; however, only a 36% inhibition was detectable, compared with Con A- and rIL-2 (25 U/ml)-stimulated cells without core protein.

Interestingly, addition of nonphysiologic doses of rIL-2 (50 and 100 U/ml) partially reversed the core-induced T cell proliferation, with only 12 and 7% inhibition observed, when compared with cells treated with Con A and rIL-2 (50 and 100 U/ml) in the absence of core protein. Partial reversal of the core-induced inhibition of T cell proliferation by the addition of high doses of rIL-2 suggests that HCV core might affect cell-surface expression and/or function of the IL-2R on activated T cells.

**HCV core inhibits cell-surface expression of high-affinity IL-2R in activated T cells by down-regulating IL-2Ra chain**

Upon TCR engagement, IL-2 is secreted by activated T cells, acts in an autocrine manner, and is critical for T cell proliferation by promoting cell cycle progression. The high-affinity IL-2R for binding IL-2 consists of \( \alpha \)(CD25)-, \( \beta \)(CD122)-, and \( \gamma \)(CD132)-chains, while the low-affinity IL-2R consists of the \( \beta \)- and \( \gamma \)-chains only.
expression of IL-2R/H9251 and IL-2R chains in anti-CD3/CD28-stimulated T cells in the presence or absence of C1q by flow cytometry analysis. As shown in Fig. 4A, we did not detect any difference of IL-2Rγ chain expression between C1q-treated cells and control cells. In contrast, the expression of IL-2Rα chain was inhibited in C1q-treated cells, as compared with the control cells, and there was moderate inhibition of IL-2Rβ expression in C1q-treated cells. These results suggest that down-regulation of IL-2Rα chain expression in C1q-treated cells may affect the expression of the high-affinity IL-2R complex.

Similar to C1q, inhibition of IL-2Rα and IL-2Rβ chain expression was also observed in HCV core-treated cells (Fig. 4B). The cell-surface expression of IL-2R on PBMC activated with anti-CD3/CD28 Abs, in the presence of β-gal core, revealed lower expression of IL-2Rα chain (10.2 vs 20.4% at 24 h; 23.6 vs 46.6% at 48 h after stimulation) and IL-2Rβ chain (6.1 vs 10.7% at 24 h; 20.4 vs 38.6% at 48 h after stimulation), as compared with the β-gal control. However, there was no significant difference in IL-2Rγ chain expression between core-treated cells and cells treated with control protein (0.7 vs 0.8% at 24 h; 0.8 vs 3.0% at 48 h after stimulation). Similar results for the effect of HCV core on inhibition of IL-2Rα chain expression were observed for cells stimulated with Con A (data not shown). These results suggest that HCV core interferes with IL-2R-mediated signaling by down-regulation of the high-affinity IL-2R complex.

To determine whether the core-induced inhibition of IL-2Rα and IL-2Rβ chain expression directly results from the interaction between HCV core and the complement receptor, gC1qR, we examined the ability of anti-gC1qR Ab to block the core-induced inhibition of IL-2R expression by preventing core protein from binding to the T cell surface. As shown in Table I, cells were stimulated with Con A in the presence or absence of HCV core protein for 24 and 48 h, and the levels of cell surface IL-2Rα and IL-2Rβ chain expression were measured by flow cytometry analysis. Both the IL-2Rα and IL-2Rβ chains were down-regulated in the presence of core protein, when compared with those treated with Con A alone. The addition of anti-gC1qR Ab to core-treated cells reversed the inhibition of IL-2Rα and IL-2Rβ chain expression. However, the addition of control sera failed to reverse the core-induced down-regulation of IL-2Rα and IL-2Rβ chain expression. These results suggest that the interaction between HCV core and gC1qR is directly involved in the core-induced inhibition of IL-2R expression and that the down-regulation of IL-2Rα chain by HCV core protein may be due to defect in T cell activation.

