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CD4⁺ T-cell engagement by both wild-type and variant HCV peptides modulates the conversion of viral clearing helper T cells to Tregs

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Abstract

Aim—To determine whether modulation of T-cell responses by naturally occurring viral variants caused an increase in numbers of Tregs in HCV-infected patients.

Patients, materials & methods—Human peripheral blood mononuclear cells, having proliferative responses to a wild-type HCV-specific CD4⁺ T-cell epitope, were used to quantify, via proliferative assays, flow cytometry and class II tetramers, the effects of naturally occurring viral variants arising in the immunodominant epitope.

Results—In combination, the wild-type and variant peptides led to enhanced suppression of an anti-HCV T-cell response. The variant had a lower avidity for the wild-type-specific CD4⁺ T cell. Variant-stimulated CD4⁺ T cells had increased Foxp3, compared with wild-type-stimulated cells.

Conclusion—A stable viral variant from a chronic HCV subject was able to induce Tregs in multiple individuals that responded to the wild-type HCV-specific CD4⁺ T-cell epitope.

Keywords

altered peptide ligand; HCV; human PBMCs; regulatory T cells

HCV is a positive-stranded RNA virus that circulates within the host as a heterogeneous population. The most frequently represented genome is referred to as the master or wild-type sequence [1]; the viral variants arising in the viral genome are called quasispecies [2]. The role of the viral variants in HCV pathogenesis can range from simple escape to deviation of the immune response, possibly leading to viral persistence [3–7]. The majority of circulating virus in chronic HCV subjects is the wild-type genome [8–10]. Therefore, a paradox exists in that the wild-type viral genome persists in the presence of T cells, suggesting HCV is

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

potentially able to induce immunologic tolerance by deviating an antiviral HCV-specific T-cell response to persistent virus [10–15].

The recognition of HCV epitopes, or segments of the virus, in the context of the peptide–MHC by T-cell receptors (TCRs) expressed on T cells is necessary for HCV clearance [15–19]. Previous studies have demonstrated that naturally occurring viral variants have mutations in CD4⁺ helper T-cell epitopes that are able to modulate T-cell signaling by acting as altered peptide ligands (APLs) [20–22]. APLs are capable of modulating the T-cell response through an amino acid substitution in the wild-type peptide [22]. A number of pathogens have been shown to exploit APLs, allowing persistence in the host. These include HCV [21,23,24], HBV [23], HIV [25,26] and *Plasmodium falciparum* [27]. Previous work in our laboratory identified naturally occurring viral variants containing mutations in an immunodominant CD4⁺ T-cell epitope [28]. One viral variant, S370P, was found to be stable for over 2 years, suggesting ‘selection and fixation’ of this HCV mutant, which indicates a role for this variant in HCV pathogenesis [29,30]. Recently, we have shown that S370P is able to induce Tregs in a HCV-infected patient, and the variant was able to suppress the antiviral T-cell response in an antigen-specific manner [29]. However, we did not determine whether the variant was activating another population of CD4⁺ T cells or modulating the wild-type-specific CD4⁺ T cells.

Although a variety of markers are associated with Tregs, the most accepted marker is the transcription factor Foxp3 [30]. The expression of Foxp3 is critical in the development of Tregs [31–35]. An increase in Treg markers has been shown in cohorts of chronic HCV subjects when compared with resolved and noninfected individuals, suggesting that HCV is inducing Tregs as a means of promoting viral persistence [29,36–41]. Furthermore, Foxp3 expression is dependent on IL-10 signaling in CD4⁺ T cells [42]. Comparison between resolved and chronic HCV subjects showed an increase in serum IL-10 levels in chronic HCV patients, suggesting the extracellular milieu is conducive to Treg differentiation in chronically infected HCV patients [7].

