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Frequency of gC1qR⁺CD4⁺ T cells increases during acute Hepatitis C virus infection and remains elevated in patients with chronic infection

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Abstract

CD4⁺ T cell responses are impaired in chronic HCV infection. To determine factor(s) involved in CD4⁺ T cell dysregulation, we examined the effect of extracellular core on the alteration of CD4⁺ T cell responses and the cell surface level of core-binding protein, gC1qR on CD4⁺ T cells from acute HCV patients with resolved and chronic infection. During the acute phase of infection, the frequency of gC1qR⁺CD4⁺ T cells increased in both resolved and chronic HCV infection compared to healthy controls. Notably, 6 months later, frequency of gC1qR⁺CD4⁺ T cells maintained elevated in chronic patients compared to that in resolved patients. In addition, TCR stimulation increased the frequency of gC1qR⁺CD4⁺ T cells, resulting in core-induced inhibition of T cell responses in both resolved and chronic patients. These results suggest that HCV infection expands gC1qR⁺CD4⁺ T cells, which, increase the susceptibility to core-mediated immune dysregulation and facilitate the establishment of HCV persistency.

INTRODUCTION

Hepatitis C virus (HCV) is an enormous worldwide health problem, with more than 170 million people infected globally and nearly 4 million cases within the United States. HCV persists in approximately 70% of infected individuals and leads to liver inflammation, fibrosis, and cirrhosis, as well as autoimmune disease. HCV infection is implicated in the rising incidence of hepatocellular carcinoma in many developed countries, with approximately 1–5% of chronically infected HCV patients developing hepatocellular carcinoma. While there is an available treatment, consisting of interferon- α and ribavarin, the treatment has side effects and response to treatment is variable. The mechanism by which HCV persists remains unknown,

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though the high incidence of persistence suggests that HCV may evade and possibly suppress the host immune response.

Outcome of HCV infection is largely influenced by the magnitude and breadth of T cell responses; in particular, CD4⁺ T cells are important for resolution of infection (1–6). CD4⁺ T cells are crucial for generation of antibody and maintenance of memory CD8⁺ T cell effector function. Impaired HCV-specific CD4⁺ T cell responses in the acute phase of infection lead to viral persistence, while a sustained multi-specific CD4⁺ T cell response is associated with spontaneous recovery from HCV infection (4,7). Establishment of persistent HCV infection is predicted by a failure to generate/sustain CD4⁺ T cell proliferation and production of Th1 cytokines (4,8–10). In studies of experimental HCV infection in chimpanzees, CD4⁺ T cell depletion prior to re-challenge of previously infected animals resulted in the generation of viral escape mutants and failure to resolve infection (11). Therefore, a strong CD4⁺ T cell response must be sustained beyond the point of apparent control of viral replication in order to prevent relapse and establishment of a persistent infection (2).

Despite the fact that there are defects in proliferation and cytokine production by HCV-specific CD4⁺ T cells from chronically infected individuals (12), the mechanism(s) responsible for CD4⁺ T cell dysregulation during HCV infection remains unknown. Several reports demonstrate that HCV core exhibits the immunomodulatory function to dampen T cell responses (13–15). Nonenveloped core protein is detectable in accumulates in liver cells and serum during early infection (16). HCV core is detectable in the absence of HCV antibodies during the window phase and its detection is used to diagnose early HCV infection (17–20). Extracellular core exhibits T cell inhibitory effects on human PBMC from healthy blood donors (15) and suppresses CD8⁺ T cell function in acute HCV patients (13). The complement C1q receptor, gC1qR, has been identified as a binding partner for extracellular core (14), as has TLR2 (21). gC1qR is expressed on a variety of cell types including T cells and is localized at both the cell surface and the intracellular compartment.

A number of groups have demonstrated the critical importance of CD4⁺ T cell help and function in the acute phase of infection. In addition, a recent analysis (7) showed that CD4⁺ T cell responses correlated more precisely with virologic outcome of acute HCV than CD8⁺ T cells. Based on the T cell inhibitory function of HCV core, it is possible that interaction between HCV core and gC1qR on the surface of CD4⁺ T cells may contribute to the functional impairment of CD4⁺ T cells that leads to viral persistence. To this end, we first characterized the functional impact of core on isolated CD4⁺ T cells from healthy PBMC. We then examined the potential for circulating core to influence the outcome of HCV infection by analyzing the serum core levels, as well as the frequency of gC1qR⁺CD4⁺ T cells in a tracked cohort of acutely infected subjects who subsequently developed viral persistence or spontaneously resolved HCV infection. The analysis for the effect of extracellular core on CD4⁺ T cell responses to TCR stimulation revealed that HCV core suppressed the proliferative capacity of CD4⁺ T cells. Interestingly, the percent of gC1qR⁺CD4⁺ T cells in infected patients was increased compared to healthy individuals during the acute phase of infection. Notably, at 6 months post-enrollment, the percent of gC1qR⁺CD4⁺ T cells in the periphery of resolved individuals was significantly reduced compared to that of persistently infected individuals. In the present study, we report, for the first time, the increased frequency of gC1qR expression on CD4⁺ T cells from chronic HCV patients and the potential role of gC1qR in influencing the outcome of HCV infection in humans.

