CONVERGENT SUPPRESSION AS A MECHANISM FOR PERSISTENCE OF HEPATITIS C VIRUS IN A HUMAN IN VITRO SYSTEM

by

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STATEMENT OF DISSERTATION APPROVAL

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ABSTRACT

Although a causal relationship between hepatitis C virus (HCV) persistence and regulatory T cells (Tregs) has been purported, to date, no one has demonstrated how the induction of Tregs occurs. The central question of this dissertation was to address if HCV exploits known $CD4^+$ T cell suppressive mechanisms to cause persistent infection in humans. The central finding of these studies was that naturally occurring viral variants of HCV that emerged in a major histocompatibility complex class II epitope of the non-structural-3 protein were acting as altered peptide ligands possibly leading to the differentiation of HCV specific $CD4^+$ effector T cells into Tregs. To our knowledge, this work is the first demonstration of naturally occurring viral variants inducing Tregs, thereby defining an immunological niche that HCV is exploiting for persistence in humans.

Dedicated to my little Clan; especially my wife Susanne along with Grace, Andrew, and my parents, Darby and Joseph Cusick

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LIST OF ABBREVIATIONS

Antigen presenting cell (APC)
Altered peptide ligand (APL)
Forkhead Box P3 (Foxp3)
Hepatitis B Virus (HBV)
Hepatitis C Virus (HCV)
Human Immunodeficiency Virus (HIV)
Human Leukocyte Antigen (HLA)
Hypervariable region 1 (HVR1)
Interleukin (IL)
Interferon (IFN)
Major histocompatibility complex (MHC)
Nonstructural- 3 (NS3)
Pathogen-associated molecular pattern (PAMP)
Peripheral blood mononuclear cell (PBMC)
Recombinant nonstructural 3 protein (rNS3)
Regulatory T cell (Treg)
Retinoic acid-inducible gene-I (RIG-I)
Ribonucleic acid (RNA)
T cell receptor (TCR)
T helper cell (T_H)
Toll-like receptor (TLR)
Transforming growth factor β (TGFβ)

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CHAPTER 1

INTRODUCTION

The Hepatitis C Virus (HCV) infects approximately 3% of the world's population and is considered a global epidemic by the World Health Organization (WHO) (1). HCV is the most prevalent blood borne pathogen in the United States, and carries with it the potential risk for liver cirrhosis and hepatocellular carcinoma, making HCV the foremost reason for liver transplantation (2). To date, the only approved treatment available for chronic HCV subjects is a combination of pegylated interferon-α (IFN-α) and ribavirin therapy, but this method is successful in less than 50% of patients, because the outcome is wholly contingent upon the patients' HCV genotype (3). This course of treatment, however, is not a long-term solution to this global epidemic due to the efficacy, side effects, and cost associated with these drugs (4). Attempts at developing alternative therapeutics and vaccines for HCV have been unsuccessful mainly due to the sequence heterogeneity of the HCV genome. This heterogeneity allows HCV to evade the immune response, or at least impart a specific tolerance in the patient, thus ensuring the virus' survival in over 80% of infected individuals through mechanisms including, but not limited to, viral escape, T cell anergy, and induction of regulatory T cells (Tregs).

HCV Genome

HCV, an enveloped positive-stranded RNA virus, is the only member of the genus *Hepacivirus* of the family *Flaviviridae*. There are 6 HCV genotypes with more than 100 subtypes (3). The virus is translated into a 3,000 amino acid poly-protein and cleaved into 10 proteins by both host and viral proteases (Figure 1.1). Further, the poly-protein is sub-divided into structural (core, envelope 1 and 2) and nonstructural (NS2, NS3, NS4A,

NS4B, NS5A, and NS5B) proteins, with a p7 protein between these two groups of proteins (Figure 1.1) (5).

HCV is a hepatotropic virus, but is nonetheless still able to reside in peripheral blood mononuclear cells (PBMC), serum, and the central nervous system (6, 7). HCV entry is dependent on numerous receptors such as sulfated heparin sulfate, low-density lipoprotein receptor, CD81, scavenger receptor BI, claudin-1, and occludin (8-14). After attaching to the cell surface, the virus is endocytosed via clathrin coated pits into the cell (reviewed in (2)). Translation of the viral genome occurs in the cytoplasm and is initiated at the internal ribosome entry site (IRES). HCV is able to form a lipid "web-like" membrane for replication and assembly (2). The structural components of the virus important for entry and assembly are flanked by NS2 to NS5b. NS2 is a protease that acts in *cis* by cleaving between NS2 and NS3 (5). NS3 has both protease and helicase activity. NS3 is responsible for the cleavage of the other nonstructural proteins with NS4A, making this protein essential for viral replication. The helicase activity of NS3 is responsible for unwinding RNA and DNA, but it is still not clear how this process is important to viral replication. The function of NS4B and NS5A is likewise unclear. However, NS5B has viral RNA-dependent RNA polymerase activity, indicating its importance in viral replication. The focus of this dissertation is on NS3, due to the importance of this protease in viral replication.

Further, the NS3 genotype used in this dissertation is genotype 1a. This genotype is the most common HCV genotype in the United States (15); its rate of replication is extremely high, at approximately 1×10^{12} virions per day (16). Similar to other RNA viruses, HCV lacks RNA-proof reading, leading to a heterogeneous population of the

viral genome. The most frequently represented genome is referred to as the wild type sequence; the viral variants arising in the viral genome are called quasispecies (17). The majority of circulating virus in chronic HCV patients appears to be the wild type population (18-20). The role of these quasispecies or viral variants in HCV pathogenesis can possibly range from simple escape from immune detection to deviation of the immune system (21, 22). The combination of high viral replication and high error rates can be deleterious for viral clearance.

Factors in HCV Clearance

HCV clearance is dependent on both a robust innate and adaptive immune response. The host's first line of defense against HCV is the innate immune response when the virus engages pathogen-associated molecular pattern (PAMP) receptors (23). During the viral replication a negative strand RNA of HCV is synthesized and it acts as a template for the synthesis of additional genomic positive-stranded RNA destined for viral progeny. The double stranded RNA is recognized by the toll-like receptor 3 (TLR3) and the retinoic acid-inducible gene I (RIG-I)-receptor, both PAMP receptors, leading to transcription and secretion of type-1 IFNs, thereby creating an antiviral state. The process of HCV being largely asymptomatic in the early onset of infection has led to a major challenge in studying the innate immune response to HCV; also, having few model systems has led to a deficit in knowledge as to how the human immune system is able to clear the virus during the acute phase of infection.

Although the innate immune system's role in HCV clearance is not clearly defined, in general, the innate immune system is able to initiate or "set the stage" for the

adaptive immune response during viral infections. The adaptive immune response is triggered when T cells are activated. T cell activation occurs when a T cell receptor (TCR) binds to cognate or specific peptide bound to major histocompatibility complex (MHC) molecules on the surface of antigen presenting cells (APCs) (24). The engagement of the TCR to pMHC is necessary for the activation of $CD4^+$ and $CD8^+$ T cells, thereby leading to an effective adaptive immune response against an invading pathogen (Figure 1.2) (25). If co-stimulatory molecules associated with T cell activation are not activated along with TCR engagement, the T cells can become unresponsive.

The recognition of viral epitopes by T cells is critical for the clearance of viruses (26). In the case of HCV infection, clearance of the virus has been found to be dependent upon the quality of the $CD4^+$ T cell response in up to 30% of infected individuals (27-30). Although $CD8^+$ T cells are responsible for killing virus-infected cells, it is now accepted that $CD4^+$ T cells are also critical for HCV clearance (31). $CD4^+$ T cells help direct the adaptive immune response to viral infections by providing help for $CD8^+$ T cells, enhancing antigen presentation, and playing a role in B cell maturation. The most direct correlation showing the importance of HCV-specific $CD4^+$ T cells was a study demonstrating antibody-depletion of $CD4^+$ T cells from chimpanzees prior to reinfection (28). The depletion of $CD4^+$ T cells resulted in persistent infection, despite the presence of memory HCV-specific $CD8^+$ T cells (28). Although this experiment clearly demonstrated the importance of $CD4^+$ T cells in HCV clearance, it did not reveal a specific role for these cells in viral clearance. *In vitro* data using chronic and resolved HCV PBMC show an attenuation of or absent of HCV-specific $CD4^+$ T cells in chronic subjects, while the T cell responses in resolved subjects were broad and vigorous (32).

Although *in vitro* studies demonstrate a role for $CD4^+$ T cells in HCV clearance, it has not been determined how HCV is able to subvert these T cells in the case of viral persistence.

A major component of an effective immune response against HCV is the breadth and magnitude of the HCV-specific $CD4^+$ T cell responses against the HCV NS3 protein (21). Due to the importance of this protein, the focus of this dissertation is on the NS3 protein. This protein contains a number of immunogenic epitopes that have been identified in both humans and chimpanzees (18, 20, 27, 29, 33-36). Although there are a number of studies that have found an association between the numbers of epitopes recognized in resolved *vs.* chronic subjects (29, 37), the only accepted difference between these two groups is the sustainability of the antigen-specific T cell response throughout the course of infection in the resolved subjects. A genetic component that could be important for viral clearance is the MHC; these molecules are responsible for presenting the epitope or peptide fragment to T cells, leading to the hypothesis that the MHC could be a factor in viral clearance. There are certain MHC class II alleles in humans that have an association with clearance, indicating that $CD4^+$ T cells are important in HCV clearance (reviewed in (30)). These studies are still incomplete and need further investigation.

As discussed above, the $CD4^+$ T cell responses are found to be narrow and attenuated in chronic HCV subjects. In general, $CD4^+$ T cell responses are phenotypically characterized by the type of cytokines secreted along with the expression of certain transcription factors when activated. To date, there are four characterized $CD4^+$ T cell subsets. Also, the development of $CD4^+$ T cells is directed by cytokines produced by the

innate immune system. The predominant model for $CD4^+$ T cell responses is the T_H1 – Th2 paradigm (38). The differentiation of naïve $CD4^+$ T cells to effector/ memory T_H1 cells is important in clearance of intracellular pathogens. Development of T_H1 cells is dependent on IFNγ and (interleukin-12) IL-12 and secrete IL-2 and IFNγ (39, 40). The development of Th2 cells is dependent on IL-4 and this subset clears parasites by driving the humoral immune response and secretes cytokines IL-4, IL-5, IL-10, and IL-13 (41). Recently, $CD4^+$ T cells with a suppressive phenotype have been characterized and are called Tregs (42). Tregs are able to prevent autoimmunity and immunopathology associated with an infection, either through contact mediated mechanisms or secretion of IL-10 (43). Treg's differentiation is dependent on IL-10 and transforming growth factor β (TGFβ) (44). The fourth $CD4^+$ T cell subset is T_H17 cells. These cells have a role in bacterial clearance and they are differentiated into IL-17A, IL-17F, IL-21, and IL-22 secreting cells through the cytokines IL-6 and TGFβ, but the role of these cells in HCV has not been reported (reviewed in (45)). Also, a subpopulation of $CD4^+$ T cells is able to directly kill virus-infected cells through perforin and granzyme B mechanisms. Patients with chronic HCV have been shown to have higher serum levels of IL-10 and IL-4, suggestive of either Tregs or T_H2 T cells (19, 46). The induction of T_H1 responses is related to clearance of the virus (Figure 1.3) (21, 27).

Previous work in our laboratory demonstrated a functional induction of IL-10 in $CD4^+$ T cells in chronic HCV subjects, indicative of inducible Tregs, as opposed to resolved HCV subjects, which secreted T_H1 cytokines (21, 30). Furthermore, screening for immunodominant epitopes in one chronic HCV subject, using an array of synthetic peptides, found a T_H1 epitope, $NS3_{358\text{-}375}$, showing a distinct cytokine profile in contrast

to the rNS3 protein-stimulated PBMC (47). In a longitudinal study tracking viral variants in a chronic HCV subject, we identified viral variants consistent with selective immune pressure (48). One variant, S370P, was noted to be stable for over 2 years, indicating selection and fixation of this HCV viral isolate (22, 48). Simple escape and redirection of the immune response does not explain, however, the maintenance of an abundant population of wild type HCV sequences in infected patients, even years into an ongoing infection. The paradox is that viral genomes persist in the presence of T cells, which should be able to specifically recognize and help to clear virus-infected cells. This suggests that there may be another level of immunoregulation that is modulated by the viral infection (49-53). Based on these observations, we hypothesize that a population of Tregs specifically suppresses the response of the effector T cells to the HCV antigens, and this Treg-mediated suppressive activity is induced by naturally occurring viral variants that accumulate mutations in an important viral epitope recognized by T cells. This suppressive mechanism does not preclude the virus' ability to evade clearance through mechanisms such as escape and T cell exhaustion.

Tregs in HCV Infection

Recent studies on HCV have described an increase in Treg markers in cohorts of chronically infected patients when compared to resolved and noninfected individuals, possibly leading to viral persistence (Figure 1.4) (19, 54-59). Although these studies suggest a correlation between numbers of Tregs and HCV clearance, it has not been determined if Tregs are induced in an antigen-specific manner, or if they are up regulated to inhibit immunopathological damage associated with a chronic infection (Figure 1.4).

There are two main subsets of Tregs: (I) thymically selected natural Tregs (nTreg), which are phenotypically defined as $CD4^{+}$ $CD25^{hi}$ $Foxp3^{+}$, and (II) "inducible" Tregs, activated in the periphery, termed either Tr1 or Th3, and defined as secreting IL-10, TGFβ and possibly IL-4 (60, 61). A variety of markers are available to define Tregs, but the most generally accepted marker is the expression of Foxp3 (42). This expression positively correlates with the development of Tregs that have the capacity to suppress the *in vitro* and *in vivo* proliferation and function of effector T cells (62-66). Recent Studies have found a correlation between α-chain of IL7R (CD127) and Treg cells (67). Golden-Mason et al. (68) also found a correlation between CD127 expression and the virological outcome of acute HCV, suggesting a relationship between HCV persistence and an increase in Tregs. The mechanism of the induction of Tregs has not been delineated and is a major focus of this dissertation.

Role of Altered Peptide Ligands (APLs) in Viral Infection

Single amino acid substitution in the cognate peptide, termed APLs, can elicit a gradient of effector function in a specific T cell (Figure 1.2) (69). One such mechanism that HCV may be able to exploit for persistence includes APLs, which have been observed within the hypervariable region 1 (HVR1) of HCV (70, 71), and in other pathogens, such as, Hepatitis B Virus (HBV) (26, 72), Human Immunodeficiency Virus (HIV) (73, 74), and *plasmodium falciparium* (75). Our previous studies identified and tracked viral variants arising in a single HCV chronic individual and further determined the effect these variants had on the *in vitro* immune response (22, 48, 76). Although HCV circulates in the host as a quasispecies, it appears that such variation does not lead to viral

escape, but rather that some variants are able to suppress the immune response in an antigen-specific manner.

Convergent Suppression

Although the induction of Tregs and naturally occurring APLs are used by HCV to evade clearance, these adaptations employed by the virus may or may not be part of a common mechanism. The central hypothesis of this dissertation is that HCV has implemented the mechanism of APLs to induce Tregs (Figure 1.5).