Recent work has demonstrated that HCV is able to exploit naturally occurring viral variants to modulate CD4⁺ T-cell responses [20,43]. Taken together, we hypothesized that one possible mechanism of induction of Tregs is through viral variants antagonizing HCV antigen-specific T cells. We performed *in vitro* assays using two HCV subjects that responded to the wild-type peptide. Both the wild-type and variant peptides enhanced suppression of a T-cell response. Using MHC class II tetramers, we tested the avidity of the variant tetramer to antigen-specific CD4⁺ T cells and found that the avidity was different in comparison to the wild-type tetramer. Further, one viral variant, S370P, induced upregulation of Foxp3 in MHC class II tetramer wild-type-positive cells. Lastly, we provide evidence that the presence of IL-10 is necessary for Foxp3⁺CD4⁺ T cells. These results demonstrate that a stable viral variant in a chronic HCV subject is able to induce Tregs in multiple individuals that are able to respond to an HCV-specific CD4⁺ T-cell epitope.

Patients, materials & methods

Patient samples

These studies have been reviewed and approved by University of Utah and Medical College of Wisconsin Institutional Review Boards. Blood was collected in acid citrate dextrose and peripheral blood mononuclear cells (PBMCs) were isolated over Lymphocyte Separation Medium (GE-Healthcare, NJ, USA) and preserved in liquid nitrogen, as previously described [3]. Human leukocyte antigen (HLA) class II typing had been performed on each patient, as previously described [3]. PB3019 is *DRB1*0701/1501* and PH1127 is *DRB1*1501/1301*. PB3019 is a chronic HCV subject infected with genotype 1a, as

previously described [3]. Hemophiliac individual PH1127 is a resolved HCV subject with detectable antibody for HCV. These two individuals were selected for these experiments due to their HLA-type and recall responses to certain NS3 peptides.

Synthetic peptides & mitogen

Synthetic peptides representing the *DRB1*1501*-restricted HCV epitope, NS3 amino acids 358–375 (amino acids 1384–1401 of the HCV polyprotein), were synthesized at the Blood Center of Wisconsin, as previously described [3,32]. The three single amino acid variants were identified in a chronic HCV patient (PB3019) [3,44]. Peptide sequences were as follows: wild-type 358–375 (NS3₃₅₈), VIKGGRHLIFCHSKKKCD; variant H369R, VIKGGRHLIFCRSKKKCD; variant S370P, VIKGGRHLIFCHPKKKCD; and variant K371E, VIKGGRHLIFCHSEKKCD. Phytohemagglutinin (PHA; *Phaseolus vulgaris*, Sigma, MO, USA) was dissolved in dimethyl sulfoxide (0.1%, Sigma) and RPMI-1640 medium (BioWhittaker, ME, USA).

Cell culture & media

PBMCs were cultured in RPMI-1640 medium supplemented with 25 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 1 mM sodium pyruvate, 5 µg/ml gentamycin (all from Mediatech Cellgro, VA, USA), 10 U/ml heparin sodium (Fisher Scientific, PA, USA) and 10% pure human serum (Atlanta Biologicals, GA, USA). Cells were cultured in a 37°C, 5% CO₂ incubator.

T-cell proliferation assay

To measure proliferative responses of PBMCs after stimulation with the indicated peptides, 1×10^5 cells/well were added to round-bottom 96-well plates (Corning Costar, NY, USA) and incubated at 37°C, 5% CO₂ at the indicated times and peptide doses. Human recombinant IL-10 (rIL-10; 5 U/ml; BD Pharmingen, CA, USA) was added into PH1127 cultures at the same time as the peptide(s) and cells. The cells were pulsed for the last 16–18 h of incubation with 1 µCi/well of tritiated (³H)-thymidine (PerkinElmer, VA, USA), and harvested onto glass fiber filters (PerkinElmer) for measurement of radiolabel incorporation by liquid scintillation spectroscopy. Results are presented as the mean counts per minute (cpm) ± standard error of the mean of triplicate cultures.

