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Phyllis T. Losikoff,¹ Sasmita Mishra,¹ Frances Terry,² Andres Gutierrez,³ Matt T. Ardito,² Loren Fast,¹,³ Martha Nevola,¹ William D. Martin,² Chris Bailey-Kellogg,⁴ Anne S. De Groot²,³ and Stephen H. Gregory¹

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2 Tables and 4 Figures; Supplemental Material: 3 Figures, 1 Table

Abbreviations. HCV, hepatitis C virus; T_{reg} cells, regulatory T cells; FoxP3, forkhead box P3; nT_{reg} cells, natural T_{reg} cells; iT_{reg} inducible T_{reg} cells; BLAST, Basic Local Alignment Search Tool; T_{eff} cells, effector T cells; AbVL, antibody/viral load; ICS, immunogenic consensus sequence; TcR, T cell receptor; PBMCs, peripheral blood mononuclear cells; CFSE carboxyfluorescein diacetate, succinimidyl ester.

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ABSTRACT

Background & Aims: Spontaneous resolution of hepatitis C virus (HCV) infections depends upon a broad T cell response to multiple viral epitopes. Most patients fail to clear infections spontaneously, however, and develop chronic disease. The elevated number and function of CD3⁺CD4⁺CD25⁺FoxP3⁺ regulatory T(reg) cells in HCV-infected patients suggest the role of T(reg) cells in impaired viral clearance. The factors contributing to increased T(reg) cell activity in chronic hepatitis C cases remain to be delineated.

Methods: Immunoinformatics tools were used to predict promiscuous, highly-conserved HLA-DRB1-restricted immunogenic consensus sequences (ICS), each composed of multiple T cell epitopes. These sequences were synthesized and added to cultures of peripheral blood mononuclear cells (PBMCs) derived from patients who resolved HCV infection spontaneously, patients with persistent infection, and non-infected individuals. The cells were collected following 5 days incubation, quantified and characterized by flow cytometry.

Results: One ICS, HCV_G1_p7_794, induced a marked increase in T(reg) cells in PBMC cultures derived from infected patients, but not patients who spontaneously cleared HCV or non-infected individuals. An analogous human peptide (p7_794), on the other hand, induced a significant increase in T(reg) cells among PBMCs derived from both HCV infected and non-infected individuals. JanusMatrix analyses determined that HCV_G1_p7_794 is comprised of T(reg) cell epitopes that exhibit extensive cross-reactivity with the human proteome.

Conclusion: A virus-encoded peptide (HCV_G1_p7_794) with extensive human homology activates cross-reactive CD3⁺CD4⁺CD25⁺FoxP3⁺ nT(reg) cells, which potentially contribute to immunosuppression and chronic hepatitis C.

Keywords: hepatitis C; regulatory T cells; cross-reactive; epitope
Introduction

Chronic hepatitis C virus (HCV) infection is a major public health concern worldwide. It is the leading cause of liver failure and reason for liver transplant in the US. Irrespective of clinical outcome, acute HCV infections are characterized by broad HCV-specific T cell responses that correlate with spontaneous viral clearance in a minority of individuals [1]. In most patients, however, this initial response fails to contain the virus and chronic disease results. Increased numbers of CD4⁺ regulatory T₉₉ cells circulating in the bloodstream and accumulating in the liver have been implicated in the pathogenesis of chronic hepatitis C [2,3].

T₉₉ cells constitute one of the major mechanisms underlying immunological homeostasis and self-tolerance [4]. In addition, T₉₉ cells play a key role in moderating the immune response to infectious diseases, suppressing host tissue and organ damage that would occur in the absence of regulation [5]. Although key to maintaining immune homeostasis, a growing body of evidence suggests that T₉₉ cells also contribute to the establishment and persistence of chronic infections, e.g., HCV [6,7]. While no single marker exists, human T₉₉ cells are classically identified by expression of the transcription factor, forkhead box P3 (FoxP3), and the cell surface expression of the interleukin (IL)-2 receptor α chain (CD25).

Two distinct T₉₉ cell subsets are described in the literature: natural (n)T₉₉ cells specific for self epitopes and generated by high-avidity selection in the thymus, and inducible (i)T₉₉ cells that derive from conventional (CD4⁺CD25⁻FoxP3⁻) T cells following stimulation in the periphery [8]. nT₉₉ cells can induce the conversion of conventional T cells to iT₉₉ cells via cytokine-dependent and -independent mechanisms, a process called infectious tolerance [9,10]. Notably, the factors that affect expansion of the T₉₉ cell population in cases of chronic hepatitis C remain to be fully delineated. Nonetheless, the consensus supports the heterogeneous nature of the expanded T₉₉ cell population composed of both nT₉₉ and iT₉₉ cell subsets [11].
Here, we describe a unique viral peptide derived from HCV p7 protein (HCV_G1_p7_794) that promotes a T_{reg} cell response among PBMCs derived from patients with persistent HCV infection. This peptide exhibited human homology when evaluated using GenBank Basic Local Alignment Search Tool (BLAST) [12]. Further analysis using a new bioinformatics tool, JanusMatrix [13,14], demonstrated that this HCV peptide cross-reacts with HLA matched peptide sequences located within hundreds of human proteins. Our data suggest that HCV_G1_p7_794 engages preexisting nT_{reg} cells as a consequence of this homology, induces infectious tolerance and the expansion of an iT_{reg} cell population, which contributes to suppression of effector T_{eff} cell activity in cases of chronic HCV infection.
Materials and Methods

