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

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

**Abbreviations.** HCV, hepatitis C virus; T<sub>reg</sub> cells, regulatory T cells; FoxP3, forkhead box P3; nT<sub>reg</sub> cells, natural T<sub>reg</sub> cells; iT<sub>reg</sub> inducible T<sub>reg</sub> cells; BLAST, Basic Local Alignment Search Tool; T<sub>eff</sub> 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.

**Disclosures.** Anne De Groot and William Martin are senior officers and majority shareholders at EpiVax, Inc. These authors acknowledge a potential conflict of interest and attest that the work contained in this report is free of any bias that might be associated with the commercial goals of the company. None of the remaining authors have conflicts to disclose.

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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<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> regulatory T<sub>reg</sub> cells in HCV-infected patients suggest the role of T<sub>reg</sub> cells in impaired viral clearance. The factors contributing to increased T<sub>reg</sub> 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<sub>reg</sub> 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<sub>reg</sub> cells among PBMCs derived from both HCV infected and non-infected individuals. JanusMatrix analyses determined that HCV\_G1\_p7\_794 is comprised of T<sub>reg</sub> 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<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> nT<sub>reg</sub> 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<sup>+</sup> regulatory T<sub>reg</sub> cells circulating in the bloodstream and accumulating in the liver have been implicated in the pathogenesis of chronic hepatitis C [2,3].

T<sub>reg</sub> cells constitute one of the major mechanisms underlying immunological homeostasis and self-tolerance [4]. In addition, T<sub>reg</sub> 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 T<sub>reg</sub> cells also contribute to the establishment and persistence of chronic infections, e.g., HCV [6,7]. While no single marker exists, human T<sub>reg</sub> 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  $\alpha$  chain (CD25).

Two distinct T<sub>reg</sub> cell subsets are described in the literature: natural (n)T<sub>reg</sub> cells specific for self epitopes and generated by high-avidity selection in the thymus, and inducible (i)T<sub>reg</sub> cells that derive from conventional (CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup>) T cells following stimulation in the periphery [8]. nT<sub>reg</sub> cells can induce the conversion of conventional T cells to iT<sub>reg</sub> cells via cytokine-dependent and -independent mechanisms, a process called infectious tolerance [9,10]. Notably, the factors that affect expansion of the T<sub>reg</sub> cell population in cases of chronic hepatitis C remain to be fully delineated. Nonetheless, the consensus supports the heterogeneous nature of the expanded T<sub>reg</sub> cell population composed of both nT<sub>reg</sub> and iT<sub>reg</sub> 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<sup>+</sup>VL<sup>+</sup>), patients who spontaneously resolved infection (Ab<sup>+</sup>VL<sup>-</sup>) and HCV-seronegative, non-infected (Ab<sup>-</sup>VL<sup>-</sup>) 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; WPLLLLLLALPQRAYAQ), 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<sup>+</sup>CD4<sup>+</sup>FoxP3<sup>+</sup> 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<sup>-</sup>VL<sup>-</sup>), patients who spontaneously cleared HCV infection (Ab<sup>+</sup>VL<sup>-</sup>) and infected patients in whom viremia persisted (Ab<sup>+</sup>VL<sup>+</sup>). HCV\_G1\_p7\_794 induced a marked increase in CD3<sup>+</sup>CD4<sup>+</sup>FoxP3<sup>+</sup> cells when added to PBMC cultures derived from infected, Ab<sup>+</sup>VL<sup>+</sup> patients (Figure 1A). In contrast, HCV\_NS4B\_1941 (AARVTQILSSLTITQLLKRLHQWI; 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; ISGIQYLAGLSTLPGNPA) failed to elicit a CD3<sup>+</sup>CD4<sup>+</sup>FoxP3<sup>+</sup> cell response (data not shown). CD3<sup>+</sup>CD4<sup>+</sup>FoxP3<sup>+</sup> cells, induced by the addition of HCV\_G1\_p7\_794 to Ab<sup>+</sup>VL<sup>+</sup> PBMC cultures, expressed both CD25 (IL-2 receptor  $\alpha$  chain, constitutively expressed by T<sub>reg</sub> cells) and CD39, a cell ectonucleotidase associated with T<sub>reg</sub> cell function (Figure 1B) [6,19].