CD4⁺ T-cell line

PBMCs from both PH1127 and PB3019 were labeled with 0.5 µM carboxyfluorescein succinimidyl ester (CFSE; Invitrogen, CA, USA) and stimulated with NS3₃₅₈ synthetic peptide. On day 7, the cells were stained with anti-CD4-allophycocyanin (APC; Invitrogen) and sorted at the University of Utah cell-sorting core facility. The CD4⁺ T cells were sorted by either CFSE^{high} (nonproliferating) or CFSE^{low} (proliferating). The cells were washed and expanded by CD3/CD28 Dynabeads® (Invitrogen) with 2 ng/ml of human rIL-2 (BD Pharmingen). The CD3/CD28 beads were removed 3 days prior to testing T-cell responses. CD4⁺ CFSE cells, at 2×10^4 cells/well, were stimulated with increasing concentrations of NS3₃₅₈ peptide presented by a mixture of chronic and resolved HLA-DR15 antigen-presenting cells treated with 10 µg/ml mitomycin C from *Streptomyces caespitosus* (Sigma) at 1×10^5 cells/well for 3 h prior to incubation (Supplementary Figure 1; see online at www.futuremedicine.com/doi/suppl/10.2217/FVL.13.49).

Foxp3 staining

Cultures were stained with the 358–375-phycoerythrin (PE) tetramer (NIH Tetramer Facility at Emory University, GA, USA) for 1 h. CD4-Pacific Blue (BD Bioscience, CA, USA), CD3-Amcyan (BD Bioscience), CD25-APC (BD Bioscience) and 7-amino-actinomycin D

(7-AAD; BD Bioscience) were added for the last 20 min at 4°C and then the cells were washed two times with staining buffer (BD Pharmingen). Using a Foxp3 staining kit (eBioscience, CA, USA), the cells were fixed and permeabilized for 1 h at 4°C, and washed two times in permeabilization buffer. Normal rat serum was added (2 µl/100 µl) for 15 min and then the cells were stained with anti-Foxp3-FITC (eBioscience) for 1 h at 4°C, washed two times with staining buffer and analyzed on a BD FACSCanto™II. To account for nonspecific fluorescence and antibody staining, we performed fluorescence-minus-one with isotype control. These fluorescence-minus-one controls contained all of the antibody conjugates used in the experiment except Foxp3-FITC, with the addition of a FITC-isotype control. This was performed for each culture condition. Flow cytometry data analysis was performed using FlowJo software (Tree Star, OR, USA).

Tetramer staining

T cells were stained with tetramers (NIH Tetramer Facility at Emory University): wild-type 358–375-PE, variant H369R-APC, variant S370P-APC or variant K371E-APC for 1 h at 37°C. Then, 7-AAD, anti-CD4-Pacific Blue, anti-CD3-Amcyan or anti-CD8-FITC (eBioscience) were added for 15 min at 4°C. The cells were washed with flow-staining buffer (BD Bioscience) and analyzed by flow cytometry. Negative controls consisted of staining cells with a nonspecific peptide, CLIP-DR15 tetramer, labeled with either PE or APC. In addition, CD4⁺CFSE^{low} T cells specific for tropomyosin were used as controls to determine the specificity for each tetramer (Supplementary Figures 2–4) [29]. Flow cytometric analysis was carried out using FlowJo software.