Subjects

HCV-seropositive subjects with persistent viremia (Ab*VL*), patients who spontaneously resolved infection (Ab*VL*) and HCV-seronegative, non-infected (Ab\'VL\') individuals were recruited from the Rhode Island Adult Corrections Institution to participate in this study. Patients with serological evidence of co-infection with hepatitis B virus or human immunodeficiency virus, reported history of any other immunocompromising condition or prior treatment for HCV were not included. Details of recruitment and the population are described elsewhere [15]. PBMC obtained from subjects were cryopreserved for use in the investigations presented herein. The Institutional Review Boards of the Miriam Hospital, Rhode Island Department of Corrections and Office of Human Research Protection approved this study. All HCV infected subjects were infected with HCV genotype 1a or 1b (Versant HCV genotype assay2, Siemens Healthcare Diagnostics Inc.). Hartford Hospital Transplantation Research Laboratory (Hartford, CT) performed HLA typing. Demographic and serologic data are shown in Table 1.

JanusMatrix analysis

Crystal structure analyses of ternary, MHC:epitope:T cell receptor (TcR) complexes indicate that certain amino acid residues of a T cell epitope contact the MHC molecule while other residues contact the TcR [16]. JanusMatrix, a new bioinformatics tool, interrogates a potential T cell epitope from both its HLA-binding and TcR-facing aspects, and assesses TcR cross-reactivity with T cell epitopes encoded by other genomes [13,14]. Those epitopes from two different genomic sources, e.g., HCV and human, that bind the same HLA molecules and present identical amino acids to the TcR are designated potentially cross-reactive, capable of stimulating the same TcR and triggering the same T cells to respond. In the analysis undertaken here, JanusMatrix divided the HCV HLA-DRB1-restricted epitopes (comprising the ICS described above) into TcR-facing and MHC-binding amino
acid residues. The human protein database (UniProtKB) was then searched for TcR-facing epitopes that cross-react with the HCV epitopes.

**HLA-DRB1-restricted epitope selection, peripheral blood mononuclear cell (PBMC) culture, flow cytometric and statistical analyses.**

Detailed descriptions are provided in Supplementary Materials.
Results

A peptide sequence in HCV p7 exhibits human homology

Sustained, virus-specific CD4 and CD8 T cell responses are associated with successful control of HCV infection. Therapeutic vaccination offers a rational approach to stimulating host resistance and overcoming viral persistence in cases of chronic disease. Toward this end, 20 promiscuous HCV (genotype 1) ICS, each predicted to contain multiple T cell epitopes, were synthesized and validated by demonstrating their ability to bind a panel of the eight common alleles HLA-DRB1 alleles: DRB1*0101, *0301, *0401, *0701, *0801, *1101, *1301, and *1501, representing essentially the entire human population [17,18]. One highly conserved ICS, found in >90% genotypes 1a and 1b isolates and located within HCV p7 protein (HCV_G1_p7_794; WPLLLLLALPQRAYAQ), exhibited significant human homology (>70% shared identities) determined by GenBank BLAST analysis; none of the remaining ICS exhibited the same homology.

HCV_G1_p7_794 induces a CD3+CD4+FoxP3+ T cell response in HCV-infected patients

A series of experiments was undertaken to determine and compare the HCV_G1_p7_794-specific responses of PBMCs obtained from non-infected control individuals (Ab−VL−), patients who spontaneously cleared HCV infection (Ab−VL+) and infected patients in whom viremia persisted (Ab+VL+). HCV_G1_p7_794 induced a marked increase in CD3+CD4+FoxP3+ cells when added to PBMC cultures derived from infected, Ab+VL+ patients (Figure 1A). In contrast, HCV_NS4B_1941 (AARVTQILSSLTITQLKLHQWI; an ICS that exhibits little human homology) failed to promote a comparable increase. Similarly, other highly conserved HCV ICS with negligible human homology (e.g., HCV_G1_NS4b_1769; ISGIQLAGLSTLPGNPA) failed to elicit a CD3+CD4+FoxP3+ cell response (data not shown). CD3+CD4+FoxP3+ cells, induced by the addition of HCV_G1_p7_794 to Ab+VL+ PBMC cultures, expressed both CD25 (IL-2 receptor α chain, constitutently expressed by T\text{reg} cells) and CD39, a cell ectonucleotidase associated with T\text{reg} cell function (Figure 1B) [6,19].
While a significant increase in CD3^+CD4^+FoxP3^+ cells was determined in PBMC cultures derived from HCV-infected patients, neither HCV_G1_p7_794 nor the control peptide (HCV_G1_NSBU_1941) added to PBMC cultures derived from non-infected individuals or from patients who spontaneously cleared HCV infection induced an increase (Figure 2A). Notably, Ab^"VL"^+ patients had a higher baseline level of CD3^+CD4^+Foxp3^+ cells compared to clearers or non-infected controls, a finding consistent with the literature [20].