While a significant increase in CD3<sup>+</sup>CD4<sup>+</sup>FoxP3<sup>+</sup> cells was determined in PBMC cultures derived from HCV-infected patients, neither HCV\_G1\_p7\_794 nor the control peptide (HCV\_G1\_NSB4\_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<sup>+</sup>VL<sup>+</sup> patients had a higher baseline level of CD3<sup>+</sup>CD4<sup>+</sup>Foxp3<sup>+</sup> cells compared to clearers or non-infected controls, a finding consistent with the literature [20].

#### *HCV\_G1\_p7\_794 activates cross-reactive nT<sub>reg</sub> cells*

A human analog of HCV\_G1\_p7\_794 (p7\_794, PLLLLLSLPPRA), identified by GenBank BLAST analysis, was synthesized in an effort to provide a clearer understanding of the nature of the T<sub>reg</sub> cells that respond to HCV\_G1\_p7\_794. Like the HCV-encoded homolog, the human analog induced a significant increase in CD3<sup>+</sup>CD4<sup>+</sup>FoxP3<sup>+</sup> 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<sup>+</sup>CD4<sup>+</sup>FoxP3<sup>+</sup> cells in PBMC cultures derived from non-infected (Ab<sup>-</sup>VL<sup>-</sup>) individuals, indicative of the capacity to elicit an nT<sub>reg</sub> cell response.

#### *HCV\_G1\_p7\_794 suppresses the mitogenic response of T cells to anti-CD3 and IL-2 treatment*

The hallmark of CD3<sup>+</sup>CD4<sup>+</sup>FoxP3<sup>+</sup> T<sub>reg</sub> cells is suppressor activity. The addition of HCV\_G1\_p7\_794 to Ab<sup>+</sup>VL<sup>+</sup> 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 [<sup>3</sup>H]-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<sup>+</sup>CD4<sup>+</sup> T cells obtained from these same patients. This latter finding indicates that HCV\_G1\_p7\_794 induces the conversion of conventional CD3<sup>+</sup>CD4<sup>+</sup>FoxP3<sup>-</sup> T cells to CD3<sup>+</sup>CD4<sup>+</sup>FoxP3<sup>+</sup> iT<sub>reg</sub> cells (i.e., infectious

tolerance) rather than stimulates the proliferation of  $T_{reg}$  cells already present. An increase in  $CD3^+CD4^+FoxP3^+$   $T_{reg}$  cells that lack cell-surface neuropilin-1 supports this suggestion.

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

#### *JanusMatrix confirms $T_{reg}$ cell epitopes shared by HCV\_G1\_p7\_974 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  $T_{reg}$  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- $\gamma$  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- $\gamma$  assessed by ELISpot

assay, i.e., 64 + 75 versus 655 + 591 (mean  $\pm$  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- $\gamma$  production supports our speculation that, rather than activate T<sub>eff</sub> cells, HCV\_G1\_p7\_794 activates a cross-reactive nT<sub>reg</sub> cell population that normally functions to suppress autoimmune responses to a large number of human proteins, which contain a common peptide sequence (epitope).

## Discussion

Resolution of primary HCV infections is dependent upon the vigorous response of CD4<sup>+</sup> and CD8<sup>+</sup> 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 T<sub>reg</sub> cell-mediated suppression [2,3,6,7,24,25]. The increased frequency of T<sub>reg</sub> 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 T<sub>reg</sub> 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 T<sub>reg</sub> cells or the nonspecific consequence of chronic inflammation and liver disease [7].

The ability of HCV-derived epitopes to stimulate T<sub>reg</sub> 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 T<sub>reg</sub> 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 T<sub>reg</sub> cell response in a wide, HLA-diverse human population, such as the infected patient population shown in Table I. Unlike the T<sub>reg</sub> 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<sup>+</sup>CD4<sup>+</sup>FoxP3<sup>+</sup> cells [20,26]. In addition to expressing CD25, characteristic of T<sub>reg</sub> cells, the vast majority of these cells

expressed CD39, a marker that distinguishes FoxP3<sup>+</sup> T<sub>reg</sub> cells from activated T<sub>eff</sub> 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<sup>+</sup>CD4<sup>+</sup>FoxP3<sup>+</sup> 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<sub>reg</sub> 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<sub>reg</sub> 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<sub>reg</sub> 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<sub>reg</sub> 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<sup>+</sup>CD4<sup>+</sup>FoxP3<sup>+</sup>CD25<sup>+</sup> cells (Supplementary Figure 2). It is pertinent to note, in this regard, that HCV genotype 3 encodes a peptide sequence: LALLVLLLQRAYAW, 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  $T_{\text{eff}}$  cell responses by exploiting similarity to self and activating  $nT_{\text{reg}}$  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  $T_{\text{reg}}$  cell epitope content.