Results

Both NS3₃₅₈ & variant S370P peptides enhanced T-cell suppression

Previous work by us demonstrated that variant S370P was able to attenuate T-cell responses to HCV NS3₃₅₈ peptide [21]. To determine whether viral variant S370P was able to suppress T-cell responses to a mitogen, PBMCs from PB3019 were cultured with either each individual peptide (1 µM) or both peptides together, and then the cells were stimulated with 2 µg/ml PHA (Figure 1). PHA is a plant-derived protein that can cross-link glycosylated proteins on the surface of T cells, leading to nonspecific activation of human T cells. PBMCs preincubated with NS3₃₅₈ and subsequently stimulated with PHA ($121,192 \pm 10,280$ cpm) showed no effect on proliferation in comparison to PHA-induced proliferation ($98,280 \pm 8105$ cpm; Figure 1). PBMCs preincubated with S370P had a significant decrease in proliferation ($66,149 \pm 11,244$ cpm Figure 1). The addition of NS3₃₅₈ and PHA to PBMCs preincubated with S370P led to a marked suppression of proliferation ($48,696 \pm 6581$ cpm). As a control, PBMCs were preincubated with S370P at various concentrations and then stimulated with either an influenza antigen or a different HCV epitope, and S370P had no effect on the influenza or other HCV epitope T-cell proliferative responses (data not shown). Therefore, the use of a nonspecific activator of T cells (PHA), in conjunction with S370P, most likely led to the engagement of NS3₃₅₈-specific T cells. Conversely, nonspecific antigens, such as influenza proteins, and a different HCV epitope were not able to engage NS3₃₅₈-specific T cells. These results suggest that the suppressive phenotype was enhanced in the presence of both the viral variant and wild-type peptide, and that suppression was dependent on the peptide–MHC engaging the TCR on wild-type HCV-specific T cells. These results led us to investigate the optimal concentrations of variant and wild-type peptides that, in combination, caused inhibition of T-cell proliferation.

Variant S370P acts as an antagonist

To determine whether variant S370P was an antagonist, CD4⁺CFSE^{low} T cells were stimulated with either NS3₃₅₈ or S370P peptides in a dose-dependent manner (Figure 2A).

Variant S370P had an attenuated T-cell response. When the antigen-presenting cells were prepulsed with variant S370P (1 μ M) and the CD4⁺CFSE^{low} T cells were then cultured with an increasing concentration of NS3₃₅₈ peptide, S370P reduced the proliferative response to the NS3₃₅₈ peptide (Figure 2B). However, a lack of proliferation was not found at lower NS3₃₅₈ peptide concentrations, suggesting that S370P's inhibitory effect is dependent on NS3₃₅₈-specific T cells reaching a certain level of activation for suppression of proliferation to occur. Taken together, these results demonstrate that S370P was able to attenuate the T-cell response in an antigen-specific manner; however, NS3₃₅₈ peptide stimulation was able to overcome the suppressive effect of the variant S370P (Figure 2B).

Variants S370P & K371E have lower avidities in comparison to NS3₃₅₈ & H369R

To determine if the viral variants could bind to the TCR of NS3₃₅₈-specific CD4⁺ T cells, MHC class II tetramer staining dilution assays were performed (Figure 3). Nonspecific CLIP-tetramer staining, at 12 μ g/ml, was performed as a control for each peptide-specific tetramer staining dilution experiment (Figure 3A, representative flow plots). The frequency of tetramer-positive cells was similar between H369R and NS3₃₅₈; however, S370P and K371E variants had a lower frequency of tetramer-positive T cells when staining NS3₃₅₈-specific T cells expanded from both PB3019 and PH1127 (Figure 3B). The median fluorescence intensity had a similar trend as the frequency of tetramer-positive cells, in that wild-type and H369R were similar and variants S370P and K371E had a fourfold lower median fluorescence intensity (Figure 3C). To confirm these results, tetramer competition assays were performed using CD4⁺ NS3₃₅₈-specific T cells. The CD4⁺ T-cell lines expanded from both PH1127 and PB3019 were stained with each variant and NS3₃₅₈ tetramer. Each tetramer was added at the same time. Variant H369R had a much higher avidity at 2 μ g/ml than the NS3₃₅₈ tetramer, indicating that this variant could potentially be anergizing these cells (Table 1 & Supplementary Figures 2–4). However, the S370P and K371E variant tetramers were able to bind to NS3₃₅₈-specific T cells with similar frequencies as the wild-type tetramer, suggesting that the timing of when NS3₃₅₈-specific T cells engage the variant is important in determining if the cells are antagonized (Table 1). Taken together, the variant peptides have different avidities for the same TCR, which suggests that the variants are potentially able to modulate NS3₃₅₈-specific CD4⁺ T cells.