**HCV_G1_p7_794 activates cross-reactive nT_reg cells**

A human analog of HCV_G1_p7_794 (p7_794, PLLLLLLSLPPRA), identified by GenBank BLAST analysis, was synthesized in an effort to provide a clearer understanding of the nature of the T_reg cells that respond to HCV_G1_p7_794. Like the HCV-encoded homolog, the human analog induced a significant increase in CD3^+CD4^+FoxP3^+ cells in PBMC cultures derived from patients with persistent viremia (Figure 2B). In contrast to HCV_G1_p7_794, however, the human analog also induced an approximate three-fold increase in CD3^+CD4^+FoxP3^+ cells in PBMC cultures derived from non-infected (Ab^-VL^-) individuals, indicative of the capacity to elicit an nT_reg cell response.

**HCV_G1_p7_794 suppresses the mitogenic response of T cells to anti-CD3 and IL-2 treatment**

The hallmark of CD3^+CD4^+FoxP3^+ T_reg cells is suppressor activity. The addition of HCV_G1_p7_794 to Ab^-VL^- PBMC cultures resulted in a modest, albeit reproducible, decrease in the proliferative response to anti-CD3 monoclonal antibody treatment assessed in terms of a loss in CFSE staining intensity (Figure 3A). Similar results were obtained when [³H]-thymidine incorporation was used to monitor cell proliferation, i.e., reduced proliferation in response to either anti-CD3 or IL-2 in those cultures that contained HCV_G1_p7_794 (Figure 3B). Importantly, the addition of HCV_G1_p7_794 alone (in the absence of anti-CD3 or IL-2) had no effect on the proliferation of CD3^+CD4^+ T cells obtained from these same patients. This latter finding indicates that HCV_G1_p7_794 induces the conversion of conventional CD3^+CD4^+FoxP3^- T cells to CD3^+CD4^+FoxP3^+ iT_reg cells (i.e., infectious
tolerance) rather than stimulates the proliferation of T\textsubscript{reg} cells already present. An increase in CD3\textsuperscript{+}CD4\textsuperscript{+}FoxP3\textsuperscript{+} T\textsubscript{reg} cells that lack cell-surface neuropilin-1 supports this suggestion.

CD304 (neuropilin-1) is expressed by a subset of FoxP3\textsuperscript{+} T\textsubscript{reg} cells in humans [21]. In mice, CD304 expression differentiates natural (CD304\textsuperscript{+}), from inducible (CD304\textsuperscript{-}), T\textsubscript{reg} cells [22]. While a similar distinction has yet to be reported in humans, it is pertinent to note that the bulk of CD3\textsuperscript{+}CD4\textsuperscript{+}FoxP3\textsuperscript{+} cells contained among Ab\textsuperscript{+}VL\textsuperscript{+} PBMCs cultured in the absence of HCV\_G1\_p7\_794 expressed CD304 indicative of nT\textsubscript{reg} cells (Figure 4A). In contrast, the vast majority of CD3\textsuperscript{+}CD4\textsuperscript{+}FoxP3\textsuperscript{+} cells among Ab\textsuperscript{+}VL\textsuperscript{+} PBMCs cultured in the presence of HCV\_G1\_p7\_794 were CD304\textsuperscript{-} characteristic of iT\textsubscript{reg} cells (Figure 4B). Taken together, these findings support our speculation that HCV\_G1\_p7\_794 recognition by nT\textsubscript{reg} cells following HCV exposure promotes infectious tolerance and the production of FoxP3\textsuperscript{+} iT\textsubscript{reg} cells from conventional CD3\textsuperscript{+}CD4\textsuperscript{+}FoxP3\textsuperscript{-} T cells. This contention is supported by experiments demonstrating the failure of HCV\_G1\_p7\_794 to promote an increase in CD3\textsuperscript{+}CD4\textsuperscript{+}FoxP3\textsuperscript{+} cells among Ab\textsuperscript{+}VL\textsuperscript{+} PBMCs depleted of constitutive, CD25-expressing (nT\textsubscript{reg}) cells prior to culture (Supplementary Figure 1).