MATERIALS AND METHODS

Study Population

The study group was comprised of acutely HCV-infected patients recruited from multiple sites. The study protocol was approved by all appropriate institutional review boards. Acute HCV was diagnosed based on HCV antibody (Ab) seroconversion in a subject with previously negative HCV testing, seroconversion in a subject with new-onset risk factors and alanine aminotransferase (ALT) levels 10-fold greater than normal, or HCV RNA positivity with HCV antibody negativity. Twenty treatment-naïve patients (11 male, 9 female), mean age of 34.6 years, were selected from a larger cohort for the present study. The majority (17) of patients were Caucasian. Spontaneous viral resolution (n=10) and chronicity (n=10) were defined as the absence or presence of HCV RNA at 6 months post-enrollment with at least two viral determinations. The clinical details of the acutely infected cohort are described in Table 1. The median time post-infection at time of enrollment (Month 0) for acute-to-resolved and acute-to-chronic patients in this study was 151 days and 150 days respectively. Additionally, PBMCs from 10 patients (6 male, 4 female), mean age of 49.9 years, persistently infected with HCV for greater than 5 years ("long-term chronic") were evaluated in this study.

For subjects who lacked known iatrogenic exposures but who reported symptoms, the acquisition date was defined as 6 weeks before the onset of symptoms. Among the remaining participants, the midpoint between the last negative antibody test result and the earliest of the first positive antibody test or positive HCV RNA test result was used as the date of acquisition, as detailed in a recent manuscript (30,31). The average interval between acquisition and enrollment was not different for acute-resolved versus acute-chronic patients.

HCV core ELISA

Circulating HCV core levels were determined from serum collected at the time of enrollment (Month 0) and approximately six months post-enrollment (Month 6) from several patients in this study cohort. Additionally, serum core levels from three of the long-term chronic patients were determined. Ortho® HCV core Ag ELISA test kit (Wako Chemicals, Richmond, VA) was used to quantify serum core levels as per manufacturer's protocol.

Sample preparation and storage

Peripheral blood was drawn from the acutely infected cohort at time of enrollment (Month 0) and 6 months after enrollment (Month 6), from various collection sites. PBMC were isolated by Ficoll (Amersham Biosciences, Piscataway, NJ for HCV-infected donors) and cryopreserved (20% dimethyl sulfoxide in fetal bovine serum (FBS)) for subsequent analysis. Peripheral blood from healthy donors was drawn at Virginia Blood Services (Richmond, VA) at a single time point (n=14). PBMCs from healthy donors were isolated by density centrifugation with Lympholyte-H (Cedarlane Labs, Burlington, NC) and cryopreserved for subsequent analysis.

Cells and culture conditions

Human PBMC from healthy, acutely HCV infected, resolved, and chronically HCV infected patients were cultured with RPMI 1640 (Life Technologies, Gaithersburg, MD) containing 10% (vol/vol) FBS (Mediatech, Manassas, VA), penicillin-streptomycin (Invitrogen, Carlsbad, CA), L-glutamine (2mM) (Invitrogen, Carlsbad, CA), and 2-mercaptoethanol (Invitrogen). CD4⁺ T cells were isolated from PBMC of healthy donors using CD4-microbeads (Miltenyi Biotec, Auburn, CA) as per the manufacturer's protocol. Briefly, CD4⁺ cells were obtained by passing the cell mixture over MiniMACS magnetic separation columns (Miltenyi Biotec) and collecting the magnetically captured cells.

To activate PBMC and purified CD4⁺ T cells, 96-well round bottom tissue culture plates were pre-coated with anti-CD3 antibody (0.5ug/ml Hit3a clone, eBioscience, San Diego, CA) and anti-CD28 (5ug/ml 28.2 clone, eBioscience) antibody. Wells were washed and purified PBMC or purified CD4⁺ lymphocytes (2×10⁵ cells per well) were added in 200ul of culture medium, with 10U/ml of recombinant human IL-2 added to purified CD4⁺ T cell cultures. To determine PBMC or purified CD4⁺ lymphocyte responsiveness to HCV core protein, 2.5 µg/ml of HCV core [recombinant, N-terminus fused β-galactosidase (β-gal), Virogen, Watertown, MA] or 2.5 µg/ml of β-gal control protein were added to human cells stimulated with anti-CD3/CD28 antibodies (14,22). Media was replenished on the third day of culture and 10U/ml of recombinant human IL-2 was added to purified CD4⁺ T cell cultures.