Preview of Dissertation

This dissertation demonstrates a mechanism by which HCV is able to impart tolerance to itself and possibly leading to persistence. We demonstrate *in vitro* induction of Tregs capable of suppressing antigen-specific T cell responses. We postulated that previously defined viral variants in an immunodominant epitope could be responsible for the induction of Tregs based on the cytokine shift and attenuated T cell response (21). Furthermore, we demonstrate that chronically infected subjects exhibit significantly lower T cell responses in comparison to resolved subjects. These attenuated T cell responses correlated with the induction of the Treg lineage-specific markers in HCV-specific $CD4^+$ T cells. Our initial findings indicate that variants in at least one epitope of HCV NS3 could induce Tregs and attenuate T cell responses *in vitro*.

Testing the specificity of these naturally occurring viral variants, we used T cell clones and found that these variants antagonized these T cells. These results suggested that naturally occurring variants within an immunodominant epitope may act as APLs,

leading to changes in the quality of T cell responses, which could allow for viral persistence.

The third part of the thesis is a "convergence" of Treg induction and naturally occurring viral variants acting as APLs. The suppression imparted by these variants was dependent on both the wild type and variant peptide being present. This finding sheds light on how the wild type population is able to persist in the host as the dominant quasispecies, despite being detected by the host. Lastly, one viral variant, S370P, induced an up regulation of Foxp3 in MHC class II tetramer wild type positive cells. These results demonstrate 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. These results clearly demonstrate that one mechanism of HCV persistence is through naturally occurring APLs that change effector viral clearing $CD4^+$ T cells into Tregs.

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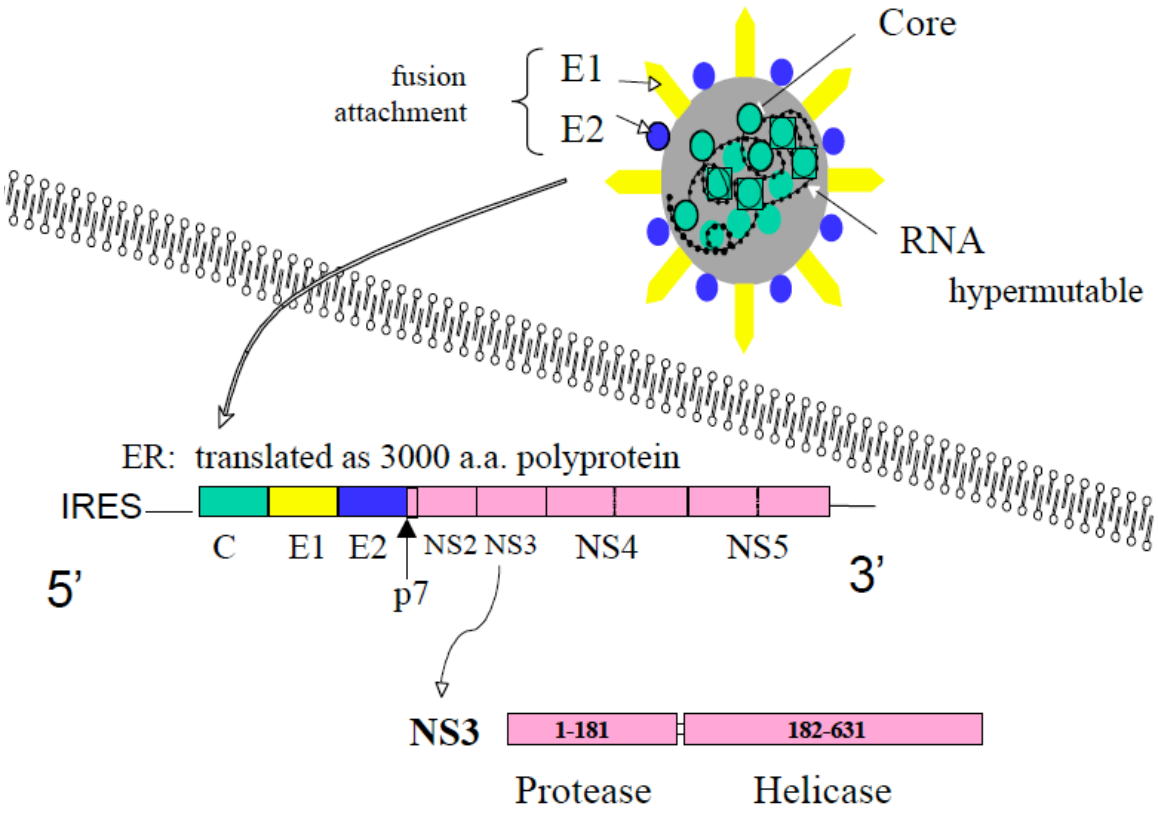


Figure 1.1. Schematic of the Hepatitis C Virus (HCV) viral life cycle. The nonstructural-3 (NS3) protein is a protease and helicase that is critical for both viral infection and replication.

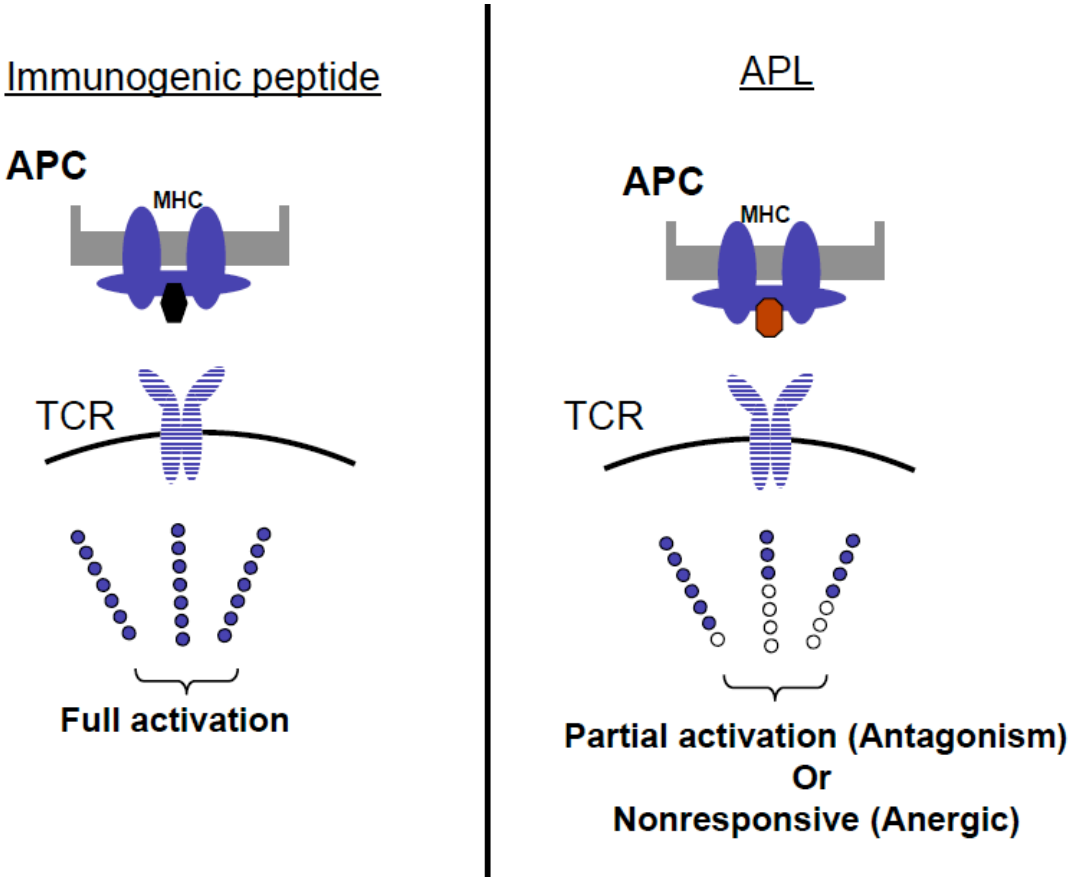
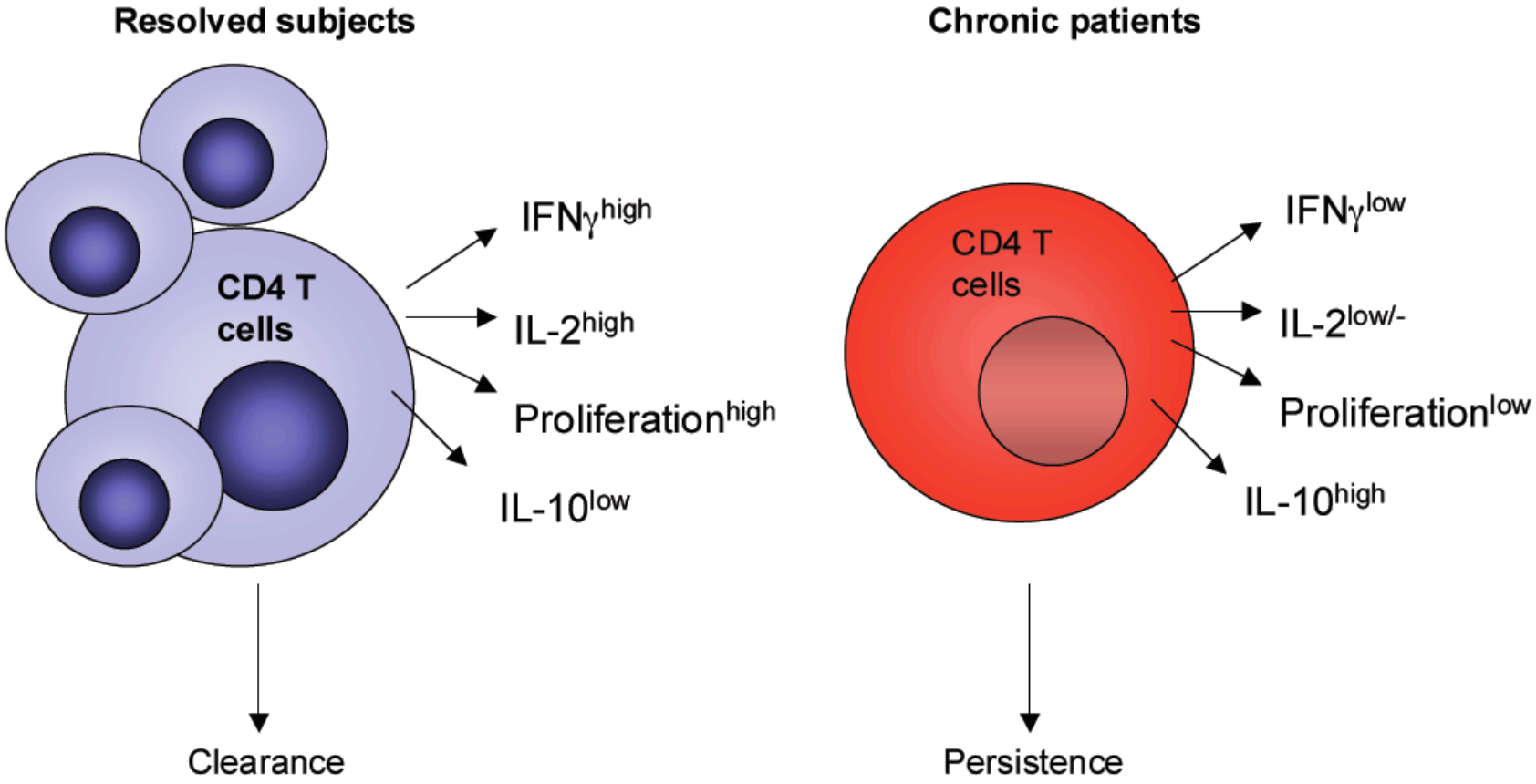


Figure 1.2. Altered peptide ligands (APLs) lead to partial activation or nonresponsiveness in $CD4^+$ T cells. The immunogenic peptide (black) is able to fully activate the antigen-specific $CD4^+$ T cells when the immunogenic peptide loaded onto the major histocompatibility complex (MHC) is presented to the T cell. APLs (red) are defined as amino acid changes in the immunogenic peptide that are able to bind to the same T cell receptor (TCR) as the immunogenic peptide, leading to partial activation or nonresponsive T cells.

Figure 1.3. Resolved subject's $CD4^+$ T cell response to NS3 protein in comparison to chronic patients $CD4^+$ T cell response to NS3 protein. Resolved HCV subjects have a $CD4^+$ T_H1 T cell response to HCV protein NS3. Chronic HCV patients have an attenuated $CD4^+$ T_H1 T cell response to HCV protein NS3.


Resolved subjects
CD4 T cells
IFNγhigh
IL-2high
Proliferationhigh
IL-10low
Clearance
Chronic patients
CD4 T cells
IFNγlow
IL-2low/-
Proliferationlow
IL-10high
Persistence

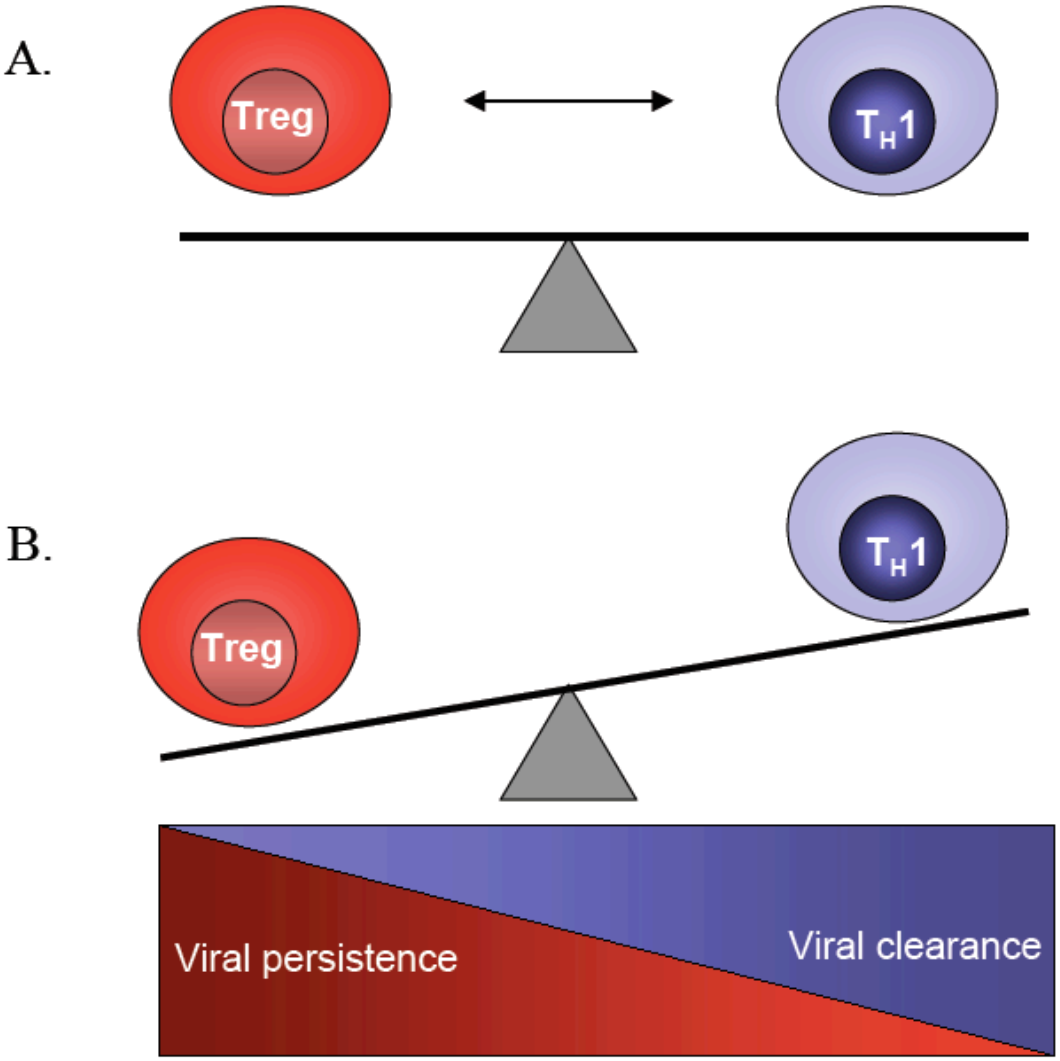


Figure 1.4. Regulatory T cell (Treg) frequency is higher in chronically infected patients when compared to resolved and noninfected subjects. A. Tregs are critical for maintaining homeostasis of the immune system. B. Chronically infected HCV patients have an imbalance of Tregs, suggesting that HCV is able to induce Tregs for viral persistence.