**JanusMatrix confirms T\textsubscript{reg} cell epitopes shared by HCV\_G1\_p7\_794 and the human proteome**

By comparing HCV\_G1\_p7\_794 with peptide sequences found in the human proteome, JanusMatrix analysis provided further insight into the capacity of HCV\_G1\_p7\_794 to induce a T\textsubscript{reg} cell response by PBMCs derived from HCV-infected patients. As illustrated in Table 2, HCV\_G1\_p7\_794 contains five T cell epitopes, which cross-react with 152 putative human T cell epitopes contained in 264 different human proteins. Similarly, the human analog, p7\_794, cross-reacts with putative T cell epitopes located within several hundred human proteins. In contrast, neither HCV\_G1\_NS4b\_1941 (control ICS often used in the ex vivo experiments described above) nor any of the 18 other putative ICS, validated by their ability to elicit immune recognition and IFN-\textgamma production by naïve human T cells ex vivo [17], exhibited significant cross-reactivity with the human proteome. Moreover, HCV\_G1\_p7\_794 induced substantially fewer naïve T cells to produce IFN-\textgamma assessed by ELISpot
assay, i.e., 64 + 75 versus 655 + 591 (mean ± SD number of spots/10^6 T cells, n=8) for HCV_G1_p7_794 and HCV_G1_NS4b, respectively; values are significantly different (p=0.021; Mann-Whitney rank sum test). The relative inability to induce IFN-γ production supports our speculation that, rather than activate T_{eff} cells, HCV_G1_p7_794 activates a cross-reactive nT_{reg} cell population that normally functions to suppress autoimmune responses to a large number of human proteins, which contain a common peptide sequence (epitope).
Resolution of primary HCV infections is dependent upon the vigorous response of CD4+ and CD8+ T cells to multiple viral epitopes [23]. HCV persists in the majority of infected patients, however, by modifying and/or evading the host immune response. Purportedly, a variety of factors contribute to the diminished T cell responses observed in chronically infected patients including: viral mutation and escape linked to both CD4 and CD8 T cell failure, CD4 T cell anergy, CD8 T cell exhaustion, impaired dendritic cell function, and Treg cell-mediated suppression [2,3,6,7,24,25]. The increased frequency of Treg cells found in the liver and circulating in the peripheral blood of chronically-infected patients, confirmed in the current study (Figure 2), provided an initial indication of the role of Treg cells in the pathogenesis of chronic hepatitis C [2,3,5,11]. It remained unclear until recently, however, whether this increase represented the HCV epitope-specific response of Treg cells or the nonspecific consequence of chronic inflammation and liver disease [7].

The ability of HCV-derived epitopes to stimulate Treg cell responses is well documented; epitopes associated with both structural and non-structural HCV proteins have been reported [20,26]. Using HLA class II-peptide tetramer complexes, other investigators quantified and characterized the response of Treg cell specific for single HCV epitopes [20,26]. The study described herein is the first, however, to identify a promiscuous HCV peptide sequence (HCV_G1_p7_794) that exhibits extensive human homology and the ability to elicit a Treg cell response in a wide, HLA-diverse human population, such as the infected patient population shown in Table I. Unlike the Treg cell epitopes described previously, HCV_G1_p7_794 is an ICS composed of multiple HLA binding motifs able to bind a number of DRB1 alleles. Unfortunately, this precludes construction of an HLA-peptide tetramer complex and quantitation of the HCV_G1_p7_794-responsive cells. In accord with the literature, HCV_G1_p7_794 added to PBMCs cultures derived from HCV-infected patients, but not non-infected individuals or patients who cleared infection, induced a marked increase in CD3+CD4+FoxP3+ cells [20,26]. In addition to expressing CD25, characteristic of Treg cells, the vast majority of these cells
expressed CD39, a marker that distinguishes FoxP3+ T_{reg} cells from activated T_{eff} cells that transiently express FoxP3 [6,19].

In contrast to HCV_G1_p7_794, the human peptide analog (p7_794) elicited a significant increase in CD3+CD4+FoxP3+ cells in PBMC cultures derived from non-infected individuals with no evidence of prior HCV exposure, as well as HCV-infected patients. This finding is congruent with the suggestion that viral epitopes with human homology influence the pathogenesis of chronic HCV by activating preexisting, cross-reactive nT_{reg} cells [11]. Indeed, extensive homology between the HCV polyprotein and proteins that comprise human proteome is well documented [27,28]. JanusMatrix, a bioinformatics algorithm that interrogates potential T cell epitopes from both their HLA-binding and TcR-facing aspects, confirmed the existence of significant homology between HCV_G1_p7_794 and proteins that compose the human proteome. The results of this analysis demonstrate the potential efficacy of JanusMatrix in identifying pathogen-encoded epitopes that elicit the activity of nT_{reg} cells, which normally function to suppress autoimmune reactivity to self-antigens (proteins). In this regard, it is pertinent to remark that HCV_G1_p7_794 is comprised of epitopes that are homologous to those found in hundreds of human proteins. This suggests the autoimmune response to a large number of proteins is inhibited by a single or limited number of nT_{reg} cell clones responsive to a common peptide sequence, rather than a large number of clones each responsive to a unique sequence in a single protein only. The oligoclonality of the T_{reg} cell response to HCV_G1_p7_794 and its human analog is a matter of ongoing investigation.