Although immunosuppression by  $T_{\text{reg}}$  cells is readily demonstrated in mice, demonstrating the suppressor activity of human  $CD3^+CD4^+CD25^+FoxP3^+$   $T_{\text{reg}}$  cells has proven problematic [31]. Recent studies indicate that the nature of the responder T cells ( $CD4^+CD25^-$  versus  $CD4^+CD25^{\text{low}}$ ) and the ratio of  $T_{\text{reg}}$  cells to responder cells exert significant effects on the outcome of suppression assays [32,33]. Nonetheless, the HCV\_G1\_p7\_794 responsive  $T_{\text{reg}}$  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  $T_{\text{reg}}$  cells, i.e., infectious tolerance, a suggestion supported by studies demonstrating the inability of  $nT_{\text{reg}}$  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  $FoxP3^+$   $T_{\text{reg}}$  cells in humans and associated specifically with  $nT_{\text{reg}}$  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- $\alpha$  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<sub>reg</sub> cells expressing neither CD304 nor Helios, markers associated with nT<sub>reg</sub> 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.

## **Acknowledgment**

We are grateful to Joseph Desrosiers (URI) for conducting the flow cytometric analyses shown.

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**Table I. Human Subjects**

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	↓	↓	+	03,11
222	25	Clearer	↓	↓	+	03,15
246	26	Clearer	↓	↓	+	01,04
267	23	Clearer	↓	↓	+	03,11
264	27	Clearer	↓	↓	+	03,11
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>	WPLLLLLLALPQRAYAQ	5.0	264
Human p7_794 <sup>b</sup>	PLLLLLSLPPRA	4.0	325
HCV_G1_NS4b_1941 <sup>c</sup>	AARVTQILSSLTITQLLKRLHQWI	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<sup>+</sup>CD4<sup>+</sup>FoxP3<sup>+</sup> 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<sup>+</sup>CD4<sup>+</sup>FoxP3<sup>+</sup> 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.