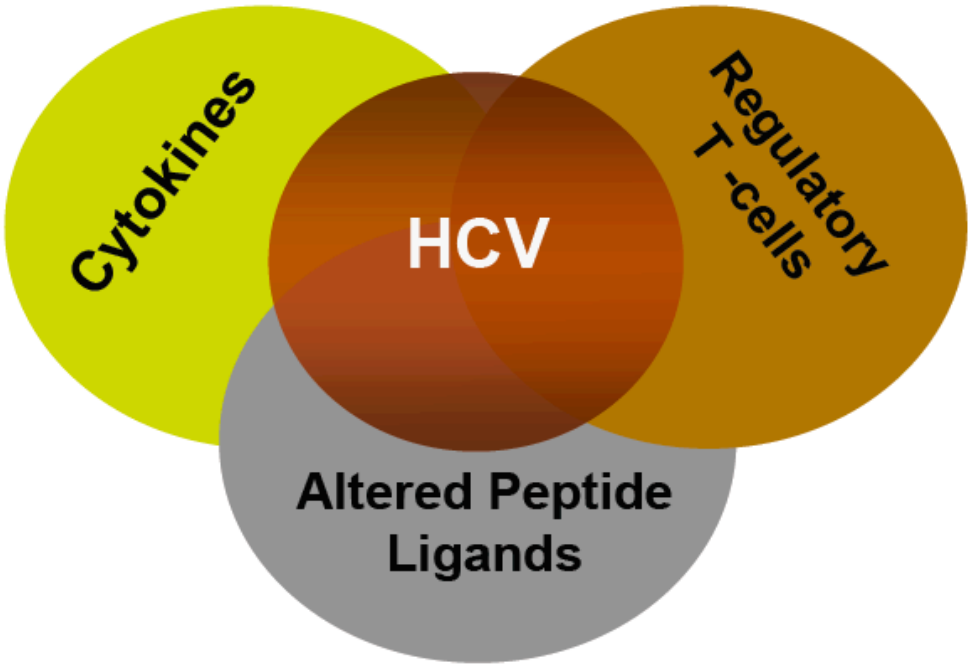


Figure 1.5. Convergent suppression. The hypothesis of the dissertation that HCV is able to exploit known $CD4^+$ T cell suppressive mechanisms to cause persistent infection in humans.

CHAPTER 2

HEPATITIS C VIRUS (HCV) INDUCES REGULATORY T CELLS BY NATURALLY OCCURRING VIRAL VARIANTS TO SUPPRESS T CELL RESPONSES

To be published: "Hepatitis C Virus (HCV) induces regulatory T cells by naturally occurring viral variants to suppress T cell responses; Matthew F. Cusick[1], Jennifer J. Schiller[2], Joan C. Gill[2], David D. Eckels; manuscript accepted into Clinical and Developmental Immunology (open access)"

Abstract

Regulatory T cell markers are increased in chronically infected individuals with the hepatitis C virus (HCV), but to date, the induction and maintenance of Tregs in HCV infection has not been clearly defined. In this study, we demonstrate that naturally occurring viral variants suppress T cell responses to cognate $NS3_{358-375}$ in an antigen specific manner. Of four archetypal variants, S370P induced regulatory T cell markers in comparison to $NS3_{358-375}$ stimulated CD4 T cells. Further, the addition of variant specific CD4 T cells back into a polyclonal culture in a dose dependent manner inhibited the T cell response. These results suggest that HCV is able to induce antigen-specific regulatory T cells to suppress the antiviral T cell response in an antigen specific manner, thus contributing to a niche within the host that could be conducive to HCV persistence.

Introduction

Hepatitis C Virus (HCV) may evade the immune response or impart a specific tolerance to itself to ensure its survival in over 80% of infected individuals through mechanisms such as, but not excusive to, viral escape, T cell anergy and induction of regulatory T cells (Treg).

Recent studies on hepatitis C virus (HCV) have described an increase in Treg markers in cohorts of chronically infected patients when compared to resolved and noninfected individuals, possibly leading to viral persistence (1-7). Although these studies suggest a correlation between Treg cell numbers and HCV clearance, it has not been determined if Tregs are induced in an antigen-specific manner, or upregulated to inhibit immunopathological damage associated with a chronic infection.

There are two main subsets of Tregs: (I) thymically selected natural Tregs (nTreg) which are phenotypically defined as $CD4^{+}$ $CD25^{hi}$ $Foxp3^{+}$ and (II) "inducible" Treg cells, activated in the periphery, termed either Tr1 or Th3 defined as secreting IL-10, TGFβ and possibly IL-4 (8, 9). A variety of markers are available to define Tregs, but the most generally accepted marker is the expression of Forkhead Box P3 (Foxp3). This expression positively correlates with the development of Treg cells that have the capacity to suppress the *in vitro* and in vivo proliferation and function of effector T cells (10-14). Recent studies have found a correlation between α-chain of IL7R (CD127) and Treg cells (15). Golden-Mason et al. also found a correlation between CD127 expression and the virological outcome of acute HCV suggesting a relationship between HCV persistence and an increase in Treg cells (16).

Previous work in our laboratory demonstrated a functional induction of IL-10 in CD4 T cells in chronic HCV subjects, indicative of inducible Treg cells, as opposed to resolved HCV subjects which secreted IL-2 and IFNγ (17, 18). Further, screening for immunodominant epitopes in one chronic HCV subject using an array of synthetic peptides, found an IFNγ and IL-2 producing epitope $NS3_{358\text{-}375}$ showing a distinct cytokine profile in contrast to the rNS3 protein stimulated PBMC (19). In a longitudinal study, tracking viral variants in a chronic HCV subject, we identified viral variants consistent with selective immune pressure (20). One variant, S370P, was noted to be stable for over 2 years indicating selection and fixation of this HCV viral isolate (20, 21). Simple escape and redirection of the immune response does not explain, however, the maintenance of an abundant population of wild type HCV sequences in infected patient's even years into an ongoing infection. This paradox is that viral genomes persist in the

presence of T cells, which should be able to specifically recognize, and help to clear virus infected cells and suggests there may be another level of immunoregulation that is modulated by the viral infection (22-26). Based on these observations, we hypothesize that a Treg population specifically suppresses the response of the effector T cells to the HCV antigens, and this Treg-mediated suppressive activity is induced by naturally occurring viral variants that accumulate mutations in an important viral epitope recognized by helper T cells.

In the present study, we evaluated the role of naturally occurring viral variants in the suppression of T cell responses to cognate $NS3_{358-375}$ *in vitro*. Of four archetypal variants, the S370P variant induced regulatory T cell markers in comparison to $NS3_{358-375}$ stimulated CD4 T cells. Further, adding variant specific CD4 T cells back into a polyclonal culture, in a dose dependent manner, inhibited the T cell response to cognate $NS3_{358-375}$. These results suggest that HCV may be able to induce regulatory T cells to suppress the antiviral T cell response in an antigen specific manner; potentially creating a niche within the host that could be conducive to HCV persistence.

Materials and Methods

Patients

Blood was collected in acid citrate dextrose and processed for PBMC isolation over Lymphocyte Separation Medium and preserved in liquid nitrogen, as previously described (27). DNA was isolated from whole blood and sent for HLA typing at the University of Utah (Table 2.1) and the lymphocytes were incubated with various concentrations of rNS3 to test for T cell responses. Quantitative RT-PCR and HCV

genotyping on all serum samples were sent to ARUP laboratories (Salt Lake City, UT). All chronic HCV subjects used in this study are genotype 1a (Table 2.1). If the subjects had no detectable viral load, the samples were screened for HCV antibodies by recombinant immunoblot assay (RIBA) carried out at ARUP laboratories. These studies have been reviewed and approved by University of Utah Institutional Review Board and the Medical College of Wisconsin Institutional Review Board.

Cell Culture and Media

Culture of PBMC was in RPMI 1640 tissue culture medium (BioWhittaker, Walkersville, ME) 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, Herndon, VA), 10 U/ml heparin sodium (Fisher Scientific, Pittsburgh, PA) and 10% pure human serum (Atlanta Biologicals, Lawrenceville, GA). Cells were cultured in a 37ºC, 5% CO_2 incubator.

Synthetic Peptides and Protein

In vitro PBMC were stimulated with synthetic peptides representing one human leukocyte antigen DR15 (HLA-DR15) restricted epitope surrounding HCV NS3 amino acids 358-375 (a.a. 1384-1401 of the HCV polyprotein). The three single amino acid variants were identified in a chronic HCV patient (P.B3019), and recombinant nonstructural protein 3 (rNS3) protein was purified as previously described (17, 28). Recombinant H3 (A/Phillipines/1992) and H5 (A/Vietnam/2004) were obtained from Protein Sciences. Peptides were synthesized using Fmoc chemistry and HPLC purified,

dissolved in a small amount of DMSO, and then adjusted to 1 mg/ml stock solutions in RPMI 1640. Peptide sequences were as follows: wild type 358-375, VIKGGRHLIFCHSKKKCD; variant H369R, VIKGGRHLIFCRSKKKCD; variant S370P, VIKGGRHLIFCHPKKKCD; variant K371E, VIKGGRHLIFCHSEKKCD.

T Cell Proliferation Assay

To measure proliferative responses of PBMC following stimulation with wild type peptide $NS3_{358-375}$ and several variants, 1 x 10^5 PBMC were plated in round-bottom 96-(29)incubated at 37ºC, 5% CO_2 for either 4 or 6 days, as indicated, pulsed overnight with 1 μCi/well of titrated thymidine (^{3}H-TdR), and harvested onto glass fiber filters for measurement of radiolabel incorporation by gas scintillation spectroscopy. Results are presented as the mean ± SEM of at least triplicate cultures (typically 6 wells/sample were analyzed), and samples were compared using Student's unpaired *t*-test. Data were considered significant at $P < 0.05$. Proliferation data was transformed using a previously described algorithm: $\log_{10}\Delta cpm = \log_{10}[\bar{X}_{exp} - \bar{X}_{exp}]$ (29).

Foxp3 Staining

PBMC were analyzed by flow cytometry to evaluate the frequency of Foxp3 in an expanding $CD4^+$ T cell population when stimulated with various antigens in both HCV chronic and resolved subjects. Carboxyfluorescein succinimidyl ester (CFSE) staining protocol was adapted from Quah et al. (30). Briefly, 0.5μM CFSE was added to PBMC in complete media + 10% PHS for 5 min. at 37°C, washed 3 times, and stimulated with the appropriate antigen(s) for 7 days. Cultures were then stained with CD4-Pacific Blue,

CD8-Amcyan, CD25-APC, and CD127-percp-cy5.5 (BD Pharmingen, San Diego, CA) for 20 min. at 4˚C and then washed 2x with stain buffer (BD Pharmingen, San Diego, CA). Using eBioscience Foxp3 staining kit, the cells were fixed and permeabilized for 1hr at 4˚C, washed 2x in permeabilization buffer. Normal rat serum was added (2μl/100μl) for 15 min. and then stained with Foxp3-PE (eBioscience, San Diego, CA) for 1hr at 4˚C, washed 2x with stain buffer and analyzed on a BD FACS Canto II. To account for fluorescence spill over and nonspecific staining, we performed fluorescence-minus-one (FMO) with isotype control. More specifically, FMO controls contain all of the antibody conjugates used in the experiment except one, with the addition of the appropriate isotype control of the fluorochrome initially excluded. This was performed for each fluorochrome and each unique culture condition. For PH1127, the addition of IL-10 was added 3 hrs. after the appropriate antigen was added to culture at 2ng/ml. Results were compared using Student's *t*-test. Flow cytometry data analysis was performed using Flow Jo software (Tree Star).

Magnetic Cell Sorting

To deplete $CD4^+CD25^+$ Treg cells from the PBMC, magnetically labeled microbeads were used and selected for using an autoMACS Separator (Miltenyl Biotec, Auburn, CA). First, non-$CD4^+$ cells were labeled by incubation with a cocktail of biotin-labeled anti-human antibodies against CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCRγ/δ, and glycophorin A, followed by addition of anti-biotin microbeads, as recommended by the manufacturer of the $CD4^+CD25^+$ regulatory T cell isolation kit (Miltenyi Biotec). The non-$CD4^+$ cells were then depleted with the autoMACS separator,

resuspended in culture media, and set aside. CD25 microbeads were added to the remaining $CD4^+$ cell pool, and positively selected with the autoMACS. The resulting CD25-depleted $CD4^+$ cells were pooled with the non-$CD4^+$ cells, and cultured with peptides as described above for proliferation assays.

Tetramer Staining

All tetramers were obtained from the NIH tetramer facility at Emory University. PBMC were stained immediately following thawing and washing. The $CD4^+$ T cells specific for $NS3_{358}$ were amplified by stimulating PBMC from PB3019 with 1μM of cognate peptide and incubating the cells at 37ºC, 5% CO_2 for 7 days. The cells were then sorted at the University of Utah cell sorting core facility. PBMC were stained with CD4-APC (BD bioscience) and sorted under sterile conditions by gating on $CD4^+$ $CFSE^{low}$ cells. The sorted cells were expanded with CD3/CD28 Dynal beads (Invitrogen) and 10U/ml of rIL-2 (BD Bioscience) at 37ºC, 5% CO_2. Prior to staining with either wild type 358-375- Phycoerythrin (PE), or variant H369R-Allophycocyanin (APC); variant S370P-APC; variant K371E –APC tetramers, the CD3/CD28 beads were magnetically removed from the cultures and the cells were stained with 2μg/ml of tetramer at 37ºC for 1hr in complete media plus 10%PHS. Extracellular surface staining was performed by adding 7-AAD cell viability probe, CD4-pacific blue (BD bioscience), CD3-Amcyan (BD bioscience), CD8-FITC (eBioscience). Negative controls consisted of staining cells with nonspecific peptide, CLIP-DR15 tetramer, labeled with either –PE label or –APC, respectively, which were performed with each experiment and noninfected individuals.

Tetramer Depletion and Add Back Assays

Tetramer staining (10μg/ml) with the wild type 358-375- Phycoerythrin (PE), variant H369R-Allophycocyanin (APC); variant S370P-APC; variant K371E –APC were incubated with PBMC (1 X 10^6 cells/50μl) in a 96 round well bottom plate for 90 min. at RT temperature in the dark. The cells were washed, and MACS beads specific for either anti-APC or anti-PE were incubated with their respective tetramer for 15 min. at 4°C. The Cells were washed, and then applied to MS MACS column and tetramer depleted. Tetramer positive cells were then collected and washed. Next, the cells were washed 2x in complete media + 10%PHS and then stimulated with the appropriate antigens. In the appropriate cultures tetramer positive cells were added to cultures in a dose dependent manner based on volume. A CLIP loaded tetramer for both PE and APC fluorochromes used as a control for nonspecific staining.