The majority of individuals in our patient population were infected with HCV genotype 1, the derivation of HCV_G1_p7_794. The addition of HCV_G1_p7_794 to PBMC cultures derived from a single HCV genotype 3-infected patient, however, also resulted in a marked increase in CD3+CD4+FoxP3+CD25+ cells (Supplementary Figure 2). It is pertinent to note, in this regard, that HCV genotype 3 encodes a peptide sequence: LALLVLPLLQRAYAW, which exhibits HCV_G1_p7_794 homology.
Conceivably, other viral pathogens that cause chronic disease, e.g., herpes simplex, Epstein-Barr, human immunodeficiency and cytomegalovirus, also avoid or reduce T eff cell responses by exploiting similarity to self and activating nT_{reg} cells [29,30]. Indeed, recent analyses indicated that Epstein-Barr virus and cytomegalovirus contained fewer T cell epitopes and exhibited higher cross-reactivity with the human genome than did either Ebola or Marburg virus [14]. Ebola and Marburg viruses, on the other hand, were composed of significantly fewer peptide sequences that were cross-reactive with human and expressed a larger number of predicted T cell epitopes. Thus, viruses that cause acute disease and viruses such as HCV, which adapt to humans and cause chronic infection, may differ substantially in terms of their T_{reg} cell epitope content.

Although immunosuppression by T_{reg} cells is readily demonstrated in mice, demonstrating the suppressor activity of human CD3^+CD4^+CD25^+FoxP3^+ T_{reg} cells has proven problematic [31]. Recent studies indicate that the nature of the responder T cells (CD4^+CD25^+ versus CD4^+CD25^{low}) and the ratio of T_{reg} cells to responder cells exert significant effects on the outcome of suppression assays [32,33]. Nonetheless, the HCV_G1_p7_794 responsive T_{reg} cells suppressed the mitogenic response of cells derived from HCV infected patient in the experiments reported here despite the fact that the CD3^+CD4^+FoxP3^- responder cells far outnumbered CD3^+CD4^+CD25^+FoxP3^+ suppressor cells among total PBMCs by a greater than 10:1 ratio.

Notably, the addition of HCV_G1_p7_794 alone to PBMC cultures derived from HCV-infected (Ab^+VL^+) patients failed to induce cell proliferation despite a marked (3- to 4-fold) increase in CD3^+CD4^+CD25^+FoxP3^+ cell number. This finding indicates that HCV_G1_p7_794 induces the conversion of conventional CD4^+FoxP3^- T cells to T_{reg} cells, i.e., infectious tolerance, a suggestion supported by studies demonstrating the inability of nT_{reg} cells to proliferate in response to their cognate antigen in vitro [34]. Furthermore, in contrast to Ab^+VL^+ PBMCs cultured in medium alone, only a minority of CD3^+CD4^+FoxP3^- cells cultured in the presence of HCV_G1_p7_794 expressed CD304 (neuropilin), which is expressed by a subset of FoxP3^+ T_{reg} cells in humans and associated specifically with nT_{reg} cells in mice [21,22]. While it has been suggested alternatively that the
expanded T_{reg} cell population in chronic, HCV infected patients is composed of cells phenotypically similar to nT_{reg} or iT_{reg} cells [11,20], our results concur with the consensus that the expanded T_{reg} cell population in chronic HCV-infected patients is heterogeneous, composed of both T_{reg} cell subsets.

HCV p7, a 63-amino acid polypeptide chain that spans the endoplasmic reticulum membrane of infected cells, is essential for viral replication [35]. Hydrophobicity is a common characteristic of hepatitis C viral epitopes [36]. As such, the hydrophobic nature of HCV_G1_p7_794 is unlikely to account for the disparate responses of CD3^{+}CD4^{+}FoxP3^{+} T_{reg} cells in infected and non-infected individuals. Moreover, while glycosylation can impair T cell recognition [36], there is no evidence to suggest that HCV p7 is glycosylated or that glycosylation affects the differential response to HCV_G1_p7_794. Indeed, the same non-glycosylated synthetic peptide (HCV_G1_p7_794) added to cultures of infected and non-infected PBMCs elicited different outcomes. Rather, the findings reported herein suggest that HCV_G1_p7_794 is a unique peptide sequence recognized by nT_{reg} cells that function normally to suppress the autoimmune response to hundreds of human proteins that contain the p7_794 sequence. Upon HCV_G1_p7_794 recognition, these nT_{reg} cells induce the conversion of conventional T cells to iT_{reg} cells (i.e. infectious tolerance). We speculate that the nT_{reg} cells and iT_{reg} cells responsive to HCV_G1_p7_794 contribute to the elevated T_{reg} cell population found in HCV-infected patients, and play a role in immunosuppression and viral persistence.