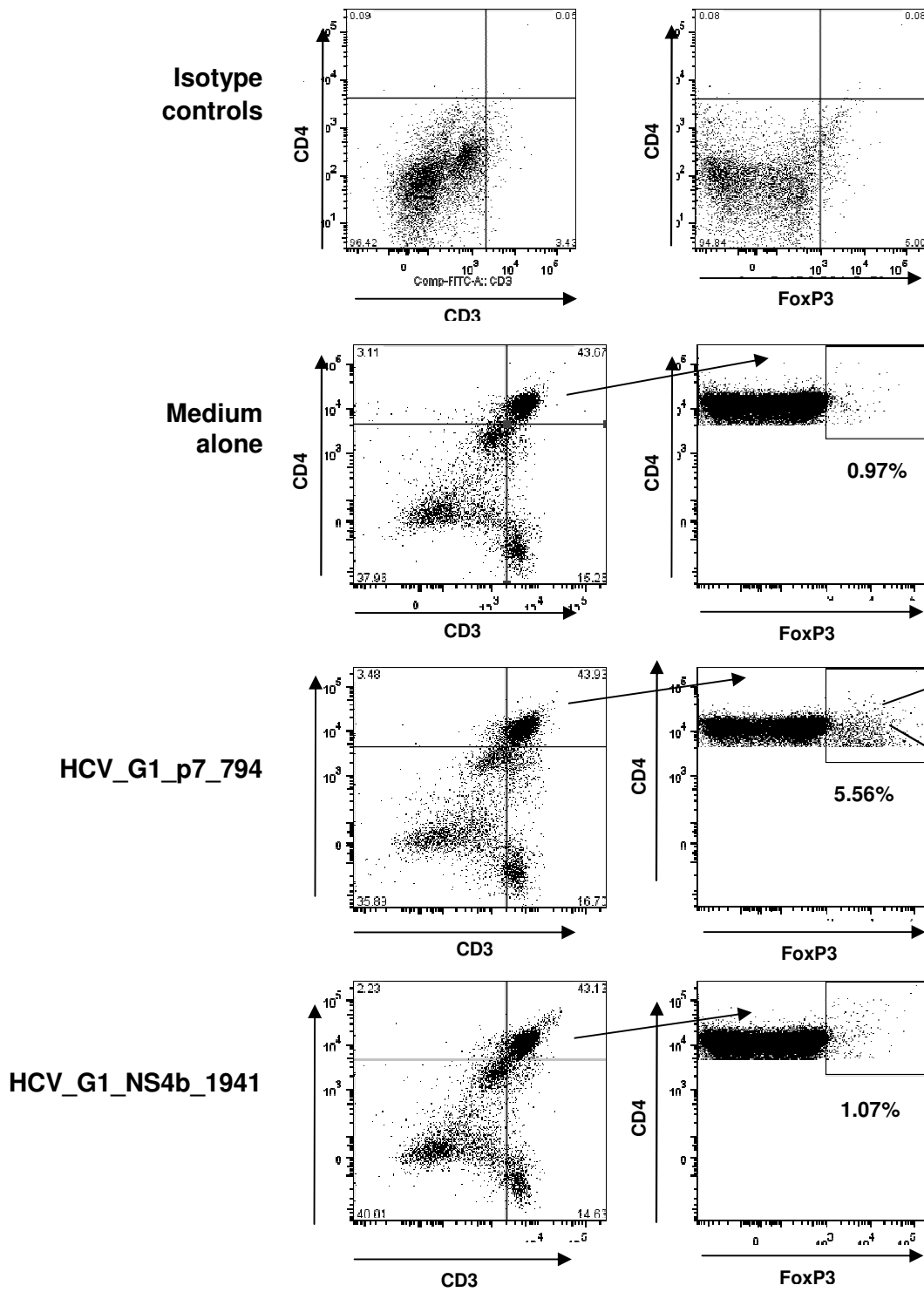
**Fig. 2. HCV\_G1\_p7\_794 fails to elicit an increase in CD3<sup>+</sup>CD4<sup>+</sup>FoxP3<sup>+</sup> cells among PBMCs derived from non-infected individuals.** PBMCs obtained from infected patients (Ab<sup>+</sup>VL<sup>+</sup>, n= 4), patients who clear infection (Ab<sup>+</sup>VL<sup>-</sup>, n= 6) and non-infected controls (Ab<sup>-</sup>VL<sup>-</sup>, 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<sup>+</sup>VL<sup>+</sup> patients (n= 4) and Ab<sup>-</sup>VL<sup>-</sup> controls (n= 4) were cultured in the presence or absence of the human p7\_794 analog (B). Significantly more CD3<sup>+</sup>CD4<sup>+</sup>FoxP3<sup>+</sup> cells were recovered from PBMC cultured with the analog than medium alone: \* $P=0.001$ ; \*\* $P=0.048$ .

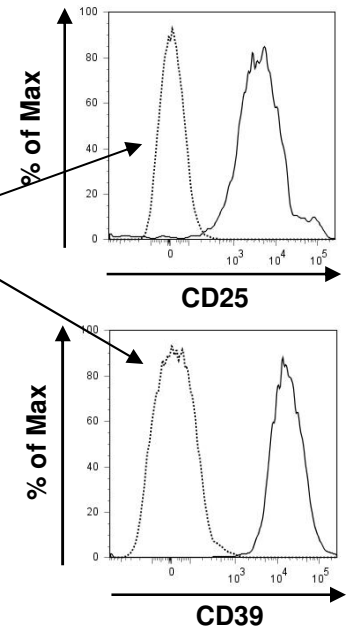
**Fig. 3. HCV\_G1\_p7\_794 suppresses the proliferation of PBMCs derived from HCV-infected patients.** CFSE-labeled, Ab<sup>+</sup>VL<sup>+</sup> PBMCs were cultured with medium alone, or medium containing anti-CD3, HCV\_G1\_p7\_794, or anti-CD3 and HCV\_G1\_p7\_794. Cells were collected after 5 days; 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 [<sup>3</sup>H-thymidine incorporated during the last 18 hours of incubation (B).

**Fig. 4. Fewer HCV-G1\_p7\_794-responsive T<sub>reg</sub> 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<sup>+</sup>FoxP3<sup>+</sup> cells in each population that expresses CD304.