T cell proliferation analysis

PBMC and purified CD4⁺ lymphocytes were cultured as stated above. After 5 days of culture, cells were pulsed with 1 µCi [³H]-thymidine for 18 hours. The cells were harvested by using semi-automated cell harvester 96 (TOMTEC, Hamden, CT), and the amount of incorporated [³H]-thymidine was measured by using a Wallac MicroBeta liquid scintillation counter (Trilux, Turku, Finland). Data were expressed as the mean ± standard deviation of cpm from triplicate cultures. Percent inhibition is calculated as follows: 100% – [(Average cpm of core-treated, stimulated PBMCs/Average cpm of stimulated PBMCs)*100].

Cell death analysis

To determine HCV core cytotoxicity, PBMC and purified CD4⁺ T cells from healthy donors were cultured in the presence of 2.5ug/ml of HCV core antigen or the control protein β-gal in conjunction with anti-CD3/CD28 antibody stimulus. After 4 hours or 4 days of exposure to HCV core, cell death in PBMC cultures was determined by incorporation of 7AAD (BD Biosciences, San Jose, CA) per supplier instructions. Briefly, cells were washed twice in cold PBS, resuspended, and then 5 ul of 7AAD were added to each well. Cells were gently vortexed and incubated for 15 min at 25°C in the dark. Cells were analyzed immediately by flow cytometry. To determine cell death of purified CD4⁺ lymphocytes, after three days of culture, cells were incubated with LIVE/DEAD Fixable Dead Cell Stain Kit (Invitrogen) as per the manufacturer's instructions. Briefly, cultured cells were washed with PBS and resuspended in 100ul of PBS. Reconstituted fluorescent dye was added to suspensions at 0.2ul/well and mixed. Cells were incubated on ice for 30 minutes, then washed in PBS and fixed in 100ul of BD Lysis Solution (BD Biosciences).

Flow cytometry

To determine gC1qR expression on freshly thawed and activated T cells, 4 × 10⁵ PBMC were directly surface stained or surface stained after stimulation for 72 hours with anti-CD3/CD28 antibodies. The cells were washed in FACS buffer (PBS, 2% FBS and 0.1% Sodium Azide, NaN₃) and cells were blocked with 50µl of 10% normal donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA) for 10 min on ice. 50 µl of 2ug/ml rabbit anti-human gC1qR polyclonal antibody was added and cells were incubated for 30 min on ice. Cells were washed twice in FACS Buffer and resuspended in 100 µl of PE-conjugated AffiniPure F(ab')₂ Fragment Donkey Anti-Rabbit IgG (H+L) (Jackson ImmunoResearch Laboratories) and incubated on ice for 30 minutes. The cells were washed and blocked with 10% normal mouse serum (Jackson ImmunoResearch Laboratories) for 10 minutes on ice. The cells were stained in 100µl volume with the following antibodies: FITC anti-human CD45RO clone UCHL1, PE-Cy5 anti-human CD3 clone UCHT1, APC anti-human CD4 clone RPA-T4 (eBioscience), and APC-Cy5.5 anti-human CD8 clone 3B5 (Invitrogen) for 30 minutes on ice. The cells were washed twice in FACS buffer and fixed in 100ul of BD Lysis Solution (BD Biosciences). The cells were washed and resuspended in FACS Buffer. FMO controls for each

fluorochrome were used for gating purposes. Five-color multiparameter flow cytometry was performed using a BD FACSCanto instrument (BD Biosciences) compensated with single fluorochromes and analyzed using FlowJo (Tree Star, Inc., Ashland, OR).

Statistical analysis

Results of flow cytometric analysis are expressed as medians, while results of [³H]-thymidine incorporation are expressed as mean $\pm$ SD. The nonparametric, two-tailed Mann-Whitney U test was used to compare differences between patient groups and over time. The nonparametric, two-tailed Wilcoxon matched-pair signed-rank test was used to analyze individual matched pairs data. Correlation (r) between different parameters was determined using Spearman's rank test. Significance was defined as a *P* value of <0.05 . Prism 4.0c (GraphPad Software, San Diego, CA) statistical software was used.

RESULTS

HCV core inhibits the proliferative response of purified CD4⁺ T cells upon TCR stimulation

It has been previously demonstrated that HCV core is capable of modulating human T cell responses when cultured with PBMC following TCR stimulation (14). However, the direct effect of extracellular core on purified CD4⁺ T cells has yet to be determined. To examine the effect of extracellular core on inhibition of CD4⁺ T cell responses, PBMCs (Figure 1A, 5A, 5B, 5C) and purified CD4⁺ T cells (Figure 1B, 1C) from healthy donors were stimulated with anti-CD3/CD28 antibodies in the presence of HCV core protein or the control protein β -gal for 5 days. Following addition of [³H]-thymidine to the culture for 18 hours, CD4⁺ T cell proliferative responses were determined by measuring [³H]-thymidine incorporation. As shown in Figure 1B, CD4⁺ T cell proliferation is similar between TCR stimulus alone or stimulus in the presence of the control protein β -gal. In contrast, inhibition of T cell proliferation was observed in CD4⁺ T cells stimulated in the presence of HCV core (Figure 1B, 1C).