Variant S370P induces Foxp3 in NS3₃₅₈-specific CD4⁺ T cells

To address if variant S370P was inducing NS3₃₅₈-specific CD4⁺ T cells to differentiate into Tregs, Foxp3 frequency was determined in NS3₃₅₈ MHC class II tetramer-positive cells. NS3₃₅₈-specific CD4⁺ T-cell lines, derived from both PH1127 and PB3019 (Supplementary Figure 1), were stimulated with either the individual peptides or both S370P and NS3₃₅₈ peptides at 1 μ M using irradiated HLA-matched PBMCs as antigen-presenting cells. The CD4⁺ T cells were stained with the NS3₃₅₈ tetramer in combination with the indicated antibodies (Figure 4). The histograms represent 7-AAD⁻CD8⁻CD3⁺CD4⁺ NS3₃₅₈ DRB1*1501⁺ cells that are Foxp3⁺ (Figure 4). The S370P-stimulated T cells have a higher frequency of Foxp3 (Figure 4B & 4C, red histograms). Taken together, S370P drives the CD4⁺ T-cell lines to differentiate into Tregs. To our knowledge, this is the first demonstration of a naturally occurring virus variant inducing Th1 cells to differentiate into Tregs.

IL-10 is necessary for the expansion & maintenance of Foxp3

To determine if the extracellular milieu was involved in the induction of Foxp3, human rIL-10 was added into culture with PBMCs and peptide(s) (Figure 5). PBMCs from patient PH1127 were cultured under normal conditions (Figure 5B, light green bars) or with rIL-10 (2 ng/ml) (Figure 5B, dark green bars) and PBMCs were stimulated with 1 μ M of synthetic

peptide for 7 days and analyzed by flow cytometry (Figure 5). S370P-stimulated cells incubated with rIL-10 had a significantly higher percentage of Foxp3⁺ cells in comparison with NS3₃₅₈-stimulated cells (Figure 5). These results suggest that both TCR engagement and the extracellular milieu are important for the expansion of Foxp3-expressing CD4⁺ T cells.

Discussion

We have demonstrated that naturally occurring viral variants induce antigen-specific CD4⁺ T cells to differentiate into Tregs *in vitro*. Furthermore, the combination of the wild-type and variant peptide enhanced suppression of a virus-specific T-cell response. These results indicate that a stable viral variant, arising in an epitope that is conducive to viral clearance, was able to alter the differentiation of a T cell with a virus-clearing CD4⁺ T-cell response to a Treg. Thus, we propose a model in which naturally occurring viral variants are acting as APLs. Furthermore, these viral variants work synergistically with the wild-type peptides to differentiate T cells in an antigen-specific manner, as well as suppress T-cell responses nonspecifically. Therefore, we have termed this potential mechanism of viral persistence 'convergent suppression' (Figure 6).

Viral variation or quasispecies accounted for <20% of the circulating viral sequences in the chronically infected patient used in this study [8,45]. From these identified viral variants arising in the NS3₃₅₈ epitope, there was a subset of variants able to attenuate the T-cell proliferative responses to the wild-type HCV peptide. One mechanism for the 'fixation and selection' of S370P in conjunction with the wild-type peptide could be that both the variant and wild-type peptides are necessary for suppression. More specifically, we showed that the NS3₃₅₈ peptide induces IL-2 when cultured with PB3019 PBMCs [4]. S370P has been found to induce Treg phenotypic markers in T cells. TCR engagement and IL-2 signaling on a T cell can lead to transcription factors binding to the Foxp3 promoter and enhancer, suggesting that IL-2 is necessary for the induction of inducible Tregs (reviewed in [46]). Therefore, the maintenance of an IL-2-inducing viral epitope would be advantageous for viral persistence due to the importance of IL-2 in Treg differentiation in combination with TCR engagement. However, in order for the virus to evade clearance, the variant peptide(s) would have to be presented prior to the immunodominant NS3₃₅₈ peptide.