Results

Foxp3 Expression in Chronic and Resolved HCV Subjects

To compare chronic (n=8) and resolved (n=8) HCV subjects' T cell responses to rNS3, we measured recall responses *in vitro* (Table 2.1 and Figure 2.1). Responders were considered as those giving a response greater than 2 STDs above $\overline{X}_{bkg}$. Figure 2.1 shows the medium background subtracted from the triplicate response to give a Δ_{cpm} value plotted on a log scale, described in methods. As controls, we included H3 (3μg/ml), H5 (3μg/ml), and PHA (2μg/ml) (Figure 2.1). Each subject responded to PHA and there was no statistically significant difference in the T cell response for any of the control antigens. However, rNS3 induced T cell proliferation in chronic subjects (3.1 SEM ±0.17) was

significantly attenuated ($p<0.05$) compared to resolved subjects (4.01 SEM ±0.15). Similarly, flow cytometric analysis of proliferating T cells stained with CFSE (Figure 2.2) reveals a significantly ($p<0.05$) attenuated $CD4^+$ T cell response in chronic HCV subjects (0.42 SEM ±0.15) (n=7) when compared to the resolved subjects (2.9 SEM ±0.68) (n=9) (Figure 2.2, Table 2.2). Although the $CD8^+$ T cell response was not statistically significantly lower in the chronic compared to resolved T cell subjects, a trend towards lower $CD8^+$ T cell response was evident in the chronic (1.8 SEM ±1.8) compared to resolved (3.1±0.7) (Figure 2.2, Table 2.2).

To determine if CD127 (IL-7Rα) correlated with chronicity as determined by other laboratories (16), we used flow cytometric analyses to analyze the CD127 expression on both $CD4^+$ and $CD8^+$ T cells in chronic (n=7), resolved (n=10), and noninfected (NI) (n=5) on day 7 post-stimulation antigen stimulation (Table 2.3). Further, the up regulation of CD127 on $CD4^+$ T cells has been shown to inversely correlate with Tregs in humans and the negative selection of CD127 can be used as an accurate extra-cellular biomarker of Tregs as opposed to CD25 (15). The noninfected subjects had a significantly ($p<0.05$) lower expression of $CD127^-$ (26 SEM ± 4.1) cells in comparison to resolved (40 SEM ±2.8) and chronic (62.2 SEM ±4.2) subjects (Table 2.3) and the levels remained relatively consistent regardless of antigen stimulation. To test if HCV was able to induce antigen-specific Tregs, we analyzed CFSE dilution assays staining for $CD4^+$ $CD127^-$ $CFSE^{low}$ cells at 7 days post-stimulation (Figure 2.3A). Chronic HCV subjects (16.6 SEM ±5.2) had a significantly higher expression of Foxp3 in antigen specific CD4 T cells that were $CD127^-$ (0 SEM ±3.7) or negative controls stimulated with H3 (0 SEM ±2.4) (Figure 2.3B, Table 2.4). These results suggest antigen stimulation with

rNS3 causes expansion of regulatory T cells at a higher frequency in chronic HCV subjects compared to resolved HCV subjects.

Synthetic Peptide-Mix Experiments

Previous work in our laboratory identified Th1 epitopes in a single HCV chronic subject and further characterized the viral-variants that arose in one of the Th1 epitopes identified, $NS3_{358-375}$ (21, 31). Further, these variants were found not only to escape immune detection but were able to shift the cytokine profile from a Th1 cytokine pattern, which is correlated with viral clearance, to either a Th2 or Treg viral persisting response, respectively (21, 31). Because variants and "wild type" viruses exist together in the circulation, we attempted to simulate in vivo conditions by using what we have termed peptide-mixing experiments in cell-culture assays. Extensive work was performed using P.B3019 PBMC to test the effect of various peptide concentrations and kinetics that each of the variants had on the cognate T cell response (data not shown). It should be noted that our approach is very similar to previously described antagonism assays, with the exception being that we used polyclonal PBMC instead of T cell clones (32). Our preliminary experiments showed that the proliferative response of PBMC preincubated with 1μM of variant peptide 3 hrs. prior to the addition of 1μM $NS3_{358-375}$ peptide was inhibited (data not shown), which is consistent with an antagonism model. Further, if $NS3_{358-375}$ peptide was added either before or at the same time as the variant peptide, there was no effect on $NS3_{358-375}$ T cell proliferation (data not shown). Therefore, in subsequent experiments, variant peptide remained after the addition of the $NS3_{358-375}$ peptide and cultures were incubated at 37°C at 5% CO_2 for either 5 or 7 days then pulsed

with ^{3}H-Thymidine for the last 16-18 hrs. Cultures incubated with single peptide variants alone failed to stimulate as well as the wild type $NS3_{358-375}$ peptide (Figure 2.4). Further, peptide-mix cultures (variant(s) + wild type) showed reduced levels of proliferation relative to those with $NS3_{358-375}$ peptide alone (Figure 2.4).

Flow Cytometric Analysis of Inducible Tregs

To determine if variant S370P was able to induce Tregs in an antigen specific manner, we stimulated P.B3019 PBMC with the indicated antigens, incubated the cells for 7 days and analyzed the phenotype of proliferating cells in a CFSE dilution assay (Figure 2.3A). The induction of Foxp3 by variants H369R and K371E was not significantly higher in comparison to $NS3_{358}$ peptide stimulated cultures but S370P induced a large population of $Foxp3^{+}$ cells (Figure 2.5A). Multiple experiments were performed using S370P because S370P was the only variant that was stable for over 2 years in PB3019 (Figure 2.5B). PBMC from PB3019 (n=3) increased Foxp3 expression ($p<0.05$) when stimulated with S370P ($\bar{x}$= 81.8%) (Figure 2.5B) in comparison to unstimulated culture ($\bar{x}$= 55.73%) (Figure 2.5B).

$CD4^{+}CD25^{+}$ Treg Depletion

To determine if naturally occurring variants inhibit T cell proliferation, we used commercially available $CD4^{+}CD25^{+}$ regulatory T cell isolation kit to deplete $CD4^{+}CD25^{+}$ T cells from a PBMC pool prior to stimulation with the $NS3_{358-375}$ and variant peptides (Figure 2.6). Depletion of $CD4^{+}CD25^{+}$ Tregs enhanced the proliferation of T cells in response to $NS3_{358-375}$ peptide (Figure 2.6, gray vs. black stripped bars, respectively).

Furthermore, stimulation with the K371E variant alone in cultures depleted of $CD4^+CD25^+$ Treg cells, led to an increase in the proliferation level, suggesting that at least one mechanism by which the peptide variants suppress effector T cell responses is through the induction of Tregs.

MHC Class II Tetramer Staining Using Multiple HCV Subjects

To determine if DR15 MHC class II tetramers loaded with $NS3_{358-375}$, or the variant peptides H369R, S370P, and K371E, were able to bind to $CD4^+$ T cells, PBMC from multiple HCV subjects were stained for antigen-specific T cells (Figure 2.7). P.1163, a noninfected DR15 subject (Table 2.1), was used as a control. Also, DR15 CLIP –PE and –APC tetramers were used as controls in each experiment (Figure 2.7, bottom two rows). Both P.B3019 (chronic infection) and JVP008 (resolved infection) had detectable DR15-restriced $CD4^+$ T cells with varying avidities for the tetramers (Figure 2.7). Finding HCV-specific T cells in more than one HCV subject suggests that this is not an idiosyncratic phenomenon although larger numbers of patients need to be studied in order to determine whether our results reflect a more general observation.

MHC Class II Tetramer Depletion Assays

To test the specificity of variant-induced suppression, PBMC were stained with variant-loaded tetramers and magnetic beads were used to remove tetramer-positive T cells; such depleted PBMC cultures were subsequently stimulated with peptides as indicated (Figure 2.8 A-D). Both H369R and K371E tetramer-depleted cultures responded better to $NS3_{358-375}$ peptide in comparison to the nondepleted cultures (Figure

2.8 A, C). Although S370P tetramer depleted cultures did not have a statistically significantly higher T cell response in comparison to nondepleted cultures, S370P-depleted cultures consistently showed an enhanced T cell response to $NS3_{358-375}$ (Figure 2.8B). Adding back tetramer positive cells to depleted cultures restored suppression (Figures 2.8A-C, black bars). Using a nonspecific control tetramer showed no effect on T cell responses to the $NS3_{358-375}$ peptide (Figure 2.8D). The results indicate that tetramer depletion of variant-specific T cells enhances T cell proliferative response to the $NS3_{358-375}$ cognate peptide in an antigen specific manner.

MHC Class II Tetramer Add-Back Assays

To address the potency of variant-specific T cells to suppress the cognate T cell response, we added variant tetramer positive cells back into PBMC culture stimulated with $NS3_{358-375}$ peptide. After tetramer depletion, PBMC cultures were stimulated with $NS3_{358-375}$ peptide (Figure 2.9A). Because of variability in levels of proliferation, responses in the presence of each variant were normalized to WT alone; note the effect of dilution upon responsiveness at 25% (vol/vol). T cell responses in the restored presence of all variant-specific T cells were suppressed in a dose dependent manner. To control for nonspecific depletion and suppression, we used a CLIP-loaded tetramer, which resulted in no effect on the T cell response. Depletion of PBMC cultures with $NS3_{358-375}$ tetramer and subsequent add-back of $NS3_{358-375}$-specific T cells actually enhanced proliferation at lower volumes. This result is not due to toxicity because the same concentration of tetramer (10μg/ml) added into culture along with the respective peptide(s) at 1μM resulted in no inhibition of T cell proliferation (Figure 2.9B). We conclude from these

experiments that variant-tetramer-positive T cells are able to suppress T cell proliferation to wild type $NS3_{358-375}$ *in vitro*. To our knowledge this is the first demonstration of what may be antigen-specific regulatory T cells.

MHC Class II Tetramer Staining of $NS3_{358-375}$-Specific $CD4^+$ T Cells

To extend our observation that variant-specific T cells are able to bind to cells that are specific for the cognate peptide in a somewhat larger cohort of subjects, we amplified $CD4^+$ T cells specific for $NS3_{358-375}$ peptide. PBMC from HLA-DR15 subjects (Table 2.1) were prelabeled with (0.5μM) CFSE and stimulated with $NS3_{358-375}$ synthetic peptide for 7 days. The $CD4^+$ $CFSE^{low}$ cells were sorted, expanded with CD3/CD28 beads, and stained with tetramer following removal of beads. All cultures were >99% $CD4^+$ as determined by flow cytometry (data not shown). Tropomyosin #20 (HLA-DR1/3) are $CFSE^{low}$ $CD4^+$ T cells expanded in the same manner except that shrimp tropomyosin was substituted for $NS3_{358-375}$ and served as an additional negative control (Figure 2.10, first column). Nonspecific tetramer (CLIP) –APC and –PE were also used as controls in each experiment (Figure 2.10). As might be expected, $NS3_{358}$ and variant MHC class II tetramers stain PB3019, PH1127, and PH1079 $CD4^+$ antigen-specific T cells all of which share or require the HLA-DR15 restriction element. Thus, tetramers loaded with either cognate or variant peptides are likely to bind overlapping subsets of T cells found in PBMC from multiple subjects.

Discussion

We demonstrate *in vitro* induction of regulatory T cells capable of suppressing antigen-specific T cell responses. We postulated that previously defined viral variants in a Th1 epitope could be responsible for the induction of Tregs based on the cytokine shift and attenuated T cell response (17). Further, chronically infected subjects exhibited significantly lower T cell responses in comparison to resolved subjects. These attenuated T cell responses correlated with the induction of the Treg lineage-specific markers in proliferating T cells specific for rNS3.

Although Fuller et al. (33) identified and tracked HCV NS3 viral variants in MHC class II-restricted epitopes in an infected chimpanzees, similar to our previous work (20), but it was not clear if the viral-variants were able to affect the T cell response to cognate peptide. Our study demonstrated that viral variants attenuated T cell responses to cognate peptide and not unrelated peptide. Further, the specific variant, S370P, induced Foxp3 in an antigen specific manner in a chronic HCV patient. In an effort to generalize our finding from one chronic subject, we were able to detect variant-specific T cells in multiple HLA-matched subjects. The ability to detect the wild type positive T cells along with variant specific T cells suggests that the mechanism of Treg induction by naturally occurring epitope variant is likely not exclusive to one chronically infected individual, albeit the functional studies, though cumbersome, now need to be done in a larger cohort of chronic and resolved HCV patients.

Previous studies had found that depletion of $CD4^{+}CD25^{+}$ cells enhanced HCV-specific T cell response to HCV antigens; however, these studies also described enhanced T cell response to control antigens from Epstein-Bar Virus (EBV), Cytomegalovirus

(CMV), and influenza indicating that the depletion of this subset of cells is not specific for HCV (4, 34). Depletion of $CD4^+CD25^+$ cells restored PBMC proliferative responses to $NS3_{358\text{-}375}$ to levels that matched or exceeded those in the nondepleted PBMC cultures that were preincubated with variant peptide. An increase in the level of proliferation induced by variant K371E alone following $CD4^+CD25^+$ cell depletion implied a suppressive role for $CD4^+CD25^+$ Treg cells. These results suggest that nonspecific depletion of Tregs enhanced T cell proliferation.

Compelling evidence for HCV-specific Tregs by Ebinuma et al. (35) identified $CD4^+CD25^+$ $Foxp3^+$ MHC class II tetramer positive cells in peripheral blood of HCV patients. Further, Heeg et al. (7) performed a longitudinal study using MHC class II tetramer staining to track HCV-specific $CD4^+$ $Foxp3^+$ T cells during the course of HCV infection in a cohort of patients. Although Heeg's study did not find a correlation between an increase in Foxp3 expression and viremia, they did observe an attenuated antigen-specific T cell proliferative response and lowered IFNγ secretion in MHC class II tetramer positive cells. These studies did not identify viral variants arising in the epitopes analyzed, therefore giving no indication if viral variants could have an effect on the cognate T cell response (7, 35). Depleting HCV-specific T cells that bind to MHC class II variant tetramers, we found an enhanced T cell proliferative response to $NS3_{358\text{-}375}$ peptide and a restoration of suppression when the variant-specific T cells were added back. Although the depletion of S370P tetramer positive cells did not significantly enhance the proliferative response over the nondepleted culture, the variant tetramer depletion results suggest that the avidity of the tetramers for $NS3_{358\text{-}375}$- T cells are different, leading us to hypothesize that these variants could be acting as altered peptide

ligands. Consistent with this, K371E had a higher T cell response when $CD4^+CD25^+$ cells and K371E tetramer positive cells were removed indicating that this variant might affect yet a different subset of cells. Further, adding variant tetramer positive cells back into $NS3_{358\text{-}375}$ stimulated cultures had a dose-dependent suppressive effect, suggestive of Tregs. Taken together, our results suggest antigen-specific Tregs are responsible for suppression of an effector T cell response and we believe that a possible mechanism for this phenomenon is that Hepatitis C viral variants may act as APLs to induce Tregs. Previous (21, 36) and current work in our laboratory clearly demonstrates that viral variants are able to antagonize cloned T cells specific for $NS3_{358\text{-}375}$. Further, the variant peptides loaded onto MHC class II have different avidities for T cells that are specific for $NS3_{358\text{-}375}$, which suggests that these variants are acting as naturally occurring altered peptide ligands (Chapter 4).