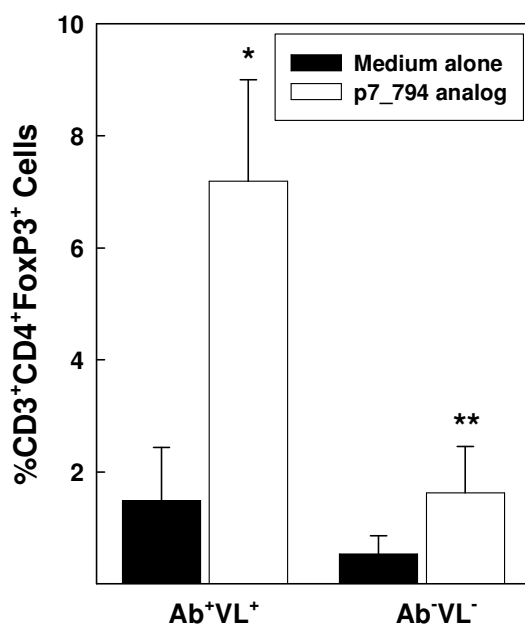
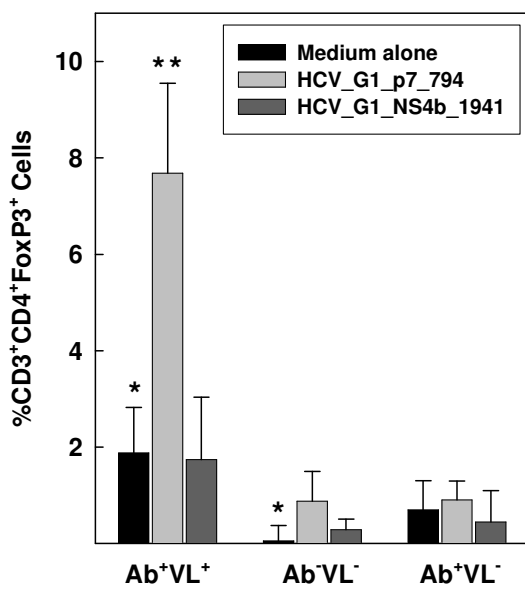
**A.**

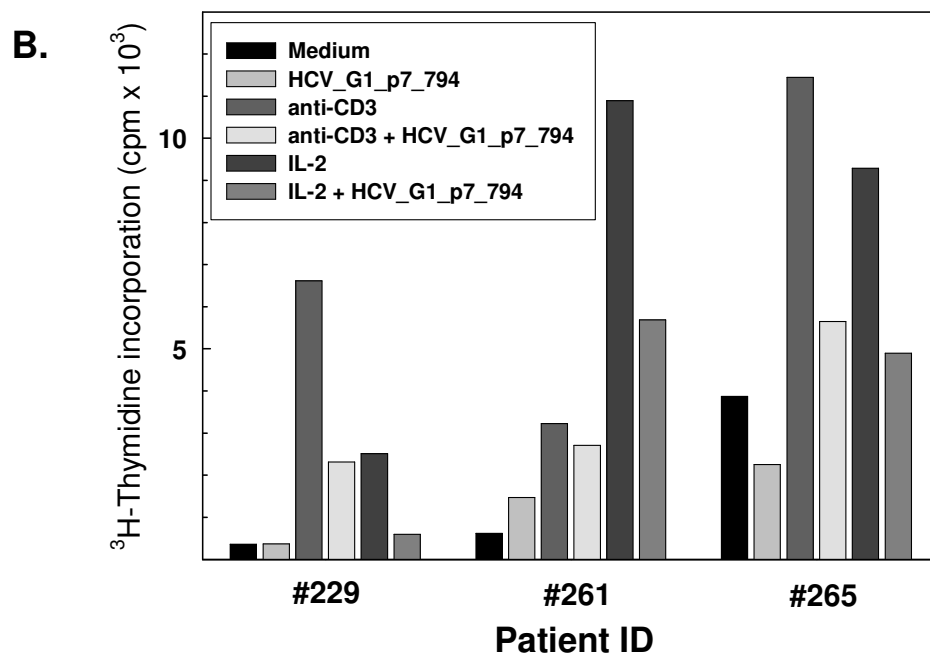
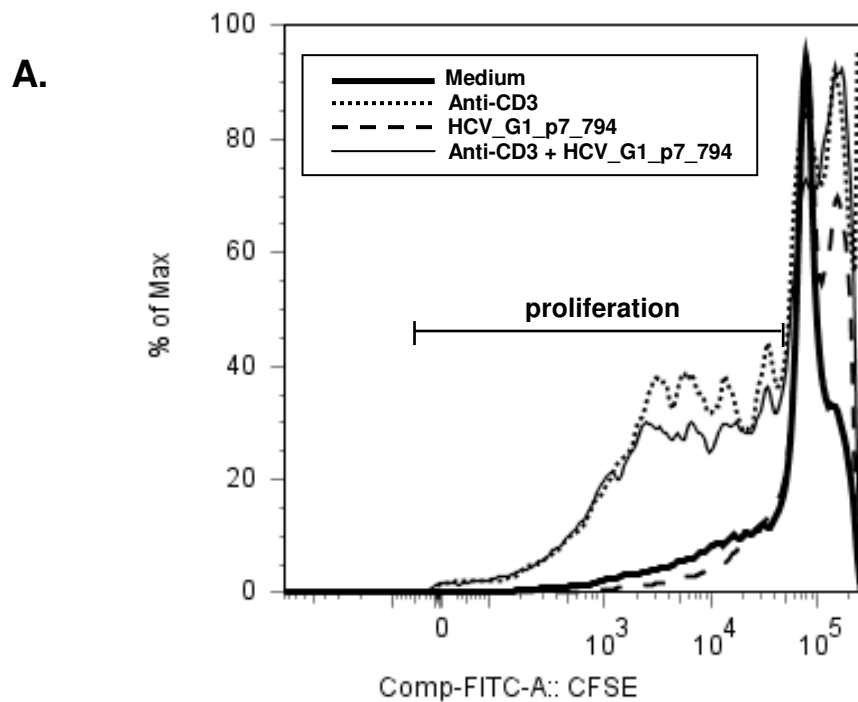