We next examined whether HCV core-mediated inhibition of T cell proliferation was due to the cytotoxicity of core protein. PBMC from healthy donors were treated with core or β -gal for 4 hours in the absence of stimulus (data not shown) or for 4 days in the presence of anti-CD3/CD28 antibodies (Figure 1D). The nucleic acid dye, 7AAD, was used to determine the frequency of cells with permeable membranes. Upon core exposure for 4 hours, PBMC from healthy donors had similar levels of 7AAD incorporation as β -gal-treated PBMC, while the positive control, UV-exposure, resulted in nearly 45% cell death (data not shown). After 4 days of exposure to HCV core protein, PBMC from healthy donors had similar levels of dead cells as β -gal-treated PBMC (Figure 1D). In addition, the analysis of potential cytotoxic effects of HCV core on CD4⁺ T cells revealed that there were similar levels of cell death of core-treated CD4⁺ T cells and β -gal-treated CD4⁺ T cells (Figure 1E). These data suggest that HCV core does not induce cell death and therefore the inhibition of PBMC and CD4⁺ T cell proliferative responses seen in Figure 1A, 1B, and 1C were not attributable to proliferation of fewer viable cells.

Higher level of circulating core protein is detectable in HCV patients who develop chronic infection

To determine the clinical relevance of HCV core-mediated impairment of CD4⁺ T cell responses during HCV infection, serum and PBMC samples were obtained from a cohort of acutely infected patients (n=20) (Table 1) and a group persistently infected for 5 years or more (long-term chronic) (n=10). For the acutely infected cohort, samples were collected at two time points; at the time of enrollment (Month 0) and approximately six months later (Month 6). Resolution or chronicity was determined at Month 6 and from here on this patient group will be referred to as acute-to-resolved and acute-to-chronic patients

To examine whether the level of circulating core protein may influence the outcome of HCV infection, we determined the serum core levels from individuals who resolved infection and individuals with chronic infection (Figure 2). Serum core levels were not determined for all individuals in the study or for each time point in the study; therefore, Figure 2 represents the serum core levels for acute-to-resolved (Resolve infection) and the serum core levels of both acute-to-chronic and long-term chronic patients (Chronic infection). Serum core levels were greater in individuals with chronic infection compared to individuals that resolve infection ($p=0.0354$) (Figure 2). This indicates that chronically infected patients have a greater amount of circulating core protein and thus may be more prone to core-mediated CD4⁺ T cell dysregulation than individuals that resolve infection.

The frequency of gC1qR⁺CD4⁺ T cells in the periphery of acute-to-chronic patients is higher than that in acute-to-resolved HCV patients

The C1q complement receptor, gC1qR, has been identified as a target protein for extracellular core (14). This HCV core/gC1qR interaction has been shown to deliver T cell inhibitory signaling (23). Given the crucial role of CD4⁺ T cells in resolution of HCV infection, we hypothesize that individuals that develop persistent infection may have a greater frequency of gC1qR⁺CD4⁺ T cells during the acute phase of infection, allowing core binding and increasing susceptibility to HCV core-mediated inhibition of CD4⁺ T cell responses. To test this possibility, we determined the frequency of gC1qR⁺CD4⁺ T cells in the periphery of the cohort of acutely infected patients described above, as well as, in the long-term chronic patients. PBMC from healthy donors, from acute-to-resolved and acute-to-chronic individuals, at both Month 0 and Month 6, and long-term chronic patients, were surface stained for CD3, CD4, CD8, CD45RO, and gC1qR expression. To determine the percent of gC1qR⁺CD4⁺ T cells, PBMC were gated on the CD3⁺ fraction, then separated into CD4⁺ and CD8⁺ populations. Representative gC1qR staining on CD4⁺ T cells at Month 0 for an acute-to-resolved and an acute-to-chronic patient are shown in Figure 3A, as well as frequencies for a healthy control.