Recently, a number of groups have demonstrated that low-dose peptide-MHC can induce naive CD4⁺ T cells to differentiate into Foxp3⁺ Tregs [47, 48]. In addition, recent work by Turner *et al.* demonstrated *in vitro* that low-dose cognate antigen presented by dendritic cells to T cells led to the induction of Tregs, and that weak TCR stimulation correlated with an increase in Foxp3 expression [49]. However, *in vivo* studies by Gottschalk *et al.* demonstrate that Foxp3 induction in antigen-specific murine CD4⁺ T cells was dependent on high peptide-MHC concentrations, suggesting that not only ligand density, but also potency of peptide and kinetics of the TCR binding to peptide-MHC complexes are important factors for T cells to differentiate into Tregs [50]. In this study, we demonstrated that pretreatment of PBMCs with S370P led to inhibition of proliferation in response to PHA (Figure 1), but not to an unrelated antigen, suggesting that S370P specifically inhibits NS3₃₅₈-specific T cells. Antagonism assays demonstrated that variant and wild-type peptide concentrations and kinetics were important for the inhibition of CD4⁺ NS3₃₅₈-specific T cells, suggesting that there is a 'sweet spot' for the inhibition of proliferation to occur (Figure 2). In addition, NS3₃₅₈-specific MHC class II tetramer-positive cells from two subjects pretreated with S370P had a higher frequency of Foxp3 expression (Figure 4). Further investigation into the role of variant peptide concentrations on the induction of Foxp3 in CD4⁺ NS3₃₅₈-specific T cells is necessary to elucidate variant ligand potency and Foxp3 expression in our system.

A defining feature of APLs is the ability of the antagonist peptide coupled to MHC to bind to the TCR. Tetramer avidity assays using MHC class II tetramers clearly demonstrated a difference in avidity between the wild-type and variants, with the exception of H369R (Figure 3). It appears that H369R has a similar avidity to the wild-type tetramer. Functional data indicate that the H369R variant epitope blunts the NS3₃₅₈-specific T-cell proliferation, which is indicative of T-cell anergy. Similar results from the tetramer avidity tests were found when each variant was added into culture with the wild-type tetramer, in that S370P and K371E were able to bind to the same T cell, but H369R had a higher avidity for the T cells due to the higher frequency of H369R-positive cells in comparison with wild-type-positive cells (Table 1). In addition, these tetramer competition assays indicated that both the variant and the wild-type tetramer were able to bind to the same T cell, thereby providing further evidence that both the variant and cognate peptides are working in concert on the same T cell (Table 1 & Supplementary Figures 2–4). These naturally occurring APLs within a protective immunodominant epitope could possibly have differential effects on TCR activation and may be used by HCV to deviate or blunt a HCV-specific T-cell response. Further investigation is needed to determine how S370P engagement of NS3₃₅₈-specific TCRs modulates TCR downstream signaling molecules, such as Akt and/or mTOR, thereby inducing Foxp3 expression.

The quality of the CD4⁺ T-cell response has been found to be critical in the clearance of HCV, but the detection and expansion of CD4⁺ T cells derived from chronically infected HCV subjects has been hampered by a lack of detection and proliferation [5,15–19]. Recently, Mueller *et al.* detected virus-specific CD4⁺ T cells in chronic HCV patients, indicating the presence of HCV-specific CD4⁺ T cells in chronic patients [51]. However, evidence that HCV-specific CD4⁺ T cells ‘disappear’ from human blood in patients that are unable to resolve HCV infection has recently been demonstrated by tracking HCV-specific CD4⁺ T cells in a cohort of HCV subjects [19]. Unfortunately, these descriptive studies do not provide any evidence as to how HCV is able to eliminate HCV-specific CD4⁺ T cells, except to hypothesize that HCV infection causes exhaustion and deletion [19]. To determine if pretreatment of PB3019 PBMCs with variant peptides caused anergy, we added human rIL-2 into culture with cells prepulsed with variant peptide and then added wild-type peptide. T-cell proliferation in IL-2-treated cultures was restored to the same level as wild-type, suggesting that one mechanism that these variants use to inhibit proliferation is by anergizing T cells (data not shown). These results also support our hypothesis that priming the immune system with this wild-type peptide in individuals with the appropriate HLA type could potentially be used therapeutically to combat HCV.