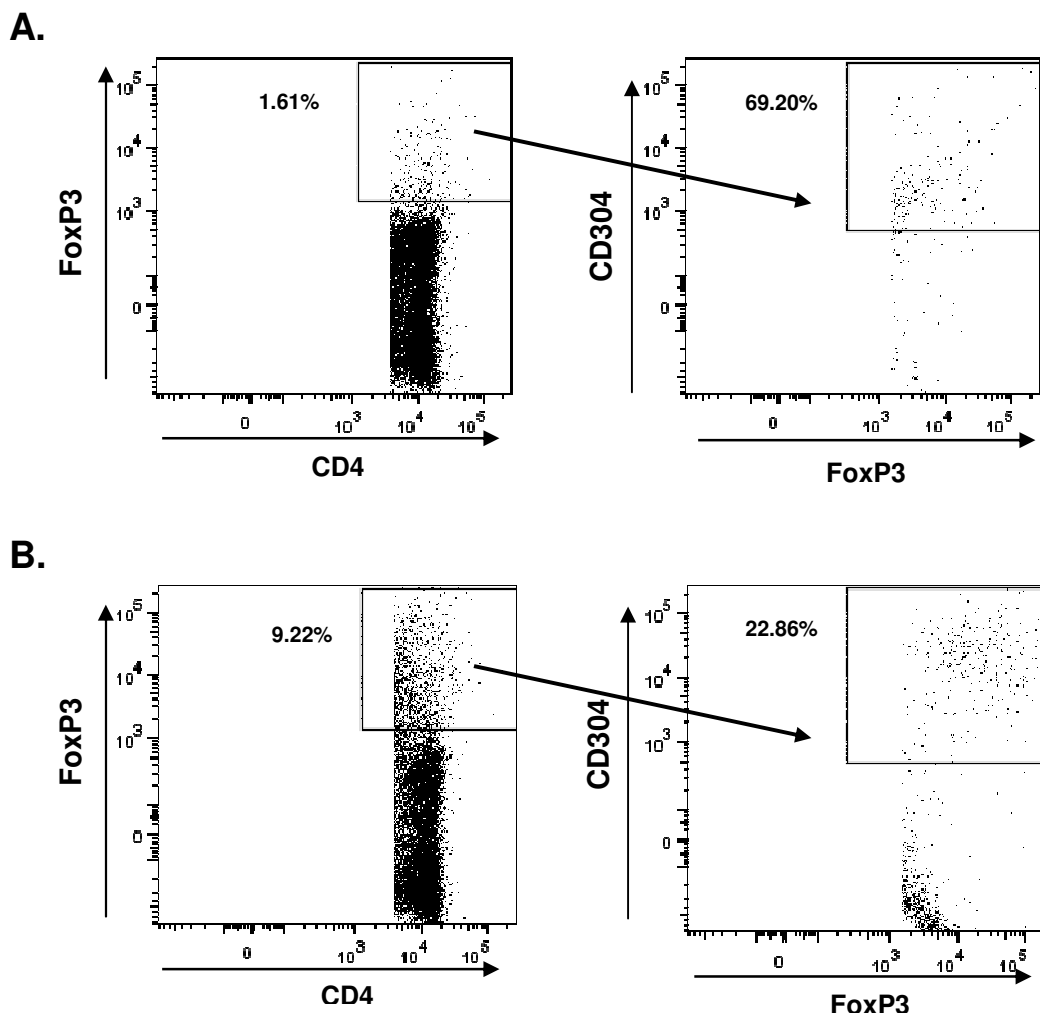
**B.**



**A.**









## 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, CO<sub>2</sub> incubator. On the following day, the PBMCs were centrifuged and resuspended in fresh medium containing 5% HuAB serum; 1 x 10<sup>6</sup> 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.

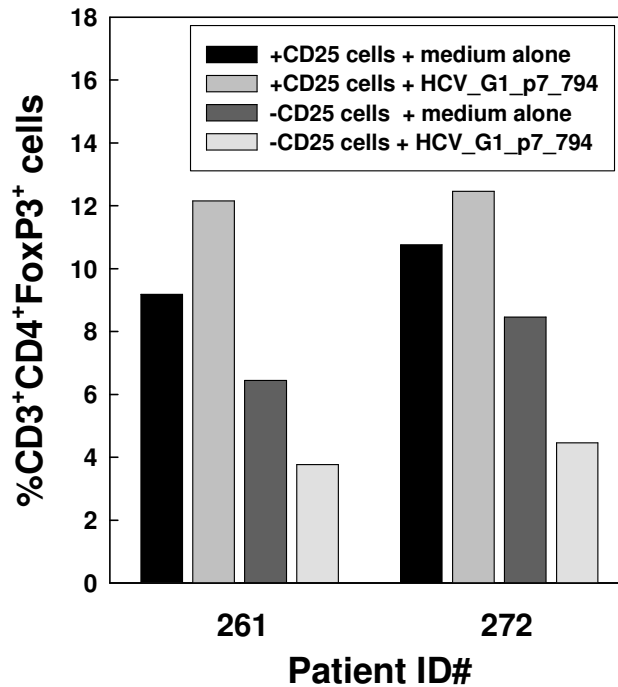
For proliferation assays, cells rested overnight were labeled with carboxyfluorescein