The mechanism(s) that underlies the elevated T_{reg} cell response to HCV_G1_p7_794 observed in HCV-infected, but not non-infected, individuals remains to be determined. We hypothesize, however, that dysfunctional interaction with antigen-presenting cells is involved. Most studies indicate that DCs are functionally impaired in patients with chronic hepatitis C [6,37]. Impairments include: decreased IFN-α and IL-12 secretion, lowered expression of co-stimulatory molecules (CD80 and CD86) and an increased ability to prime T_{reg} cells. In addition, a recent report suggests that B cells contribute to the increase in T_{reg} cells associated with chronic hepatitis C [38]. Indeed, naïve pan T cells co-cultured with purified CD19^{+} B cells in the presence of HCV_G1_p7_794, but not HCV_G1_NS4b_1941 or medium alone, exhibited a marked (3-4-fold) increase in CD3^{+}CD4^{+}FoxP3^{+}
cells (Supplementary Table I). Notably, the HCV_G1_p7_794 responsive cells were phenotypically consistent with iT\textsubscript{reg} cells expressing neither CD304 nor Helios, markers associated with nT\textsubscript{reg} cells [21,22,39]. The potential contribution of APCs to the disparate T cell responses to HCV_G1_p7_794 observed in HCV-infected and non-infected individuals is illustrated in the schematic shown in Supplementary Figure 3. The ex vivo systems described herein enable detailed analyses of the mechanisms that underlie the generation and function of these HCV_G1_p7_794 responsive cells.
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| Subject ID# | Age | HCV Status | Viral Load IU/ml | Genotype | Antibody | HLA-DRB1 |
|------------|-----|------------|------------------|----------|----------|----------|
| 229        | 28  | Infected   | 170,620          | 1        | +        | 03,11    |
| 236        | 29  | Infected   | 602,611          | 1        | +        | 01,11    |
| 260        | 28  | Infected   | 1,410,524        | 3        | +        | 03,07    |
| 261        | 25  | Infected   | 7,464,611        | 1        | +        | 01,11    |
| 265        | 22  | Infected   | 21,917,190       | 1a       | +        | 13,15    |
| 272        | 23  | Infected   | 458              | 1a       | +        | 03,11    |
| 279        | 25  | Infected   | 1,788,326        | 1a       | +        | 13,15    |
| 282        | 28  | Infected   | 86,678           | 1a       | +        | 03,04    |
| 216        | 23  | Clearer    | NA               | NA       | +        | 07:01    |
| 221        | 27  | Clearer    |                  |          |          |          |
| 222        | 25  | Clearer    |                  |          |          |          |
| 246        | 26  | Clearer    |                  |          |          |          |
| 267        | 23  | Clearer    |                  |          |          |          |
| 264        | 27  | Clearer    |                  |          |          |          |
| 248        | 26  | Non-infected|                | -        |          | 04,11    |
| 259        | 26  | Non-infected|                | -        |          | 04,11    |
| 262        | 22  | Non-infected|                | -        |          | 03,11    |
| 257        | 22  | Non-infected|                | -        |          | 03,15    |
| 263        | 26  | Non-infected|                | -        |          | 01,03    |

NA = not applicable/available.
Table II. JanusMatrix Analysis

| Description           | ICS                        | # Cross-reactive Epitopes per ICS | # Human Proteins containing Cross-reactive Epitopes |
|-----------------------|----------------------------|----------------------------------|---------------------------------------------------|
| HCV_G1_p7_794<sup>a</sup> | WPLLLLLALPQRAYAQ          | 5.0                              | 264                                               |
| Human p7_794<sup>b</sup>  | PLLLLLSSLPPRA              | 4.0                              | 325                                               |
| HCV_G1_NS4b_1941<sup>c</sup>| AARVTQILSSLTITQLKRLHQWI    | 6.0                              | 28                                                |

<sup>a</sup>Human homolog; highly conserved sequence derived from hepatitis C virus, genotype 1.

<sup>b</sup>Analog; sequence found in multiple human proteins.

<sup>c</sup>Validated promiscuous, T<sub>eff</sub> cell ICS derived from hepatitis C virus, genotype 1 [17].
Figure Legends

Fig. 1. *HCV_G1_p7_794* induces the production of CD3^+CD4^+FoxP3^+ cells in cultures of PBMCs derived from HCV-infected patients. PBMCs were cultured in the presence of medium alone, 10 μg/ml *HCV_G1_p7_794* or 10 μg/ml *HCV_G1_NS4b_1941*. Cells collected after 5 days incubation were analyzed by flow cytometry (A). The CD3^+CD4^+FoxP3^+ cells were further characterized by the expression of CD25 and CD39 where the dotted line represents the isotype control (B). The gating scheme and representative analyses are shown.

Fig. 2. *HCV_G1_p7_794* fails to elicit an increase in CD3^+CD4^+FoxP3^+ cells among PBMCs derived from non-infected individuals. PBMCs obtained from infected patients (Ab^+VL^+, n= 4), patients who clear infection (Ab^+VL^-, n= 6) and non-infected controls (Ab^VL^-, n= 4) were cultured with medium alone, *HCV_G1_p7_794* or *HCV_G1_NS4b_1941* (A). The cells collected after 5 days were analyzed by flow cytometry. *Significantly different: *P=0.014; **P<0.001.

PBMCs obtained from Ab^+VL^+ patients (n= 4) and Ab^VL^- controls (n= 4) were cultured in the presence or absence of the human p7_794 analog (B). Significantly more CD3^+CD4^+FoxP3^+ cells were recovered from PBMC cultured with the analog than medium alone: *P=0.001; **P=0.048.