When relative gC1qR expression (%gC1qR - %FMO) was examined at the time of enrollment (Month 0), HCV-infected subjects as a group had a greater percentage of circulating gC1qR⁺CD4⁺ T cells than healthy donors ($p=0.0444$) (Figure 3B). At Month 6, the time at which resolution or persistence was determined, acute-to-resolved individuals had fewer gC1qR⁺CD4⁺ T cells compared to at the time of enrollment ($p=0.0418$). In contrast, the frequency of gC1qR⁺CD4⁺ T cells was maintained in acute-to-chronic patients and was not significantly different at Month 0 and Month 6. Importantly, the frequency of gC1qR⁺CD4⁺ T cells in long-term chronic patients was greater than that in healthy individuals ($p=0.007$), acute-to-resolved individuals at Month 6 ($p=0.0068$) and acute-to-chronic individuals at Month 6 ($p=0.0220$). Additionally, gC1qR⁺CD4⁺ T cells were analyzed for expression of CD45RO, a marker of differentiation. The frequency of CD45RO⁺gC1qR⁺CD4⁺ T cells was similar among healthy and HCV infected donors at Month 0 or Month 6 (data not shown). However, long-term chronic patients had significantly greater frequencies of CD45RO⁺gC1qR⁺CD4⁺ T cells than healthy individuals or acute-to-resolved and acute-to-chronic at both Month 0 and Month 6 ($p=0.0041$, $p=0.0031$, $p=0.0010$, $p=0.0031$, $p=0.0057$ respectively) (data not shown). These results suggest that HCV infection increases the frequency of gC1qR⁺CD4⁺ T cells in the periphery and individuals with persistent infection maintain elevated levels of these cells.

Next we examined the relationship between gC1qR⁺CD4⁺ T cell frequency and viral load at Month 0, Month 6 and at all time points combined (Month 0, Month 6, and long-term chronic). There was not a significant correlation between viral load and frequency of gC1qR⁺CD4⁺ T cells in the periphery at Month 0, Month 6, and at all time points ($r=-0.3427/p=0.2756$; $r=0.1000/p=0.8100$; $r=0.0592/p=0.7692$ respectively). When the relationship between gC1qR⁺CD4⁺ T cell frequency and serum core levels was evaluated at Month 0, Month 6 and

at all time points, it was determined that there was no significant correlation for the frequency of gC1qR⁺CD4⁺ T cells with the level of circulating core ($r=-0.6429/p=0.1389$; $r=0.3364/p=0.3132$; $r=0.1832/p=0.4267$ respectively). However, long-term chronic patients appear to maintain high levels of core in their circulation in conjunction with high frequencies of gC1qR⁺CD4⁺ T cells. This trend requires further analysis to determine significance. Notably, circulating core protein is detectable late in HCV infection (Figure 2), suggesting that extracellular core protein is available during late phase HCV infection and might be involved in maintaining the impairment of CD4⁺ T cell function.

The frequency of gC1qR⁺CD4⁺ T cells is increased following T cell activation in both acute-to-chronic and acute-to-resolved individuals

It is possible that the frequency of gC1qR⁺CD4⁺ T cells present in the periphery increases upon infection as a result of TCR stimulus. To address induction of gC1qR expression following T cell activation, PBMC were stimulated with anti-CD3/CD28 antibodies for 3 days. At Month 0, the frequency of gC1qR⁺CD4⁺ T cells in acute-to-resolved (Figure 4A) and acute-to-chronic (Figure 4B) individuals did not significantly increase upon stimulation. However at Month 6, both acute-to-resolved (Figure 4C) and acute-to-chronic (Figure 4D) individuals responded to stimulus with significantly increased frequencies of gC1qR⁺CD4⁺ T cells ($p=0.0312$, $p=0.0312$ respectively). Additionally, the frequency of gC1qR⁺CD4⁺ T cells from long-term chronic patients increased upon stimulation ($p=0.0020$) (Figure 4E). These data suggest that at Month 0, when viral RNA is present in all individuals, PBMC are in a stimulatory environment and additional TCR stimulus does not significantly increase the frequency of gC1qR⁺CD4⁺ T cells. Additionally, it appears that at Month 6 and beyond, PBMC respond to TCR stimulus with significantly elevated frequencies of gC1qR⁺CD4⁺ T cells.

PBMC from acute-to-resolved and acute-to-chronic HCV patients are susceptible to HCV core-mediated inhibition of T cell proliferation

To determine whether PBMC from acute-to-resolved and acute-to-chronic individuals have differential responses to extracellular HCV core, the percent inhibition of T cell proliferation was determined for each time point. At Month 0, both acute-to-resolved and acute-to-chronic individuals had significantly greater inhibition of proliferation when exposed to HCV core protein compared to β -gal protein (Figure 5A). The same pattern was seen at Month 6 (Figure 5B), even though at this time point acute-to-resolved individuals had significantly fewer gC1qR⁺CD4⁺ T cells than during the acute phase of infection (Figure 3B). Long-term chronic patients also had significant inhibition of proliferation upon core-treatment compared to control-treated ($p=0.0312$) (Figure 5C). To determine if the capacity to respond to HCV core protein alters over time, individual matched pairs were assessed at Month 0 and Month 6 for acute-to-resolved (Figure 5D) and acute-to-chronic (Figure 5E) individuals. Capacity of HCV core to inhibit T cell proliferation was not significantly different at Month 0 and Month 6 for either acute-to-resolved ($p=0.2188$) or acute-to-chronic patients ($p=0.1641$). These data suggest that upon stimulation, PBMC from HCV infected individuals responded to extracellular core by suppression of proliferation, irrespective of time point of infection or frequency of gC1qR⁺CD4⁺ T cells.