diacetate, succinimidyl ester (CFSE: Life Technologies Corporation, Carlsbad, CA) prior to culture in the presence or absence of 10 µg/ml HCV\_G1\_p7\_794, 10 µg/ml human p7\_794 or 30 ng/ml anti-CD3 monoclonal antibody (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 [<sup>3</sup>H]-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.

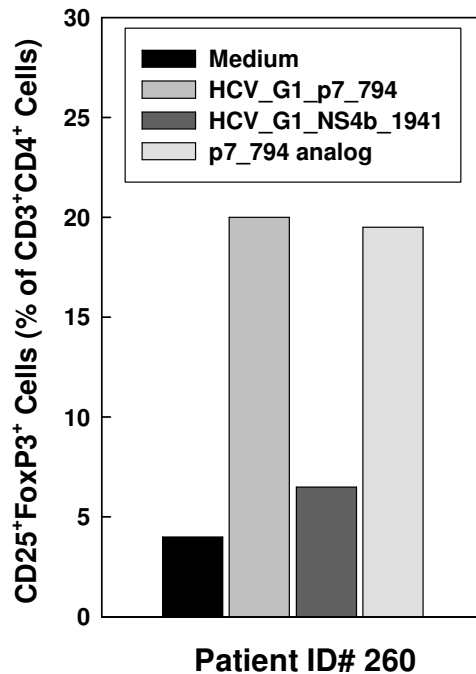
### ***Statistical Analysis***

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.