It has been observed that wild type HCV sequences remain stable in humans and chimpanzees even years into an ongoing infection (2, 31, 33, 37). We have shown that approximately 80% of the circulating virus has "wild type" 1A sequence (21, 31). Interestingly, the S370P variant was found in two isolates separated by 2 years, the variation has not impacted viral fitness negatively, yet its frequency seems not to have increased with time as might be expected with other escape models. Indeed, of all variants within the $NS3_{358\text{-}375}$ epitope tested to date, none has lost the ability to bind the DR15 class II restriction element, which is contrary to a classic evasion escape model (38). The fact that HCV epitope variants seem to induce a functional unresponsiveness in peripheral T cells implies a radically different viral strategy as well host-related immunopathogenesis. HCV seems to have developed the ability to induce a specific

tolerance to itself by exploiting natural mechanisms that operate within the host. Our data suggest the hypothesis that viral mutation leads to APL that blunts specific helper T cell responses, which thereby attenuates the usual effector mechanisms requisite for antibody and killer T cell induction.

In conclusion, we have shown for the first time that variants of an HCV immunodominant epitope, which arose during chronic infection in a human, induced Foxp3 expression in an antigen specific manner and had a dose-dependent suppressive effect *in vitro*, perhaps reflective of regulatory T cells. While the number of individuals studied to this point is small, we know that such variation occurs in other individuals and applies to the cytotoxic effector arm of the immune response as well (39); notably, this latter study was performed in an HLA-DR15-positive subject. Therefore, our *in vitro* results imply that selective immune driven viral variants do not "escape" immune detection, similar to observations by Fuller et al. (33), but rather they avoid the consequences of immune recognition by inducing antigen-specific Tregs which in turn provide "immunological cover" for wild type viral sequences including those that contain the $NS3_{358\text{-}375}$ epitope, which should otherwise be recognized, provide effective T cell help such that virus can be appropriately eliminated. Our results do not oppose other mechanisms for viral persistence, but may act in concert to subvert the adaptive immune response to HCV.

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Table 2.1. HCV and HLA genotypes of HCV subjects.

Subject ID	HCV status	Genotype	HLA- DR	AB
*RLM 037	R	-	1,15	+
*KML044	R	-	9,15	+
*ZSS035	R	-	1,15	+
*LEC028	R	-	1,8	+
*KTJ010	R	-	1,15	+
*BPB026	R	-	3,15	+
*DRB012	R	-	7,7	+
*JVP008	R	-	15,15	+
JPZ061	R	-	4,4	+
PH1127	R	-	13,15	+
PH1079	R	-	4,15	+
*AJG066	C	1A	1,8	ND
*KRW002	C	1A	8,14	ND
*MH065	C	1A	14,15	ND
*P.B3019	C	1A	15,7	ND
*NLM049	C	1A	11,13	ND
*CER014	C	1A	7,13	ND
*DRB051	C	1A	1,13	ND
*SSB007	C	1A	7,13	ND
RLW027	C	1A	4,15	ND
P.1022	NI	-	8,10	-
P.1163	NI	-	1,15	-
P.1078	NI	-	9,13	-
P.1127	NI	-	5,6	-

HCV and HLA types of chronic and resolved subjects used in this study. All subjects' PBMC were incubated with recombinant NS3 protein (rNS3) in a dose dependent manner using a proliferation assay to detect T cell responses. All subjects were screened for HCV RNA by quantitative PCR. In the case of resolved subjects, in which they had no detectable viral load, a RIBA was performed to screen for HCV antibodies. * Subjects used for T cell proliferation assay in Figure 2.1.

Table 2.2. $CD4^+$ and $CD8^+$ T cell responses to rNS3 and H3 antigens shown as frequency of $CFSE^{low}$ and the transformation of T cell proliferation data.

		Medium		NS3			H3		
Subject		CD4	CD8	CD4	CD8	log10Δmax	CD4	CD8	log10Δmax
RLM 037	R	0.97	1.5	7	7.1	4.4	1.5	4.91	0
KML044	R	0.29	0.5	4.1	11	4	0.3	0.63	0
ZSS035	R	0.6	1	4.1	2.7	4.2	3.7	1.85	4.1
LEC028	R	1.16	0.5	6	2.4	4	1.8	0.8	2
KTJ010	R	1.33	1.3	5	2.9	4	1.9	1.95	2.5
BPB026	R	0.4	0.3	1.6	1.1	3.2	0.4	0.22	2.7
DRB012	R	1.22	0.8	0.8	3.7	2.5	0.8	3.68	3.5
JVP008	R	0.4	0.5	2.6	4.1	3.2	0.8	0.47	3
JPZ061	R	1.34	1.8	1.1	1.4	3.3	1.2	1.3	0
AJG066	C	4.31	4.8	4.7	4.8	3.4	4.8	4.68	0
KRW002	C	4.79	4.6	5.3	5.2	3.8	5.4	4.75	3.5
MH065	C	6.31	7.2	7	7.3	3.6	6.6	7.3	3.2
NLM049	C	1.84	4.5	2	14	3.5	0.2	0.84	3.05
CER014	C	0.68	0.3	1	0.8	4	0.7	0.33	3
SSB007	C	22.7	29	24	32	3.9	23	28.1	0
RLW027	C	0.16	0.4	0.3	2	4.2	0.2	0.84	3.8

Frequency of $CD4^+$ and $CD8^+$ T cells in CFSE labeled lymphocytes when stimulated with rNS3 and H3 at 10μg/ml for 7 days and analyzed by flow cytometry. On day 7, the cells were stained with CD4 and CD8. The proliferative response was determined by the medium for each subject. Log10Δmax is the transformation of the T cell proliferation data for each subject and the respective antigen stimulation.

Table 2.3. $CD4^+$ and $CD8^+$ $CD127^-$ expression.

		CD127-					
		Medium		NS3		H3	
		CD4	CD8	CD4	CD8	CD4	CD8
Subject							
RLM 037	R	60.1	28	65.1	32.9	62.1	28.9
KML044	R	37.6	23.6	46.4	29.7	42.3	24.7
ZSS035	R	38.7	14.1	26.2	9.72	42.5	17.9
DOY041	R	40.7	20.3	42.7	18.1	46.1	24.3
LEC028	R	48.7	30.8	54.6	26.8	51.8	34.7
KTJ010	R	28.7	8.1	38.5	11	26.4	7.62
BPB026	R	32.8	22	37.1	9.75	33.1	10.5
DRB012	R	41	17.1	47.4	19.7	46.9	19
JVP008	R	35.9	15.4	42.4	19.7	34.2	16.2
JPZ061	R	38.6	18.7	44.6	27.9	45.5	27.3
AJG066	C	84	62.8	84	63.3	83.2	60.7
KRW002	C	62.8	54.7	63.9	57.9	64.1	57.5
MH065	C	51	54.7	44.7	45.5	51.7	56.8
NLM049	C	60.6	35.9	54.1	37.1	63.1	38.6
CER014	C	66.2	27.2	61	23.6	67	29.8
SSB007	C	59.2	33.3	52.9	23.8	61.1	35.9
RLW027	C	51.8	56.1	52.3	53.5	58.4	68
P.1022	NI	12.2	10.3	40.4	30.1	26.2	18.4
P.1163	NI	37.2	14	34.4	15.3	33.7	13.5
P.1078	NI	25.3	23.9	28.4	23.5	27	26.3
P.1127	NI	31	20.1	24.6	12.1	25.1	13.7
P.1031	NI	27.6	23.7	35.7	12.7	34.8	23.2

Frequency of $CD4^+$ $CD127^-$ and $CD8^+$ $CD127^-$ T cells in lymphocyte population when stimulated with rNS3 and H3 at 1mg/ml for 7 days and analyzed by flow cytometry.

Table 2.4. Frequency of $CD4^+$ $CD127^-$ $CFSE^{low}$ $Foxp3^+$ cells.

		$CD4^+$ $CD127^-$ $CFSE^{low}$ $Foxp3^+$		
Subject		Medium	NS3	H3
RLM 037	R	83.1	66	68.4
KML044	R	86.7	62	72.3
ZSS035	R	82.6	71.8	81.8
LEC028	R	79.3	84	85
KTJ010	R	47.4	57.7	41.9
BPB026	R	30.9	25	14.3
DRB012	R	61.4	46.4	46.4
JVP008	R	15.9	14.1	29.7
JPZ061	R	36.9	26.1	20.4
AJG066	C	52.3	66.6	57.2
KRW002	C	10.4	12.3	10.1
MH065	C	18.6	40.1	37.7
P.B3019	C	58.7	69.2	71.5
NLM049	C	16.5	16.9	31.7
CER014	C	18.6	40.1	37.7
DRB051	C	16.5	67	49.5
SSB007	C	12.3	17.3	10.9
RLW027	C	16.7	40.8	20
P.1022	NI	0	26.8	8.2
P.1163	NI	18.1	28	21
P.1078	NI	14.1	5.178	8.68
P.1070	NI	18.6	16.3	11.8
P.1031	NI	16	0	7.69

Frequency of $CD4^+$ $CD127^-$ $CFSE^{low}$ $Foxp3^+$ cells in lymphocyte population when stimulated with rNS3 and H3 at 1mg/ml for days and analyzed by flow cytometry..

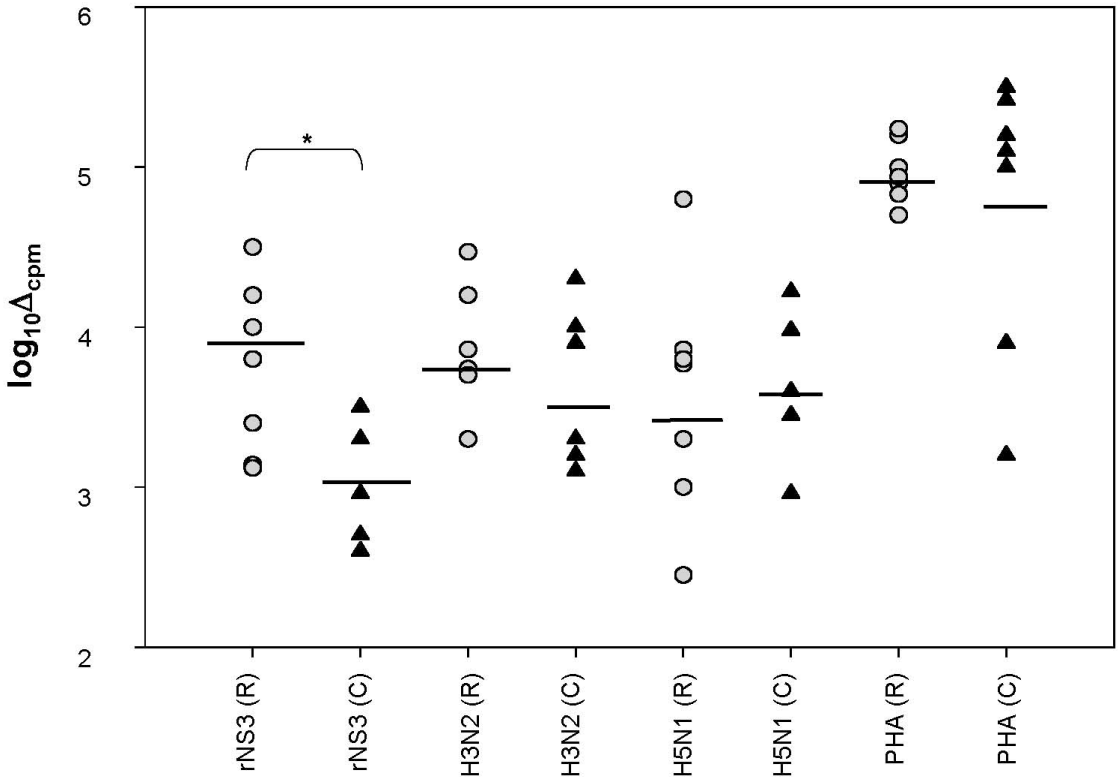


Figure 2.1. Resolved HCV subjects have a significantly higher T cell response to rNS3 than chronic HCV subjects. PBMC from both resolved subjects (gray circle) (n=8) and chronic (black triangle) (n=8) subjects (subjects used in Figure 2.1 are * in Table 2.1) were individually incubated with rNS3 (1μg/ml) and H3 (3μg/ml), H5 (3μg/ml), and PHA (2μg/ml) for 7 days. All subjects were screened in the same proliferation assay. As described in the results, the algorithm: $\log_{10}\Delta_{max}$ was used to transform the data (29). The $p<0.05^{*}$ as determined by Student's *t*-test.