Fig. 3. *HCV_G1_p7_794* suppresses the proliferation of PBMCs derived from HCV-infected patients. CSFE-labeled, Ab^+VL^+ PBMCs were cultured with medium alone, or medium containing anti-CD3, *HCV_G1_p7_794*, or anti-CD3 and *HCV_G1_p7_794*. Cells were collected after 5 days; proliferation was estimated by flow cytometry and a loss in fluorescence intensity. Data were obtained in a single experiment representative of two HCV-infected patients (A). PBMCs obtained from the three patients listed were cultured 5 days in the presence of anti-CD3 or 20 ng/ml IL-2 with or without *HCV_G1_p7_794*; cell proliferation was estimated by [³H]-thymidine incorporated during the last 18 hours of incubation (B).
Fig. 4. Fewer HCV-G1_p7_794-responsive T\textsubscript{reg} cells express CD304 (neuropilin). PBMCs obtained from an infected patient (representative of 4 patients) were incubated in medium alone (A) or medium that contained HCV_G1_p7_794 (B). The cells were collected on day 5, stained and analyzed by flow. Panels on the right indicate the percentage of CD4^{+}FoxP3^{+} cells in each population that expresses CD304.
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Figure 1

A. Isotype controls

B. Medium alone

HCV_G1_p7_794

HCV_G1_NS4b_1941

% of Max

CD25

CD39
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Figure 2

A.

%CD3\(^+\)CD4\(^+\)FoxP3\(^+\) Cells

- Medium alone
- HCV_G1_p7_794
- HCV_G1_NS4b_1941

|        | Ab\(^{+}\)VL\(^{+}\) | Ab\(^{-}\)VL\(^{+}\) | Ab\(^{+}\)VL\(^{-}\) |
|--------|---------------------|---------------------|---------------------|
| Medium | 2                   | 2                   | 2                   |
| p7_794 | 8                   | 8                   | 8                   |
| analog | 4                   | 4                   | 4                   |

**A.**

%CD3\(^+\)CD4\(^+\)FoxP3\(^+\) Cells

- Medium alone
- p7_794 analog

|        | Ab\(^{+}\)VL\(^{+}\) | Ab\(^{-}\)VL\(^{+}\) |
|--------|---------------------|---------------------|
| Medium | 2                   | 2                   |
| p7_794 | 8                   | 8                   |

**A.**
Figure 3

A. 

B. 

Patient ID

#229  #261  #265

H-Thymidine incorporation (cpm $\times 10^3$)
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Figure 4

A.  

B.  

CD4

FoxP3

CD4

FoxP3

1.61%

69.20%

9.22%

22.86%
Supplementary Materials

HCV Epitope, Homologous to Multiple Human Protein Sequences, Induces a Regulatory T Cell Response in Infected Patients

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Materials and Methods

**HLA-DRB1-restricted epitope selection**

HCV sequences were acquired from the Los Alamos database [1,2]. Nine-mer amino acid sequences, capable of fitting the binding groove of HLA class II molecules and highly conserved across HCV genotype 1a and 1b isolates, were identified and scored for potential to bind eight common HLA class II (DRB1) alleles using bioinformatics tools [3-5]. HLA class II immunogenic consensus sequences (ICS) were identified and constructed by assembling potentially immunogenic 9-mers into 18-25 amino acid sequence [3]. ICS construction improves the probability that an epitope will be presented in the context of more than one HLA allele, thus broadening the response of an HLA-diverse human population. Twenty ICS derived from HCV genotypes 1a and 1b were synthesized as peptides using 9-fluoronylmethoxycarbonyl chemistry and purified >85% by 21st Century Biochemicals (Marlboro, MA). Each of these “promiscuous” ICS contained between 5 and 28 predicted HLA binding motifs, and were bound by multiple HLA-DRB1 alleles in competitive binding assays as described previously [6,7]. Each ICS was also evaluated for human homology (>7 shared identities per 9-mer frame) using GenBank BLAST [8].

**Peripheral blood mononuclear cell (PBMC) cultures**

Cryopreserved PBMCs from participating subjects were thawed; suspended in HEPES-buffered RPMI1640 medium supplemented with 10% HuAB serum (Valley Biomedical, Winchester, VA), glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin; and rested overnight at 37°C in a humidified, CO₂ incubator. On the following day, the PBMCs were centrifuged and resuspended in fresh medium containing 5% HuAB serum; 1 x 10⁶ cells/ml were transferred to deep, flat-bottom, non-treated 48-well plates. The peptide sequence indicated in the text was added (10 µg/ml final concentration) and the cells were incubated for 5 days. PBMCs cultured in the presence of medium
with 0.1% DMSO served as a negative control. Half the spent medium was replaced on day 3. The cells were collected for analysis on day 5.