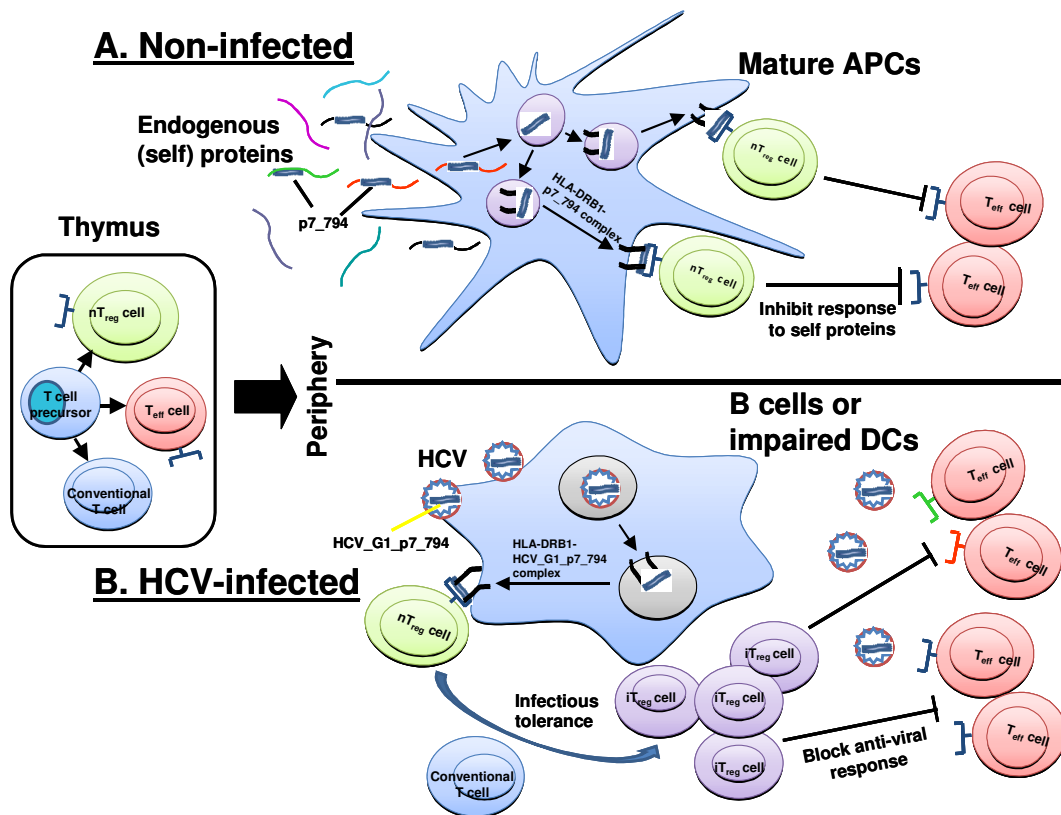


**Supplementary Fig. 1. CD25<sup>+</sup> cell depletion prior to culture abrogates the T<sub>reg</sub> cell response to HCV\_G1\_p7\_794.**

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<sup>-</sup>) and non-depleted (CD25<sup>+</sup>) 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<sup>+</sup>CD4<sup>+</sup>FoxP<sup>+</sup> T cells were quantified by flow cytometry.



**Supplementary Fig. 2. HCV\_G1\_p7\_794, derived from HCV genotype 1, induces the production of CD3<sup>+</sup>CD4<sup>+</sup>FoxP3<sup>+</sup> 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<sub>eff</sub> 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<sup>+</sup>CD4<sup>+</sup>FoxP3<sup>+</sup>CD304<sup>+</sup> nT<sub>reg</sub> cells suppress the autoimmune response of T<sub>eff</sub> 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<sub>reg</sub> cells and the subsequent conversion of conventional (CD3<sup>+</sup>CD4<sup>+</sup>FoxP3<sup>-</sup>) T cells to

CD3<sup>+</sup>CD4<sup>+</sup>FoxP3<sup>+</sup>CD304<sup>+</sup> iT<sub>reg</sub> cells. iT<sub>reg</sub> cells, in turn, block the anti-viral response of T<sub>eff</sub> cells specific for HCV\_G1\_p7\_794, as well as other viral epitopes (bystander suppression) (B).

**Table I. HCV\_G1\_p7\_794 induces CD3<sup>+</sup>CD4<sup>+</sup>FoxP3<sup>+</sup> cells in co-cultures composed of purified, naïve human T cells and B cells**

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

CD19<sup>+</sup> 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<sup>+</sup> cells were phenotyped by flow cytometry. Data are the results of a single experiment representative of two identical experiments.

<sup>a</sup>Percentage of CD3<sup>+</sup>CD4<sup>+</sup> cells.

<sup>b</sup>Percentage of FoxP3<sup>+</sup> cells.



## References

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