DISCUSSION

Resolution of HCV infection is associated with strong and multi-specific CD4⁺ T cell responses. In persistent HCV infection, the function of CD4⁺ T cells is impaired and the mechanism responsible for CD4⁺ T cell dysregulation remains to be elucidated. HCV core has been detected circulating in the bloodstream of HCV patients (24–26). Extracellular core binds to gC1qR and is capable of inducing T cell unresponsiveness, such as inhibition of proliferation and effector cytokine production in PBMC cultures (14,15,27). However, it had not been

determined whether HCV core-mediated inhibition of T cell responsiveness might contribute the outcome of HCV infection following acute HCV infection. By employing a unique cohort of acute HCV patients who resolved or failed to clear infection, we investigated if the core-binding receptor, gC1qR, on the surface of CD4⁺ T cells, is a predictor of virologic outcome. In this report, we demonstrate that extracellular core inhibits CD4⁺ T cell proliferative responses to TCR stimulation. Notably, a higher amount of circulating core protein is detectable in HCV patients with acute-to-chronic infection than acute-to-resolved HCV patients. In addition, the circulating frequency of gC1qR⁺CD4⁺ T cells that increased upon HCV infection was sustained in acute-to-chronic individuals compared to acute-to-resolved individuals. This suggests that there is more available circulating core and a higher frequency of CD4⁺ T cells expressing the core-binding receptor in acute-to-chronic individuals and thus, these individuals may be prone to core-mediated dysregulation and establishment of persistent infection.

During the course of infection in resolved individuals, viremia is controlled, while viremia is not controlled in individuals with persistent infection. High levels of circulating core protein in the periphery (16), and possibly in the liver (28), may promote the CD4⁺ T cell dysregulation seen in chronic individuals. We found that chronically infected patients had higher serum core levels than individuals that resolved infection. In chronically infected individuals, this extracellular core may be capable of inhibiting the proliferation of CD4⁺ T cells and possibly limits the expansion of HCV-specific CD4⁺ T cells. We expected that detection of core might be difficult due to formation of complexes with anti-core antibody, which is detectable in resolved and persistent patients (29). Interestingly, extracellular core was detectable both at Month 6 and after 5 years of persistent infection, which could be explained by the presence of a limited amount of anti-core antibody during the chronic phase of HCV infection, possibly due to lack of CD4⁺ T cell help. Thus, core protein circulating during late phase of infection might be responsible for impairing CD4⁺ T cell function as observed in chronic HCV patients. It will be important to use the chimpanzee model to evaluate the impact of core-mediated impairment of gC1qR⁺CD4⁺ T cell function in the liver, where core is readily detected. Additionally, we would like to continue our studies by evaluating intrahepatic gC1qR⁺CD4⁺ T cells from chronic HCV patients.

Another crucial factor for the contribution of HCV core-mediated inhibition of T cell responses could be the availability of its target protein, gC1qR, on CD4⁺ T cells from HCV patients. During the acute phase of infection, HCV infected individuals show higher levels of gC1qR⁺CD4⁺ T cells relative to healthy individuals and this is irrespective of whether the HCV subjects develop persistence or resolve spontaneously. However, at the later time point (Month 6), when persistence or recovery was determined, individuals with persistent infection maintained the level of gC1qR⁺CD4⁺ T cells seen during the acute time point while individuals that resolved infection had significantly lower frequencies of gC1qR⁺CD4⁺ T cells. This suggests that individuals that resolved infection have fewer cells available that can be dysregulated by circulating HCV core protein. Individuals with persistent infection maintain a greater frequency of gC1qR⁺CD4⁺ T cells, and therefore have more cells available that can be dysregulated by circulating HCV core protein. These data are remarkably in keeping with an independent analysis of Tregs in acute HCV infection from the same cohort of patients as used in this study (30). Specifically, 7 of the 15 acute-to-chronic patient samples were used in both studies as well as 6 of the 12 acute-to-resolved patients. As in our study, both acute HCV groups had higher circulating frequencies of FoxP3⁺ Treg cells than did healthy controls. Furthermore, the frequency of FoxP3⁺CD4⁺ T cells and their suppressive function were greater in acute HCV patients than in healthy controls. However, there was no difference of Treg activity at Month 0 between acute-to-resolved and acute-to-chronic HCV patients. Notably, at Month 6, when there is a loss of gC1qR⁺CD4⁺ T cells in acute-to-resolved patients, there is a loss of functional suppression by Treg in acute-to-resolved individuals (30). Interestingly, the elevation of gC1qR expression in chronic HCV patients could be due to the sustained

inflammatory responses. As such, one would expect to detect a higher frequency of gC1qR⁺CD4⁺ T cells in other chronic inflammatory diseases. However, we believe the dysfunction found in this study is HCV-specific due to the presence of the gC1qR-binding protein, core, which actively engages gC1qR, resulting in CD4⁺ T cell unresponsiveness. Therefore the presence of gC1qR⁺CD4⁺ T cells in other chronic diseases would not lead to CD4⁺ T cell dysfunction without the additional presence of gC1qR-binding proteins.