To further dissect the effect of HCV core on T cell activation, we examined the activation status of T cells by assessing the expression level of cell-surface CD69, an early T cell activation marker. Specifically, we quantitated the CD69-positive population in core-treated and untreated PBMC after stimulation with anti-CD3/CD28 Abs at various time points (0, 3, 6, 12, 24, 48, 72, and 96 h). PBMC stimulated with anti-CD3/CD28 was also stained with anti-CD3 and anti-CD28 Abs to determine the other T cell marker and total T cell population. There was no significant difference in the expression of either CD3 or CD28 between core-treated and control cells at any time point (data not shown). In contrast, the expression of CD69 was diminished in core-treated cells as early as 6 h after treatment, and this inhibition was sustained during the 5 days of incubation (data not shown). These results suggest that HCV core does not directly interfere with the expression of the TCR complex but does affect the intracellular signaling event in T cell activation.

**HCV core/gC1qR interaction inhibits the activation of ERK/MEK MAP kinase in anti-CD3/CD28-stimulated PBMC**

Signal transduction event through TCR initiates a cascade of intracellular biochemical changes leading to a cellular response. These early biochemical events include protein phosphorylation and activation of multiple kinases, including MAP kinase (31, 32). MAP kinase delivers signals to the nucleus and activates gene expression of transcription factors (33). ERK1/2 (p44/p42) and MEK are involved in the MAP kinase cascade, and the activation of ERK occurs through phosphorylation by an upstream kinase, MEK (34). The ERK/MEK MAP kinase plays a critical role in cellular proliferation by the activation of IL-2 and IL-2Rα gene expression (33). To determine whether HCV core protein inhibits the expression of IL-2Rα chain and CD69 by interfering with the activation of ERK/MEK MAP kinase, we examined the activation of this pathway by performing Western blot analysis using mAbs specific for phosphorylated ERK/MEK.

As shown in Fig. 5A, the expression of phosphorylated MEK and ERK was reduced in core-treated cells after 12, 24, and 48 h of stimulation, as compared with control cells. Western blot analysis using Abs recognizing unphosphorylated MEK and ERK demonstrated that there was no difference of total MEK and ERK expression between core-treated and control cells. In contrast, HCV core did not inhibit the activation of other MAP kinase, JNK,
These results suggest that HCV core specifically inhibits the ERK/MEK MAP kinase signaling cascade and the impaired activation of ERK/MEK MAP kinase by HCV core may be responsible for inhibition of T cell responsiveness upon TCR stimulation. In addition, anti-gC1qR Ab was able to reverse the core-induced inhibition of ERK/MEK MAP kinase phosphorylation (Fig. 5B), suggesting that this inhibitory effect of HCV core is directly mediated by the interaction between HCV core and gC1qR. Taken together, these results suggest that the impaired activation of ERK/MEK MAP kinase by HCV core/gC1qR interaction could be one potential mechanism for the core-induced inhibition of T cell activation/proliferation.

Because MAP kinase is responsible for the expression of early genes (i.e., IL-2, IL-2Rα chain) involved in T cell activation, we examined whether core/gC1qR-induced impairment of MAP kinase leads to the inhibition of IL-2 and IL-2Rα chain gene transcription. mRNA levels for IL-2 and IL-2Rα chain genes were inhibited by core stimulation (Table I). To further investigate the role of gC1qR in core-induced immunosuppression, we examined the ability of a polyclonal Ab against recombinant gC1qR (produced in our laboratory, ELISA titer 1/6,400–12,800) to inhibit the core-induced down-regulation of cell-surface expression of IL-2Rα and IL-2Rβ chains in PBMC. Rabbit anti-gC1qR Abs (1/10 diluted) were added simultaneously with core protein to PBMC stimulated with Con A. Prebleeding sera from the same rabbits were used as negative control. After 24 or 48 h of co-incubation, the expression of IL-2Rα (CD25) and IL-2Rβ (CD122) chains was determined by FACS analysis as described in Materials and Methods.