For proliferation assays, cells rested overnight were labeled with carboxyfluorescein diacetate, succinimidyl ester (CFSE: Life Technologies Corporation, Carlsbad, CA) prior to culture in the presence or absence of 10 µg/ml HCV_G1_p7_794, 10 µg/ml human p7_794 or 30 ng/ml anti-CD3 monoclonal antibody (clone HIT3a; BioLegend, San Diego, CA). Cell proliferation was evaluated by flow cytometric analysis according to the protocol provided by Life Technologies in which a loss in fluorescence intensity correlates directly with the extent of replication. Alternatively, non-CSFE-labeled cells were pulsed with 0.1 µCi [³H]-thymidine during the last 18 hours of the 5-day culture period; radiolabeled-thymidine incorporation was quantified by liquid scintillation counting.

**Flow cytometry**

Stimulated PBMC were quantified and characterized in accordance with methods we described previously [9]. The following dye-conjugated mouse monoclonal antibodies were purchased from BioLegend (San Diego, CA) and used: CD3, CD4 (clone OKT4), CD8a (clone HIT8a); CD39 (clone A1), and CD304 (clone 14H4); anti-human CD25 (clone M-A251) and anti-human FoxP3 (clone 236a) were purchased from BD Biosciences (San Jose, CA). Data were collected on a BD LSRII Flow Cytometer (BD Biosciences, San Jose, California) and analyzed using FlowJo software (Tree Star, Inc., Ashland, OR). All analyses were conducted using the appropriate isotype controls to correct for non-specific staining.

**Statistical Analysis**

The results were analyzed using the SigmaStat statistics program (Aspire Software International). Means were compared using a non-paired Student’s t test or a Mann-Whitney Rank Sum test. Aggregate data were compared by one-way analysis of variance; the Dunnett’s test determined which groups differed significantly.
Supplementary Fig. 1. CD25+ cell depletion prior to culture abrogates the T\(_{\text{reg}}\) cell response to HCV\_G1\_p7\_794.

PBMCs obtained from the two HCV-infected patients indicated were either depleted or not depleted of CD25 expressing cells using antibody-coated magnetic beads (Miltenyi Biotech, Auburn, CA). Depleted (CD25-) and non-depleted (CD25+) PBMCs were cultured in the presence or absence of medium that contained 10 µg/ml HCV\_G1\_p7\_794. The cells were collected after 5 days incubation and the CD3+CD4+FoxP3+ T cells were quantified by flow cytometry.
Supplementary Fig. 2. HCV_G1_p7_794, derived from HCV genotype 1, induces the production of CD3^+CD4^+FoxP3^+ cells in cultures of PBMCs obtained from an HCV genotype 3 infected patient. PBMCs were cultured in the presence of medium alone, HCV_G1_p7_794, HCV_G1_NS4b_1941 or human p7_794 analog. Cells collected after 5 days incubation were analyzed by flow cytometry.
Supplementary Fig. 3. Schematic: HCV_G1_p7_794 presentation by B cells or impaired DCs promotes infectious tolerance, loss of anti-viral T\textsubscript{eff} cell activity and persistent HCV infection.

During the normal course of protein turnover, mature antigen presenting cells (APCs) process and present the p7_794 sequence in hundreds of distinct self-proteins. Immune recognition and the activity of p7_794-specific CD3\(^+\)CD4\(^+\)FoxP3\(^−\)CD304\(^+\) nT\textsubscript{reg} cells suppress the autoimmune response of T\textsubscript{eff} cells specific for the same self-proteins (A). In HCV-infected patients, HCV_G1_p7_794 presented by B cells or impaired (phenotypically immature) DCs induces the epitope-specific response of nT\textsubscript{reg} cells and the subsequent conversion of conventional (CD3\(^+\)CD4\(^+\)FoxP3\(^−\)) T cells to
CD3^+CD4^+FoxP3^+CD304^+ iT\textsubscript{reg} cells. iT\textsubscript{reg} cells, in turn, block the anti-viral response of T\textsubscript{eff} cells specific for HCV\_G1\_p7\_794, as well as other viral epitopes (bystander suppression) (B).
CD19+ B cells and pan-T cells were purified from the PBMCs of a normal blood donor using magnetic beads (Miltenyi Biotec). Equal numbers were suspended in X-VIVO 15 medium (Lonza, Walkerville, MD) supplemented with glutamine, penicillin, streptomycin, and 50 U/ml recombinant human IL-2 (Roche Applied Science, Indianapolis, IN) and subsequently co-cultured in the presence of 0.1% DMSO, 10 µg/ml HCV_G1_p7_794 or 10 µg/ml HCV_G1_NS4b_1941. The cells were collected after 14 days incubation and the CD3+ cells were phenotyped by flow cytometry. Data are the results of a single experiment representative of two identical experiments.

|                      | 0.1% DMSO | HCV_G1_p7_794 | HCV_G1_NS4b_1941 |
|----------------------|-----------|---------------|------------------|
| FoxP3+a              | 2.12      | 7.76          | 2.23             |
| CD39+b               | 94.65     | 99.00         | 96.09            |
| CD152+b              | 0.63      | 0.55          | 1.29             |
| CD304+b              | 61.01     | 37.99         | 61.49            |
| HELIOS+b             | 3.16      | 1.61          | 4.78             |

*aPercentage of CD3+CD4+ cells.

bPercentage of FoxP3+ cells.
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