It is worthwhile to point out that an immunoregulatory function of the complement system has recently been elucidated in addition to the important role of complement and its receptors during early defense against viral infection. The complement receptors are expressed on both antigen presenting cells (APC) and T cells. Conceivably, the regulation of host immune responses by complement receptor could occur at two levels: 1) modulation of APC function leading to regulation of CD4⁺ T cell responses or 2) direct effect on CD4⁺ T cell function. Our recent studies demonstrate that the binding of extracellular core to gC1qR displayed on dendritic cells suppresses the production of TLR-induced IL-12 (32,33). The suppression of IL-12 by core-treated DCs inhibits IFN- γ production by CD4⁺ T cells, limits T_H1 differentiation and skews towards T_H2 responses. However, core-treated DCs do not affect CD4⁺ T cell proliferative expansion. This is quite a contrast to the observation in this report that extracellular core profoundly inhibited the proliferation of CD4⁺ T cells in response to TCR stimulation. It suggests that complement-mediated immunoregulation exhibits two distinct roles in regulating APC function and T cell responses. Studies are on going to determine gC1qR expression on APC from early HCV infected individuals and determination of effect of core on these cells. Because of lower expression of gC1qR on T cells compared to APC, a regulatory role of complement receptor in T cell responses has not been well recognized. However, the expression gC1qR and a complement regulatory protein (i.e. CD46) are upregulated upon T cell activation (34). Importantly, costimulation of CD4⁺ T cells with antibodies to CD3 and CD46 induced a regulatory T_R1-like phenotype, with marked production of IL-10 (34). We are currently characterizing the activation status and alteration of CD4⁺ T cell function upon exposure to extracellular core and examining whether core-treated CD4⁺ T cells exhibit regulatory function on bystander effector T cells.

In summary, we examined whether the level of gC1qR expression on CD4⁺ T cells may influence the outcome of HCV infection by analyzing the ex vivo frequency of gC1qR⁺CD4⁺ T cells on PBMC from both chronic and resolved HCV patients. Our studies revealed that HCV infection increases the frequency of gC1qR⁺CD4⁺ T cells. Importantly, individuals with persistent infection maintain this frequency at late phase of infection, while individuals that resolve HCV infection do not. This suggests that CD4⁺ T cells from chronic HCV patients might be more susceptible to HCV core-mediated immune dysregulation than resolved HCV patients. It implies that agent(s) to deplete circulating HCV core protein in the bloodstream of HCV patients might help to improve host immune responses against HCV infection, perhaps increasing the likelihood of recovery. Additionally, our studies will be further strengthened by evaluating intrahepatic gC1qR⁺CD4⁺ T cell frequency in a larger cohort that would also allow us to analyze the impact of core-mediated dysregulation on CD4⁺ central and effector memory subsets.

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Abbreviations

Ab

antibody

FBS

Fetal bovine serum

FMO

fluorescence minus one

PBMC

peripheral blood mononuclear cell

TCR

T cell receptor

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Figure 1. Extracellular core inhibits proliferation of purified CD4⁺ T cells

A) PBMC and B) purified CD4⁺ T cells from representative healthy donors were stimulated with anti-CD3/CD28 antibodies in the presence or absence of core or control protein β -gal for 5 days. Proliferation was determined by incorporation of [³H]-thymidine after 18 hours of culture. * indicates p-value significance is $p < 0.05$. C) Percent inhibition of proliferation of CD4⁺ T cells from 5 healthy donors. Mann-Whitney analysis. P-value significance is $p < 0.05$. D) PBMC cell death was determined by staining cells with 7AAD after 4 days of culture with anti-CD3/CD28 antibodies in the presence or absence of core or control protein β -gal. E) CD4⁺ T cell death after HCV core-treatment or β -gal-treatment for 3 days was determined by staining with LIVE/DEAD dye. Representative of 5 healthy donors. PHA-treated cells acted as positive controls for cell death.

Figure 2. HCV patient serum core levels

Serum core levels of individuals that resolve infection and that fail to resolve infection determined at Month 6, including long-term chronic patients. Horizontal bar indicates median. Mann-Whitney analysis. P-value significance is $p < 0.05$.