Table I. Anti-gC1qR Abs reverse the core-induced inhibition of IL-2Rα-IL-2Rβ chain expression

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>IL-2Rα (CD25)</th>
<th>IL-2Rβ (CD122)</th>
</tr>
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<tbody>
<tr>
<td>24 h</td>
<td>58.1 ± 9.7</td>
<td>42.2 ± 7.1</td>
</tr>
<tr>
<td>48 h</td>
<td>58.5 ± 12.4</td>
<td>49.2 ± 9.1</td>
</tr>
<tr>
<td>Con A</td>
<td>19.5 ± 0.7</td>
<td>18.2 ± 1.7</td>
</tr>
<tr>
<td>Con A + Core</td>
<td>21.2 ± 3.8</td>
<td>28.1 ± 8.3</td>
</tr>
<tr>
<td>Con A + Core + Anti-gC1qR</td>
<td>47.7 ± 19.4</td>
<td>52.0 ± 21.2</td>
</tr>
<tr>
<td>Con A + Core + Control Sera</td>
<td>19.8 ± 2.1</td>
<td>30.0 ± 11.2</td>
</tr>
</tbody>
</table>

*p38 data not shown. These results suggest that HCV core specifically inhibits the ERK/MEK MAP kinase signaling cascade and the impaired activation of ERK/MEK MAP kinase by HCV core may be responsible for inhibition of T cell responsiveness upon TCR stimulation. In addition, anti-gC1qR Ab was able to reverse the core-induced inhibition of ERK/MEK MAP kinase phosphorylation (Fig. 5B), suggesting that this inhibitory effect of HCV core is directly mediated by the interaction between HCV core and gC1qR. Taken together, these results suggest that the impaired activation of ERK/MEK MAP kinase by HCV core/gC1qR interaction could be one potential mechanism for the core-induced inhibition of T cell activation/proliferation.

HCV core/gC1qR interaction inhibits transcription of IL-2 and IL-2Rα chain genes

Because MAP kinase is responsible for the expression of early genes (i.e., IL-2, IL-2Rα chain) involved in T cell activation, we examined whether core/gC1qR-induced impairment of MAP kinase leads to the inhibition of IL-2 and IL-2Rα chain gene transcription. mRNA levels for IL-2 and IL-2Rα chain genes were
analyzed using anti-CD3/CD28-stimulated PBMC that had been treated with C1q, core protein, or β-gal. In addition, we examined the ability of anti-gC1qR Ab to reverse the core/gC1qR-induced inhibitory effect on IL-2 and IL-2α chain gene transcription. As shown in Fig. 6A, the level of IL-2 mRNA was reduced in cells treated with C1q (lane 2, 2383 ± 16 copies) or HCV core (lane 3, 1656 ± 79 copies) by 38 or 57%, compared with that in cells treated with a control protein (lane 1, 3838 ± 28 copies). The inhibition of IL-2 mRNA expression in core-treated cells is consistent with the low level of IL-2 production, as shown in Fig. 2. In addition, anti-gC1qR Ab treatment restored the core-induced inhibition of IL-2 mRNA expression (lane 4, 3338 ± 43 copies), whereas control sera (lane 5, 1548 ± 174) had no such recovery effect. Control reactions measuring levels of GAPDH mRNA showed no significant changes in the various treatment groups.

When we determined the mRNA level of IL-2α chain mRNA by allele-specific PCR analysis, IL-2α chain mRNA levels were lower in C1q-treated cells than in control cells (lane 2) and C1q-treated cells treated with an anti-CD3/CD28-stimulated PBMC (lane 3). The reduction of IL-2α chain mRNA was reversible by the addition of anti-gC1qR Ab in the culture (lane 4), but not by preimmune serum (lane 5). Control reactions measuring β-actin mRNA level showed no difference among various treatments. Analysis of IL-2β and IL-2γ chain mRNA expression revealed no significant difference in IL-2β and IL-2γ chain mRNA between core-treated cells and control cells (data not shown). Taken together, our data suggest that the core/gC1qR interaction downregulates the expression of IL-2 and IL-2Rα chain mRNA in a β-gal-dependent manner. However, the interaction of HCV core with gC1qR does not affect the expression of IL-2β and IL-2γ chain mRNA.

To further explore the mechanism of the core/gC1qR interaction, we examined the expression of phosphorylated ERK1/2 and MEK1/2. P-ERK and MEK1/2 Abs were used to examine the expression of phosphorylated ERK1/2 and MEK1/2 proteins. The blot was then stripped and reprobed with anti-ERK1/2 and anti-MEK1/2. The blot showed a decrease in the levels of phosphorylated ERK and MEK as well as total ERK and MEK proteins in C1q-treated cells (Fig. 6B, lane 3) compared with control sera (lane 1). The reduction of phosphorylation of ERK and MEK in activated T cells by HCV core/gC1qR interaction is shown in Fig. 6C.