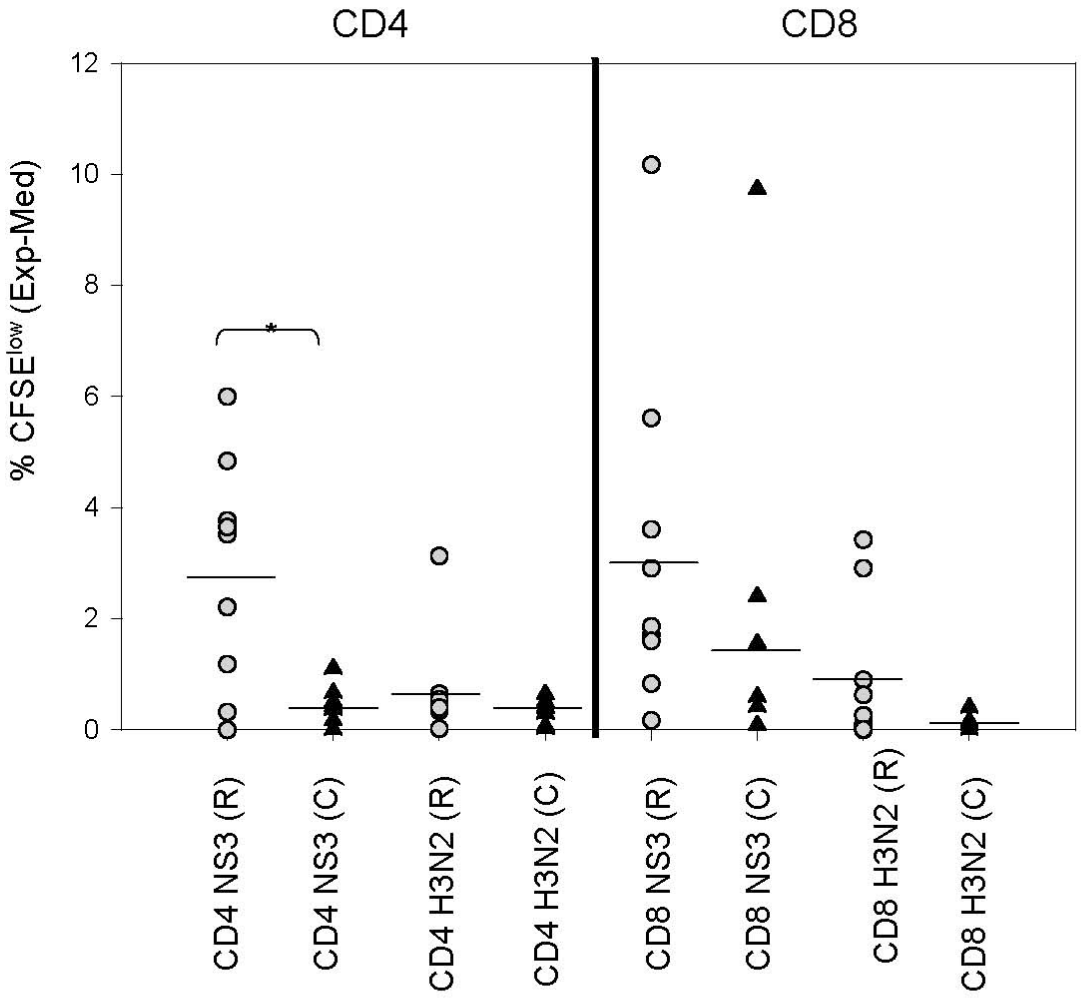
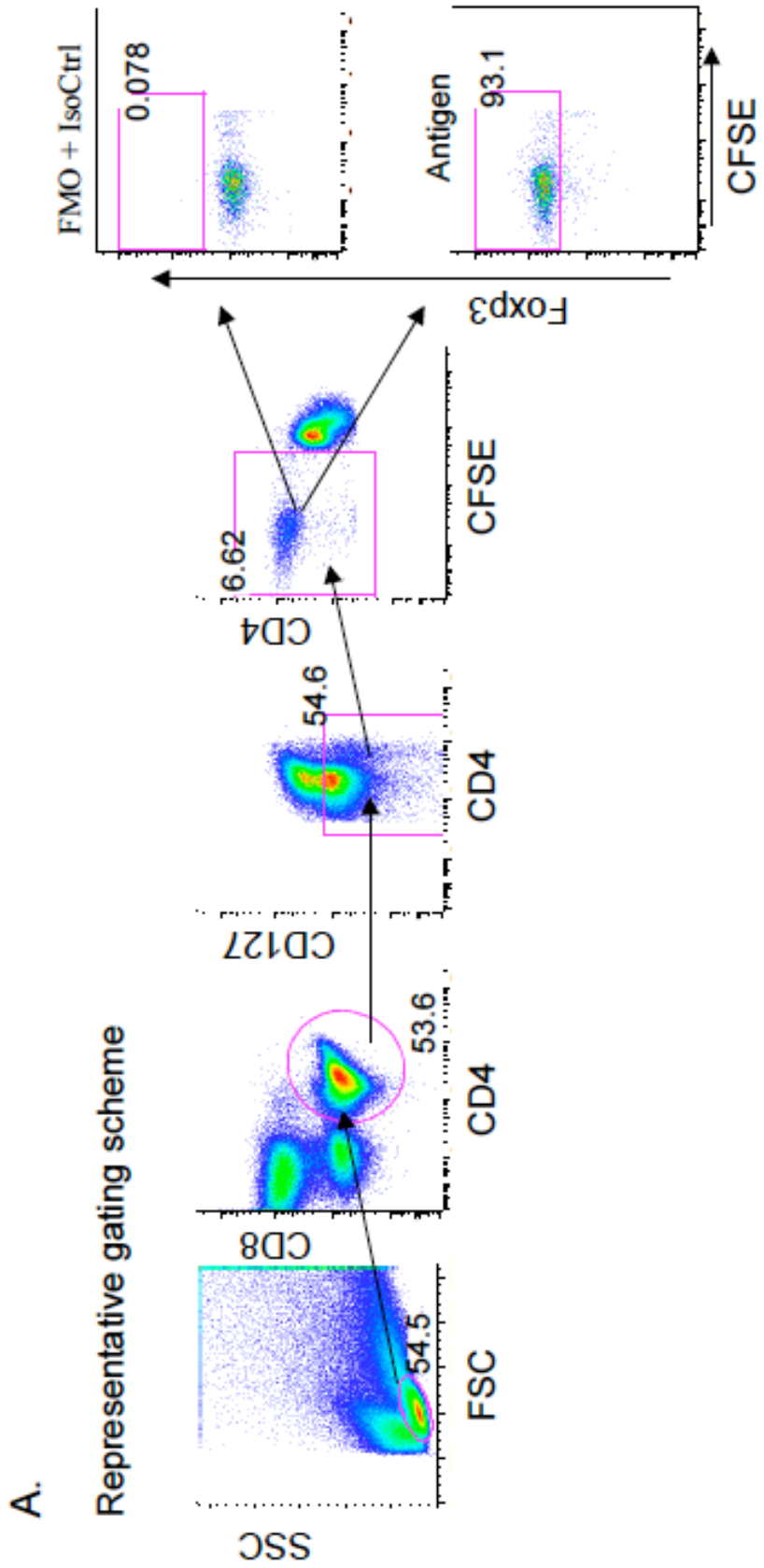


Figure 2.2. HCV subjects have an attenuated $CD4^+$ T cell response to rNS3. HCV chronic subjects (black triangle) (n=7) (Table 2.2) had a significantly lower CD4 $CFSE^{low}$ response to rNS3 (1μg/ml) in comparison to resolved subjects (gray circle) (n=9) (Table 2.2). There was no difference in the H3 (1μg/ml) response between groups. The $p<0.05^*$ as determined by Student's *t*-test.

Figure 2.3. Higher frequency of $Foxp3^+$ cells in chronic HCV subjects in comparison to resolved HCV subjects. (A) Back Gating analysis of % $Foxp3^+$ cells in $CD4^+$ $CD127^-$ $CFSE^{low}$ cells. PBMC were labeled with CFSE and then stimulated with either rNS3 (1μg/ml) or H3 (1μg/ml) and incubated for 7 days. The Fluorescence minus one (FMO) plus isotype control for Foxp3 antibody was used to determine the gate for $Foxp3^+$ cells (upper panel). An example of PBMC stimulated with an antigen is shown in the lower panel. (B) Chronic HCV patients (n=9) have a significantly higher percentage (*, $p<0.05$ as determined by Student's *t*-test) of $CD4^+$ $CD127^-$ $CFSE^{low}$ $Foxp3^+$ expressing cells in comparison to resolved HCV subjects (n=9), (Table 2.4). Δ=[Experimental - Medium]. Noninfected subjects (n=5) were stimulated with the recombinant H3 antigen as a comparison (Table 2.4).


A.
Representative gating scheme
SSC
FSC
54.5
CD8
CD4
53.6
CD127
CD4
54.6
CD4
6.62
CFSE
Foxp3
FMO + IsoCtrl
0.078
Antigen
93.1
CFSE

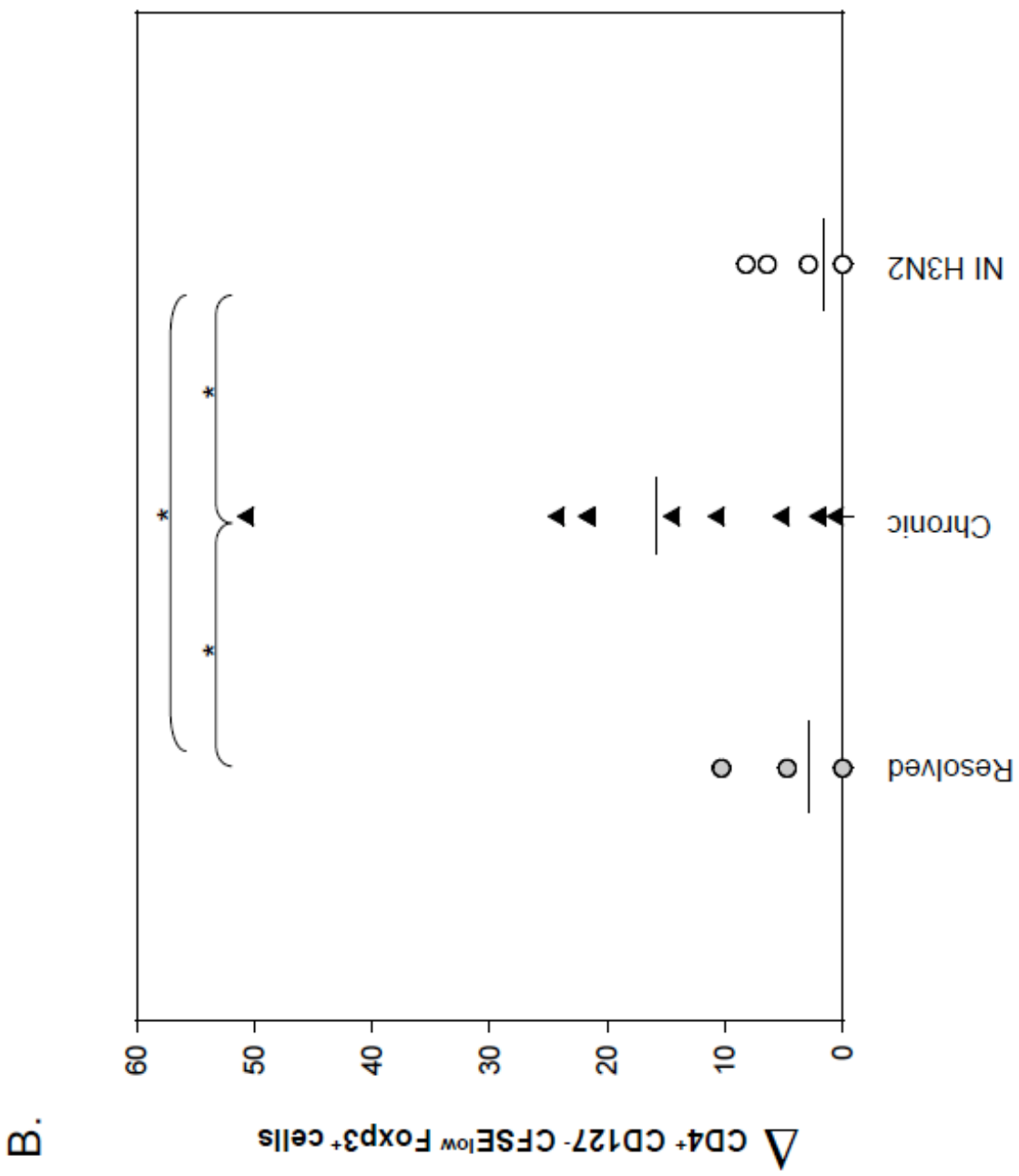


Figure 2.3 continued

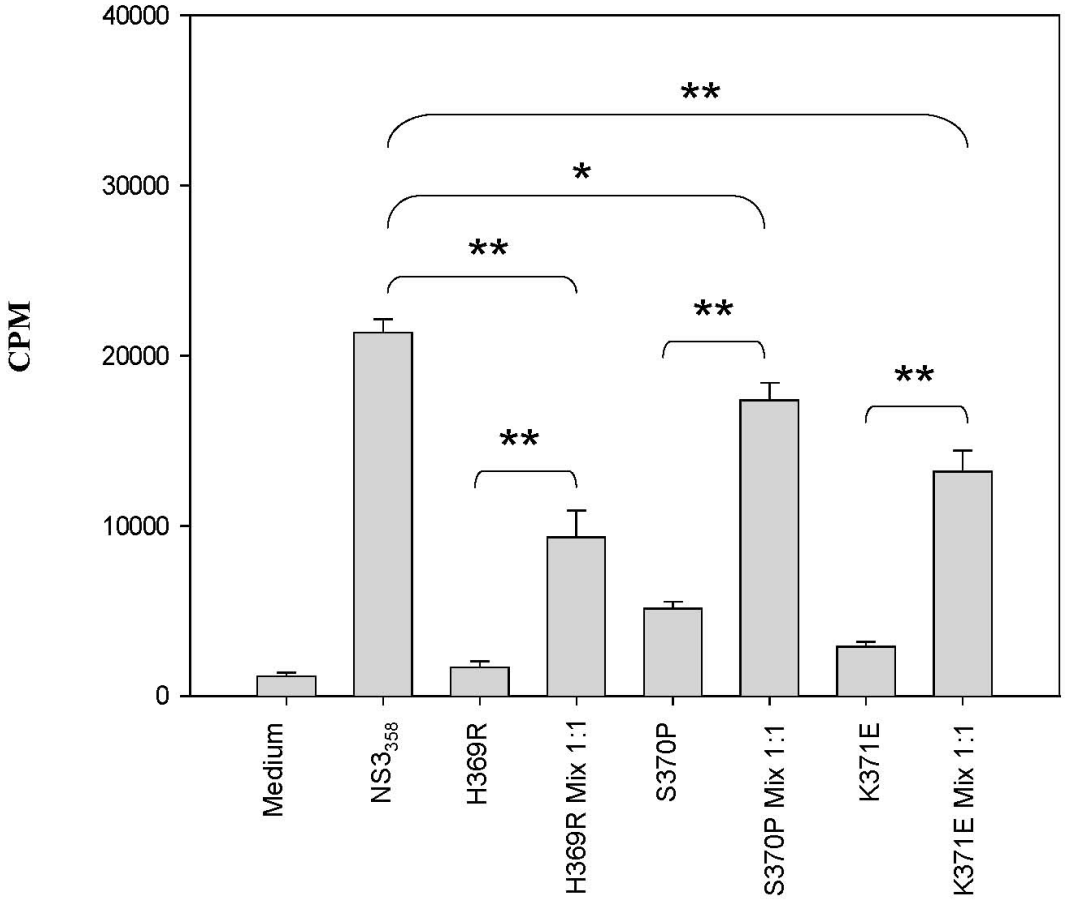
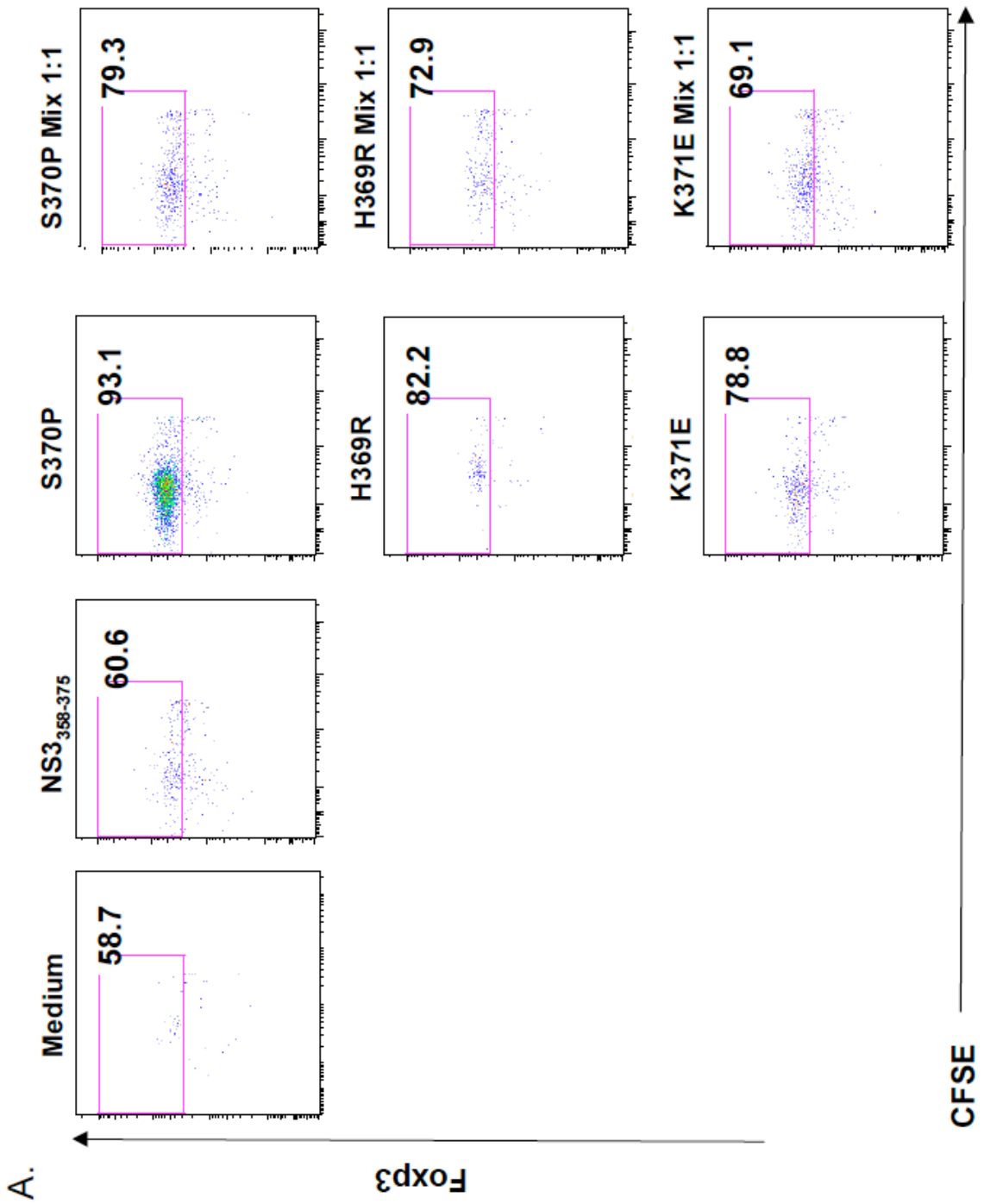


Figure 2.4. PBMC proliferative responses to $NS3_{358-375}$ peptide variants. (A) HCV subject P.B3019 PBMC incubated for 3hrs. with peptide variants at 1μM, then with the addition of 1μM wild type peptide $NS3_{358-375}$ where indicated. On day 4, proliferating cells were labeled with 1 μCi ^{3}H-TdR for the final 16 hrs. of incubation, and cells were harvested for measurement of ^{3}H-TdR incorporation on day 5. Results are shown in mean counts per minute (CPM) +/- standard error of at least triplicate cultures. *, $p<0.05$ as determined by Student's *t*-test. **, $p<0.005$. Results are representative of greater than 10 experiments.