Figure 3. Differential expression of gC1qR on CD4⁺ T cells of individuals that resolve HCV infection and those that become chronically infected

A) Representative gC1qR staining of PBMC from a healthy donor, an acute-to-resolved patient (Month 0) and an acute-to-chronic patient (Month 0) after gating down to the CD4⁺CD3⁺ lymphocytes. B) Compiled relative gC1qR expression (%gC1qR⁺ -%FMO) on CD4⁺ T cells from healthy controls, acutely infected individuals (Month 0) and those same individuals roughly six months later (Month 6), at which time chronicity or resolution were determined. Horizontal bar indicates median. Mann-Whitney analysis. P-value significance is p<0.05.

Figure 4. Activation of cells increases the frequency of gC1qR⁺CD4⁺ T cells

A) Matched pair analysis of the frequency of gC1qR⁺CD4⁺ T cells before and after stimulation of PBMC from A) acute-to-resolved patients (n=8) and B) acute-to-chronic patients (n=9) at Month 0. Matched pair analysis of the frequency of gC1qR⁺CD4⁺ T cells before and after stimulation of PBMC from C) acute-to-resolved (n=6) patients and D) acute-to-chronic patients (n=7) at Month 6. Matched pair analysis of the frequency of gC1qR⁺CD4⁺ T cells, before and after stimulation of PBMC, from E) long-term chronic patients (n=10). Wilcoxon analysis. P-value significance is p<0.05.

Figure 5. Suppressive response to HCV core in acute-to-resolved and acute-to-chronic individuals
 PBMC of acute-to-resolved and acute-to-chronic patients were stimulated with anti-CD3/CD28 antibodies for 5 days in the presence or absence of HCV core antigen or β -gal control protein. [3 H]-thymidine was added to wells on the fifth day of culture and incubated for an additional 18 hours before cell harvest. A) Percent inhibition of proliferation of healthy donors and infected patients at time of enrollment (Month 0). Mann-Whitney analysis. P-value significance is $p<0.05$. B) Percent inhibition of proliferation of healthy donors and infected patients at Month 6. C) Percent inhibition of proliferation of healthy donors and long-term chronic patients. D) Matched pairs analysis of change in inhibition of proliferation from Month 0 to Month 6 in acute-to-resolved patients ($n=7$). Wilcoxon analysis. P-value significance is

p<0.05. E) Matched pairs analysis of change in inhibition of proliferation from month 0 to month 6 in acute-to-chronic patients (n=9).

Table 1
Demographic and clinical details of the acutely HCV-infected group

C/R	Donor	Month	Gtype	Risk Factor	Age	M/F	Ethnicity	Peak ALT level	M0 HCV RNA level (IU/ml)	M6 HCV RNA level (IU/ml)
C	HS102 HS102	0 9	1b	IDU	45	M	CA	682	619,756	104,551
C	HS111 HS111	0 6	1a	IDU	20	M	CA	867	52,816	2,526,131
C	HS116 HS116	0 6	2b	IDU	24	M	CA	603	171,992	>7,692,316
C	HS124	0	1b	IDU	19	F	AA	248	52,159	137,089
C	PD106 PD106	0 6	1	IDU	45	M	CA	247	883,660	2,537,333
C	PD109 PD109	0 6	3a	Sexual	50	M	CA	468	173,432	900,963
C	TN103 TN103	0 6	1a	IDU	26	M	CA	167	6,519,507	392,303
C	TN104 TN104	0 6	1b	Sexual	47	M	AA	201	607,981	806,128
C	TN105 TN105	0 6	1a	Sex/Unk	30	M	AA	208	323,466	6,147
C	TN108 TN108	0 6	1a	Unknown	48	M	CA	68	1,145,664	1,445,738
R	HS104 HS104	0 6	1b	Needle stick	53	F	CA	3,190	5,318,925	<615
R	HS105	0	unable	IDU	21	F	CA	n/a	<615	n/a
R	HS112 HS112	0 6	1a	IDU	24	M	CA	950	199,414	<615
R	HS114 HS114	0 9	2	IDU	17	F	CA	560	<615	<615
R	HS126	0	1b	Unknown	22	M	CA	558	615	N/A
R	PD102 PD102	0 6	1a	Surgery	52	F	CA	292	<615	<615
R	PD103* PD103	0 6	1a	IDU	27	F	CA	1,461	<615	<615
R	PD105	2	ST1	IDU	35	F	CA	58	3,358	<615
R	TN106 TN106	0 6	1a	IDU	48	F	CA	35	5,185,599	<615
R	TN107 TN107	2 6	unable	Tattoo	38	F	CA	15	<615	<615

C/R	Donor	Month	Gtype	Risk Factor	Age	M/F	Ethnicity	Peak ALT level	M0 HCV RNA level (IU/ml)	M6 HCV RNA level (IU/ml)
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C, chronic; R, resolved; Gtype, genotype; ST, serotype; IDU, intravenous drug use; M, male; F, female; AA, African American; C/A, Caucasian;; ALT, alanine aminotransferase; N/A, not available

* Positive RNA at month 2