In addition, anti-gC1qR Ab treatment restored the core-induced decrease in the levels of phosphorylated ERK and MEK as well as phosphorylated ERK and MEK proteins in C1q-treated cells (Fig. 6B, lane 6) compared with control sera (lane 1). These results indicate that the core/gC1qR interaction downregulates the expression of IL-2 and IL-2Rα chain mRNA in a β-gal-dependent manner. However, the interaction of HCV core with gC1qR does not affect the expression of IL-2β and IL-2γ chain mRNA. Taken together, our data suggest that the core/gC1qR interaction downregulates the expression of IL-2 and IL-2Rα chain mRNA in a β-gal-dependent manner. However, the interaction of HCV core with gC1qR does not affect the expression of IL-2β and IL-2γ chain mRNA.
together, these results suggest that core/gC1qR interaction is directly involved in the inhibition of IL-2Rα chain expression, thereby leading to low-affinity IL-2R expression.

Discussion

In this study, we have demonstrated the immunomodulatory role of HCV core protein in the inhibition of T cell responsiveness and have further characterized the effect of HCV core on T cell activation through its interaction with gC1qR. Upon stimulation of human PBMC with either Con A or anti-CD3/CD28, we found that HCV core inhibited the proliferation of T lymphocytes in a dose-dependent manner. In addition, the production of IL-2 and IFN-γ in core-treated cells was markedly diminished, as compared with control cells. The addition of high doses of exogenous rIL-2 (50–100 U/ml) to core-treated T cell cultures partially restored the core-induced inhibition of T cell proliferation, suggesting that HCV core might interfere with the expression of IL-2R or its downstream signaling events. Indeed, HCV core affected the expression of high-affinity IL-2R by down-regulating IL-2Rα chain expression. Subsequent analysis of the effect of HCV core on the ERK/MEK MAP kinase showed that HCV core inhibited the activation of ERK/MEK MAP kinase, which led to the inhibition of IL-2 and IL-2Rα gene transcription. Importantly, anti-gC1qR Ab reversed the core-induced inhibition of ERK/MEK MAP kinase activation and IL-2Rα chain gene expression. These results suggest that the impaired activation of ERK/MEK MAP kinase due to the core/gC1qR interaction inhibits the transcription of early genes involved in T cell activation (i.e., IL-2, IL-2Rα chain) and leads to the suppression of T cell responsiveness.

It is important to point out that patients chronically infected with HCV exhibit immune dysfunction with a Th2-dominant cytokine profile, while Th1 cytokines are prominent in those with self-limited HCV infection (17, 18). The decreased levels of Th1 cytokines in the periphery of chronic HCV patients can be recovered by treatment with IFN-α and ribavirin (35). The strength and quality of both Th1 cell and CTL responses have been reported to play a crucial role in recovery from HCV infection (36, 37). The immunomodulatory function of HCV core through its interaction with the gC1qR, as described in this report, may play a critical role in the establishment of HCV persistent infection during the early viral infection by suppressing T cell responses including the IL-2 and IFN-γ production. A critical issue relating to the role of core/gC1qR-induced immune suppression in chronic HCV infection is the presence of circulating core protein in the blood of HCV-infected patients that could potentially interact with peripheral T lymphocytes. Strikingly, it has been reported that HCV core protein was secreted from tissue culture cell lines (25) and that the circulation of free HCV core protein has been detected in the plasma of HCV-infected patients (38, 39). The potential impact for circulating HCV core protein to suppress host immune responses to HCV is further supported by several reports of high levels of core protein detected during the early stage of infection before the production of anti-core Ab (40, 41). These studies support our views of the role of core-induced immune suppression in HCV persistence. During the early acute phase of HCV infection, circulating core protein (i.e., core protein free of anti-core Ab binding) could inhibit T cell responses by binding to the gC1qR on peripheral T cells. Studies on underlying mechanisms of core/gC1qR-induced immunosuppression as described in this report will provide a rational basis for developing therapeutic and immunization strategies.