Figure 2.5. Increased Foxp3 expression in an antigen specific manner by variant S370P stimulated PBMC from chronic P.B3019 (A) Representative flow plots of $CFSE^{low}$ $Foxp3^{+}$ cells from lymphocytes that were $CD4^{+}$ $CD127^{-}$ (gating scheme Figure 2.3A) stimulated with the indicated antigens at 1μM for 7 days. Peptide added to PBMC cultures is listed above the plot and the percentages are the percent of $CD4^{+}CD127^{-}$ $CFSE^{low}$ cells. (B) Variant S370P ($\bar{x}$= 81.8 ± SEM 5.7) stimulated PBMC significantly increases the expression of Foxp3 in $CD4^{+}CD127^{-}CFSE^{low}$ cells above the medium background ($\bar{x}$= 55.73 ± SEM 2.57) for P.B3019. *, $p<0.05$ as determined by Student's *t*-test.


A.
Medium
58.7
NS3358-375
60.6
S370P
93.1
H369R
82.2
K371E
78.8
S370P Mix 1:1
79.3
H369R Mix 1:1
72.9
K371E Mix 1:1
69.1
Foxp3
CFSE

B.

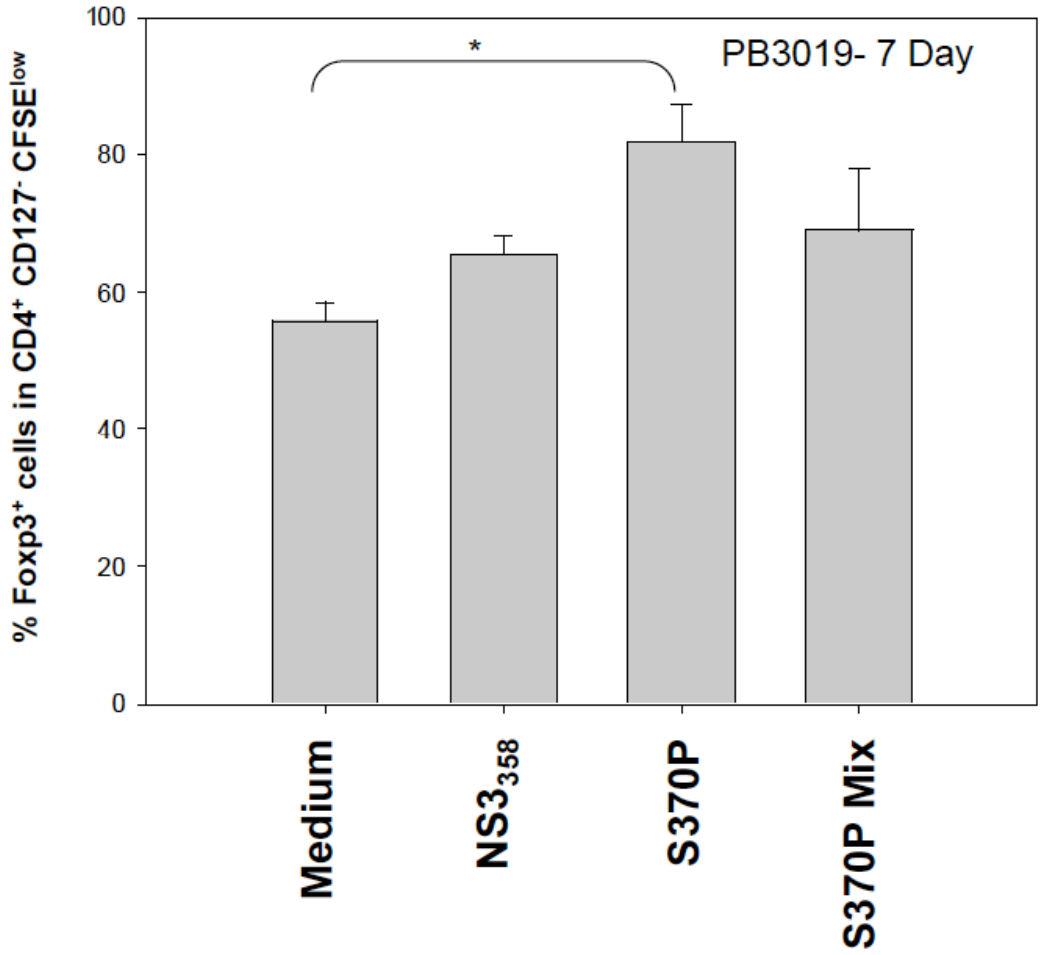


Figure 2.5 continued

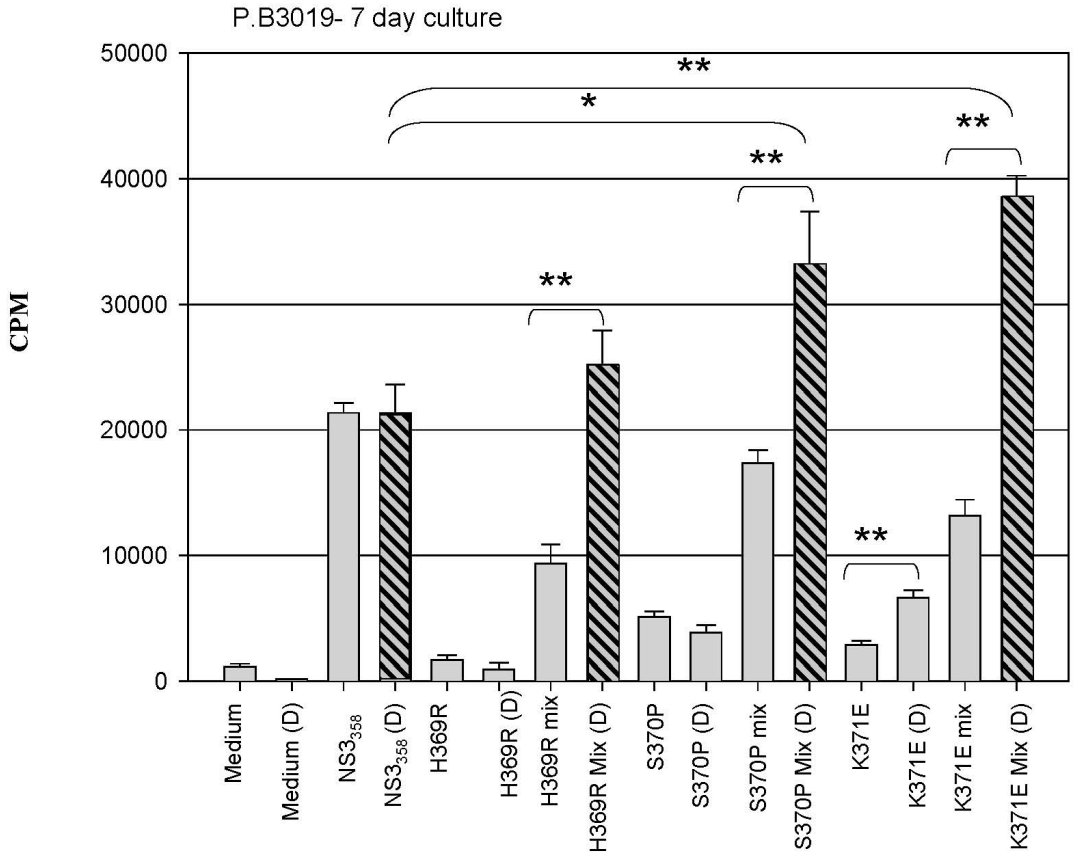


Figure 2.6. PBMC proliferative responses to $NS3_{358-375}$ peptide variants and mixes with wild type peptide in cultures depleted of $CD4^+CD25^+$ Treg cells. Subject 3019 total or Treg-depleted PBMC were stimulated with peptide variants and measured ^{3}H-TdR-uptake. Results are mean counts of six wells each +/- SEM, representative of three independent experiments. Gray bar, individual peptide; stripped bars, $CD4^+CD25^+$ Treg-depleted PMBC. *, $p<0.05$ as determined by Student's *t*-test. **, $p<0.005$.

Figure 2.7. DRB1*1501 MHC class II variant and cognate tetramers are able to bind to $CD4^+$ T cells from multiple patients. PBMC from a noninfected (P1163), chronic (P.B3019), and resolved (JVP008) were individually stained with DR15 MHC class II tetramers (2μg/ml). P.1163, noninfected DR15 subject, was used as a control to test for nonspecific labeling of each DR15 tetramer (first column). DR15-CLIP –APC and –PE tetramers were used as a control for each experiment (bottom two rows).

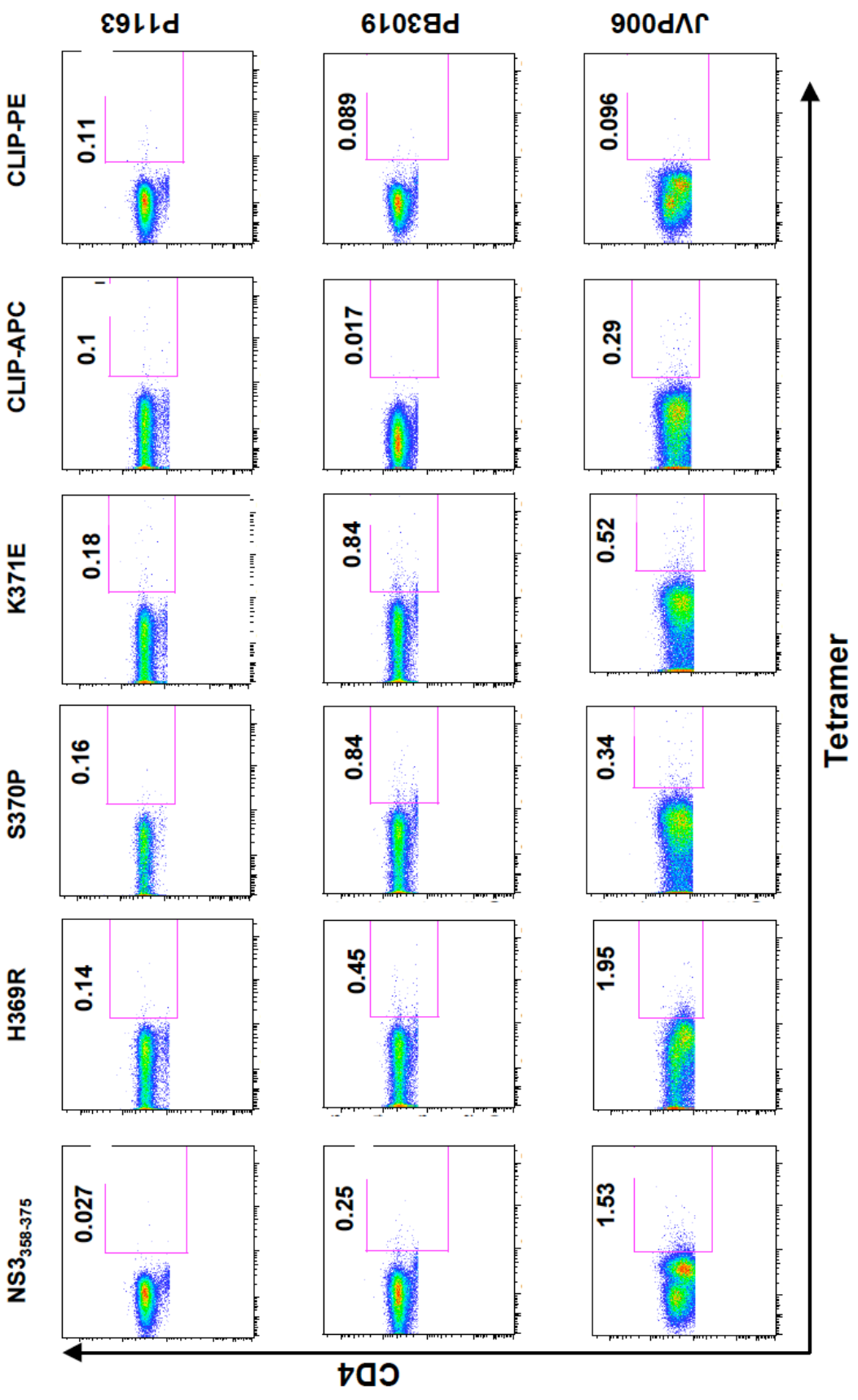
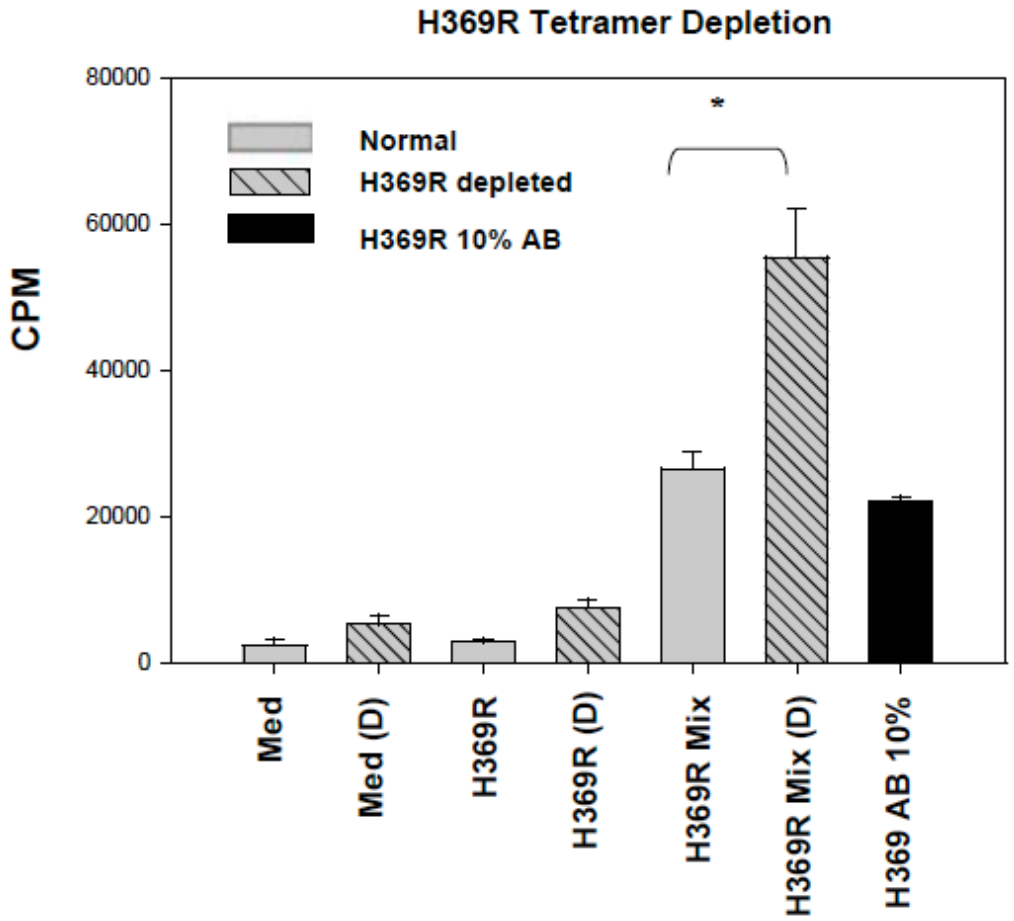