The most widely accepted Treg marker, Foxp3, is dependent on IL-10 signaling in CD4⁺ T cells [42]. Further, it has been demonstrated that IL-10 serum levels are significantly higher in chronic versus resolved HCV subjects, thereby providing an environment that is conducive to Treg differentiation and maintenance [7]. Although the levels of IL-10 have been found to be higher in the serum of chronic subjects, the cell type(s) responsible for the IL-10 secretion have not been clearly defined and need further investigation, need to include the examination of S370P-engaged NS3₃₅₈-specific CD4⁺ T cells and/or need to determine the effect on Treg induction when IL-10 is neutralized. Similarly, the viral variant S370P epitope was able to activate and maintain Foxp3 expression in comparison with the NS3₃₅₈ peptide when in the presence of IL-10, whether produced endogenously in a chronic patient or provided exogenously to cells in a resolved subject *in vitro* (Figure 5). The suppressive nature of IL-10 is nonspecific and presumably the immune system in chronic HCV subjects would be dampened, but in a cohort of subjects tested for T-cell responses to influenza antigens, it was found the T-cell response was not significantly lower when compared with resolved HCV subjects, suggesting that the suppressive effect of HCV is antigen-specific [29].

Conclusion

In our study, naturally occurring APLs alter TCR signaling in a specific population of CD4⁺ T cells, as indicated by our T-cell proliferation and tetramer avidity results. One variant, S370P, was able to induce Foxp3 expression in NS3₃₅₈-specific MHC class II tetramer-positive cells in PBMCs derived from two individuals. This mechanism of Treg induction is HCV antigen-specific, and the phenomenon of Treg induction is not exclusive to one chronic HCV subject. We have termed this mechanism of viral persistence ‘convergent suppression’ (Figure 6). These results reveal that HCV may be able to shift the immune response towards viral persistence under the right circumstances or within an immune and viral ‘sweet spot’, suggesting that priming the immune system with a viral clearing CD4⁺ T-cell response could provide protective immunity against HCV.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Executive summary

- Both cognate and variant HCV peptides enhance suppression of T-cell proliferation.
- Variant S370P antagonizes NS3₃₅₈ antigen-specific T cells.
- NS3₃₅₈ and variant tetramers have different T-cell receptor avidities using MHC class II tetramers.
- Viral variant S370P is able to induce Foxp3 expression in an antigen-specific manner.
- In conjunction with previous work from our laboratory, naturally occurring viral variants act as altered peptide ligands that work synergistically with the wild-type peptide to differentiate T cells in an antigen-specific manner, as well as suppress T-cell responses nonspecifically, a mechanism that we have termed 'convergent suppression'.
- Further characterization as to what cell types are produce IL-10 in chronically infected patients would strengthen the observation that Tregs are important in viral persistence.
- Future studies should be performed to determine if priming the immune system with viral clearing CD4⁺ T-cell response(s) provides protective immunity.

Figure 1. Both cognate and variant peptides enhance suppression of T-cell proliferation

PB3019 peripheral blood mononuclear cells (PBMCs) were stimulated with individual peptides (blue bars). The PBMCs were preincubated with either S370P or NS3₃₅₈ 3 h prior to the addition of PHA (1 µg/ml; purple bars). PBMCs were preincubated with S370P for 3 h, and NS3₃₅₈ and PHA (1 µg/ml) were added (green bars). ³H-thymidine was added at day 6 and ³H-thymidine uptake was measured 16–18 h later.

p* < 0.05; *p* < 0.005, as determined by Student's paired *t*-test.

cpm: Counts per minute;

PHA: Phytohemagglutinin.

Figure 2. Variant S370P antagonizes NS3₃₅₈ antigen-specific T cells

(A) T- cell proliferation assays with individual peptides added in a dose-dependent manner to CD4⁺CFSE^{low} T cells (2×10^4 cells/well) from both PH1127 and PB3019 patients with 1×10^5 antigen-presenting cells/well and incubated for 72 h. (B) Antigen-presenting cells (1×10^5 cells/well) were prepulsed with S370P for 3 h, washed, treated with mitomycin C and added to CFSE^{low} T cells (2×10^4 cells/well) for 72 h in the presence of NS3₃₅₈ peptide. Proliferation was measured by ³H-thymidine uptake assays (see 'Patients, materials & methods' section). Assays were performed in triplicate and data are representative of two independent experiments for both PH1127 and PB3019. Data are the averages of both PH1127 and PB3019.