The HCV core-induced inhibition of T cell responsiveness is possibly due to the impaired ERK/MEK MAP kinase activation by HCV core/gC1qR interaction. The ERK/MEK MAP kinase pathway plays a central role in downstream signaling of T cell activation and in controlling growth factor signaling. Activation of the MAP kinase pathway is responsible for the transcription of early genes involved in T cell activation (i.e., IL-2, IL-2Rα chain, CD69). Therefore, the impaired activation of ERK/MEK MAP kinase by HCV core could result in the blockage of T cell activation, such as inhibition of IL-2 and IL-2Rα chain gene expression, and lead to the inhibition of T cell proliferation in response to TCR stimulation. The inhibition of IL-2Rα chain gene transcription explained the observation that the addition of high doses of rIL-2 (50–100 U/ml) to core-treated cells partially recovered the core-induced inhibition of T cell proliferation because of formation of low-affinity IL-2Rβ and IL-2Rγ instead of high-affinity IL-2Rα, IL-2Rβ, IL-2Rγ complex. Importantly, the inhibition of IL-2Rα chain expression in core-treated T cells occurs by the direct interaction of core protein with gC1qR, similar to the complement C1q-mediated inhibition of IL-2R expression (30). It is also notable that there is similarity between core-treated cells and anergic T cells. In anergic T cells, nonphysiological quantities of IL-2 can compensate for the lack of IL-2Rα chain expression by providing signaling through the low-affinity receptor IL-2Rβ and IL-2Rγ chains (29). Consistent with the high dose of IL-2 required for restoring the proliferative response of anergic T cells, a high dose of IL-2 (50–100 U/ml) was also necessary to recover the core-induced inhibition of T cell proliferative response, suggesting that HCV core may induce T cell anergy.

The mechanism by which HCV core interferes with the ERK/MEK MAP kinase signaling cascade has yet to be elucidated. Based on the intracellular signaling of gC1qR linked to phosphatidylinositol 3 kinase (42) and protein kinase C (43), it suggests several possibilities. First, the inhibitory effect of HCV core on the MEK/ERK MAP kinase could be due to the interference of upstream signaling cascades such as phosphatidylinositol 3 kinase or protein kinase C or c-Raf/Ras (44, 45). Second, a specific phosphatase, such as CD45, may be activated by HCV core, resulting in the dephosphorylation of MAP kinase (46). However, analysis of CD45 surface expression (at 0, 3, 6, and 12 h) excludes this possibility, because we did not detect any difference between core-treated cells and control cells (Z. Q. Yao, unpublished observation). Third, HCV core protein may affect cell-cycle progression by dysregulating cell-cycle regulators, such as p21, p27, cyclin–dependent kinases, and retinoblastoma protein. We are currently investigating these issues to find out how HCV core interferes with the activation of ERK/MEK MAP kinase. Further investigation as to the effect of core protein on the intracellular signaling event of T cell activation and the expression of cell cycle regulators will provide insight into the mechanisms of core-induced inhibition of T cell activation and proliferation. Nonetheless, our finding that HCV core blocks the ERK/MEK MAP kinase pathway is of fundamental importance in understanding the role of HCV core-induced immune suppression in the establishment of HCV persistence.

The evasion of immune surveillance has been reported to play a role in the pathogenesis of other persistent infections, such as HIV. In HIV-infected patients, expression of the HIV tat protein is well correlated with suppression of host immune response and disease progression. It is important to point out that the immunomodulatory function of HIV tat is similar to HCV core, in that HIV tat also inhibits IL-2 production and interferes with the formation of the IL-2R complex (47–49). Like HCV core, HIV tat-induced inhibition of T cell proliferation is also mediated by a blockage in the activation of the MAP kinase pathway through reduced ERK/MEK phosphorylation. However, the molecular mechanism for inhibition of T cell proliferation induced by HIV tat may be distinct from that of HCV core, because the addition of a lower dose of IL-2 could reverse the HIV tat-induced inhibition of T cell proliferation. In
summary, results from the present study suggest a novel role for HCV core in immune suppression through a complement regulatory pathway. The results imply that the interaction between circulating core protein and gC1qR on peripheral T cells may inhibit the activation and proliferation of T lymphocytes during the early HCV infection and facilitate the establishment of HCV persistence.

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References