* $p < 0.05$; ** $p < 0.005$, as determined by Student's paired t -test.
cpm: Counts per minute; Med: Medium.

Figure 3. NS3358 and variant tetramers have different T-cell receptor avidities

(A) Representative histograms of NS3358-specific MHC class II tetramer staining of CD4⁺CFSE^{low} T cells expanded from PB3019 performed in decreasing concentrations of individual tetramers. (B) Frequency of tetramer-positive cells for each tetramer staining of CD4⁺CFSE^{low} T cells expanded from PB3019 and PH1127. (C) The MFI for each tetramer is shown for both PB3019 and PH1127.

MFI: Median fluorescence intensity; WT: Wild-type.

Figure 4. Viral variant S370P is able to induce FcγR3 expression in an antigen-specific manner (A) CD4⁺CFSE^{low} T cells from PH1127 and PB3019 were stimulated with either individual peptides (1 μM) or prepulsed with S370P (1 μM) and cultured for 72 h. The cells were stained with NS3₃₅₈-specific MHC class II tetramer. (B & C) Tetramer-positive cells were then stained for FcγR3. Gating for MHC class II tetramer staining was determined by control tetramer CLIP-*DRB1**1501. Darker green histograms are NS3₃₅₈-stimulated T cells and red histograms are the S370P-stimulated CFSE^{low} cells that are MHC class II tetramer-positive.

7-AAD: 7-amino-actinomycin D; ctrl: Control; FMO: Fluorescence-minus-one.

Figure 5. Foxp3 expression is induced in an antigen-specific manner with the addition of IL-10 Peripheral blood mononuclear cells from PH1127 were cultured under normal conditions (light green bars) or with rIL-10 (2 ng/ml; dark green bars) and peripheral blood mononuclear cells were stimulated with 1 μ M of synthetic peptide for 7 days and analyzed by flow cytometry. The normal and rIL-10 conditions were run in tandem and the data were normalized to medium. Error bars are representative of the standard deviation. Results are representative of two experiments.

** $p < 0.005$, as determined by Student's paired t -test.

rIL-10: Recombinant IL-10.

Figure 6. Model of convergent suppression in HCV infection

If the wild-type peptide (dark blue) is presented to the CD4⁺ T-cell prior to the variant peptide (light green), this could lead to viral clearance. If the viral variant (green) is presented to the wild-type CD4⁺ T-cell before the wild-type peptide, this could lead to viral persistence by antagonizing these antigen-specific CD4⁺ T cells to become Tregs.

APL: Altered peptide ligand; TCR: T-cell receptor.

Adapted from [22].

Table 1

Variant and NS3₃₅₈ tetramer competition for PB3019 and PH1127 CD4⁺CFSE^{low} T cells.

Tetramer	Patient	
	PB3019	PH1127
<i>NS3358 /H369R</i>		
NS3358	0.076	0.11
H369R	4.56	1.69
NS3358 + H369R	0.2	0.085
<i>NS3358/S370P</i>		
NS3358	0.059	0.078
S370P	0.31	0.52
NS3358 + S370P	0.074	0.1
<i>NS3358/K371E</i>		
NS3358	0.18	0.53
K371E	0.5	0.65
NS3358 + K371E	0.059	0.23

All units represent the tetramer-positive percentage of CD4⁺CFSE^{low} T cells.

The H369R variant had the highest avidity in comparison with the NS3₃₅₈ tetramer. Each variant tetramer was added to CD4⁺CFSE^{low} NS3₃₅₈ antigen-specific T cells at the same time as the NS3₃₅₈ tetramer (see Supplementary Figures 2–4).