P1163
PB3019
JVP006
CLIP-PE
CLIP-APC
K371E
S370P
H369R
NS3358-375
0.11
0.089
0.096
0.1
0.017
0.29
0.18
0.84
0.52
0.16
0.84
0.34
0.14
0.45
1.95
0.027
0.25
1.53
CD4
Tetramer

Figure 2.8. Tetramer depletion of T cells specific for variant peptides restores T cell proliferative response to cognate $NS3_{358-375}$. (A-C) Variant tetramer depleted listed at the top of the panels. Tetramer depletion was done using PBMC from subject PB3019. PBMC incubated with variant peptide for 3 hrs. and then stimulated with WT peptide for 7 days (Blue and red panel). Black histogram is indicative of add back experiment, in which 10% represents approximately the same number of cells as normal mix population. Each experiment was done in triplicate, replicated 3 times for a total 9 data points. (D) HLA-DR15 CLIP tetramer depleted cells shown in yellow compared to PBMC from nondepleted PBMC proliferation assay at 7 day, each experiment was done in triplicate (n=2).

A.


H369R Tetramer Depletion
CPM
80000
60000
40000
20000
0
Normal
H369R depleted
H369R 10% AB
*
Med
Med (D)
H369R
H369R (D)
H369R Mix
H369R Mix (D)
H369 AB 10%

B.

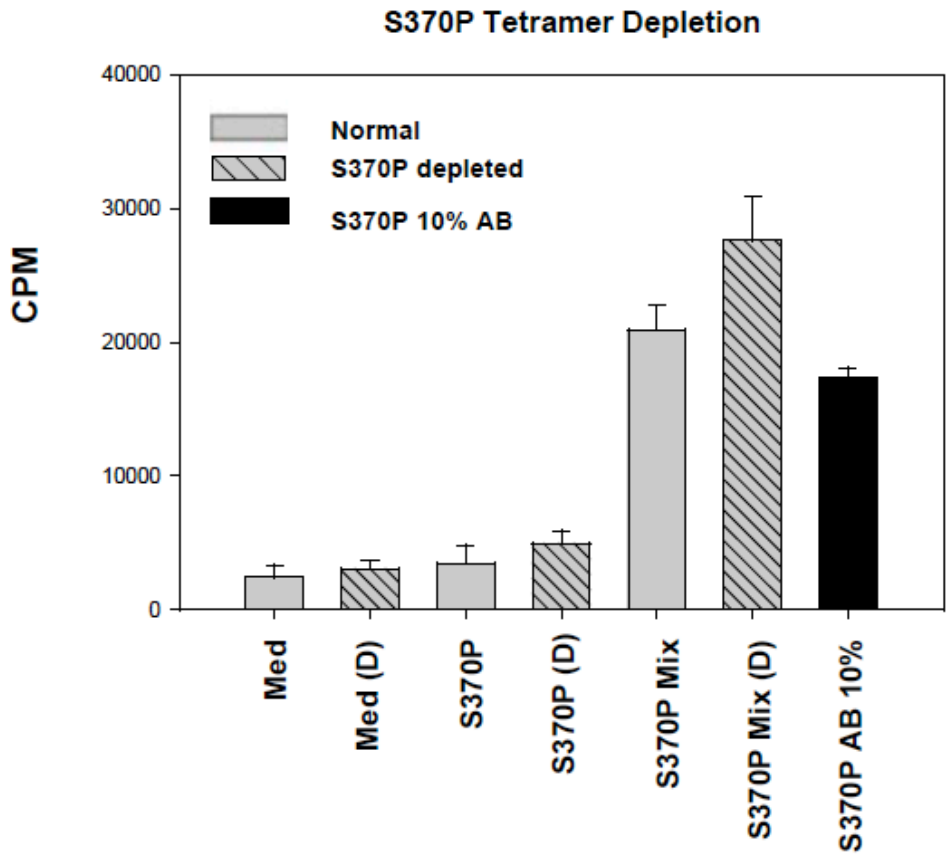


Figure 2.8 continued

C.

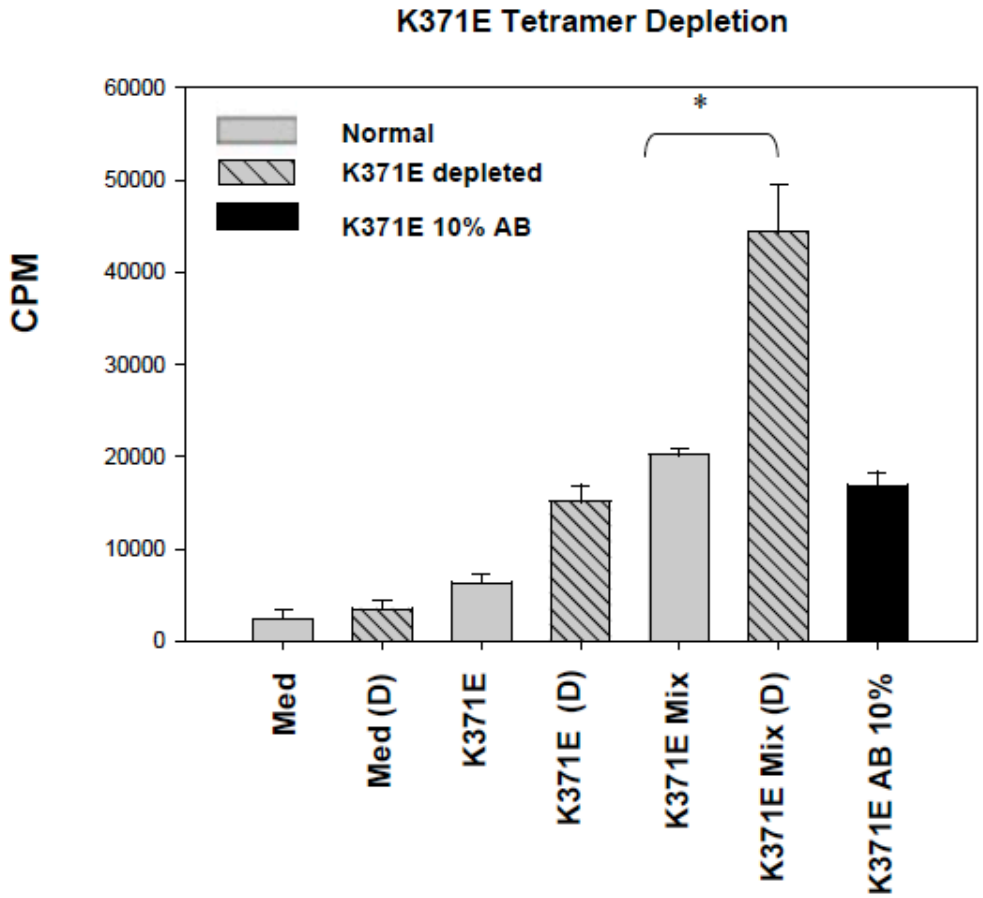


Figure 2.8 continued

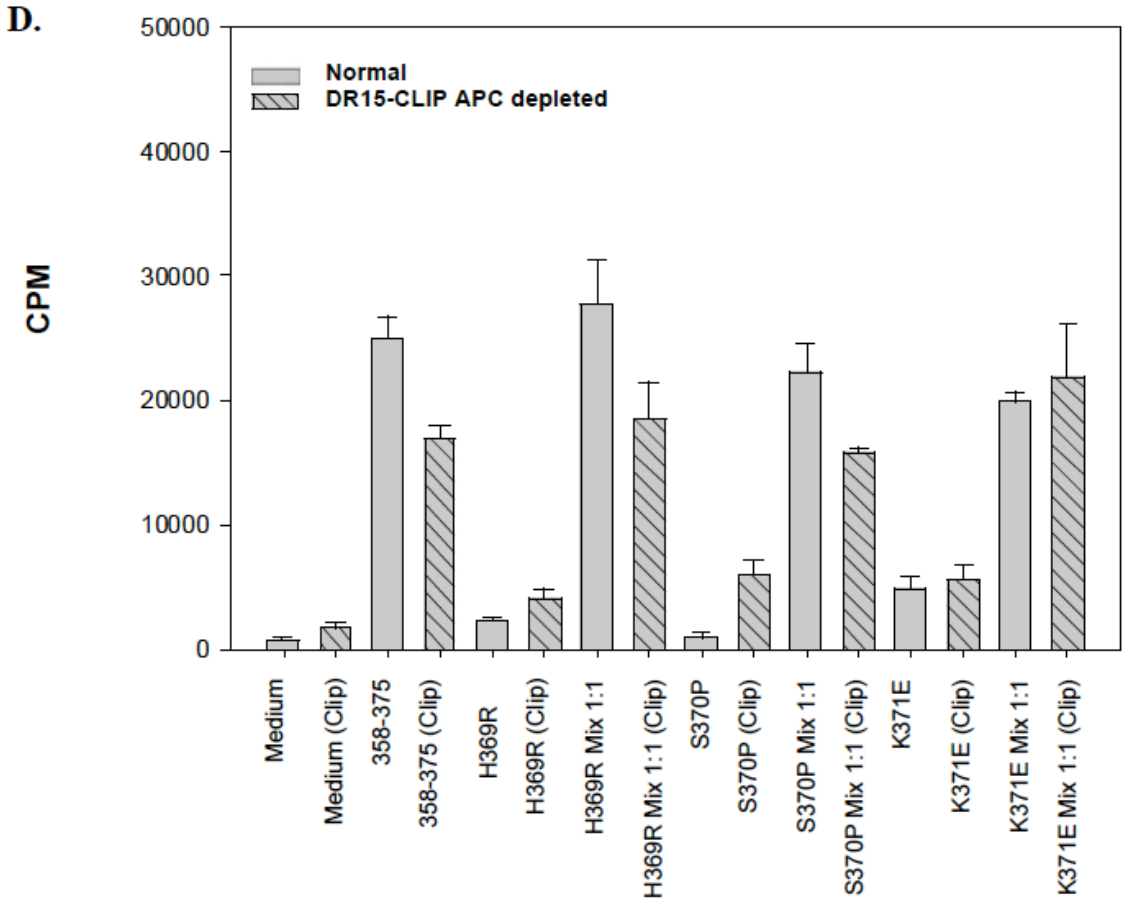
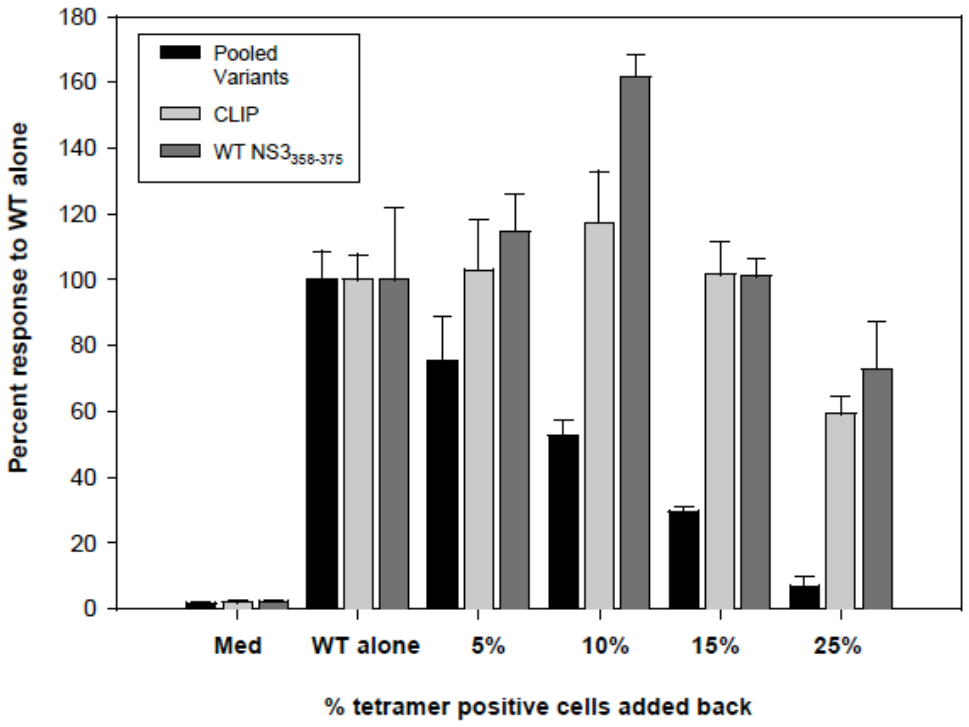


Figure 2.8 continued

Figure 2.9. Tetramer positive cells suppress T cell proliferation in a dose dependent manner. P.B3019 PBMC were stained with pooled variants of H369R, S370P, and K371E (black), CLIP (light gray), or WT $NS3_{358-375}$ (dark gray) and removed by bead depletion. After tetramer depletion, the cultures were stimulated with $NS3_{358-375}$ peptide. Tetramer positive cells were volumetrically added back into culture with P.B3019 $NS3_{358-375}$ stimulated PBMC. Data are representative of percent response to $NS3_{358-375}$ stimulated PBMC depleted with indicated tetramer(s) (WT alone) and set to 100%. Controls were CLIP (light gray) and $NS3_{358-375}$ (dark gray). (B) PB3019 PBMC cultures were stimulated with indicated peptide(s) with each tetramer added at 10μg/ml and incubated for 7 days.

A.


Pooled Variants
CLIP
WT NS3358-375
Percent response to WT alone
180
160
140
120
100
80
60
40
20
0
Med
WT alone
5%
10%
15%
25%
% tetramer positive cells added back

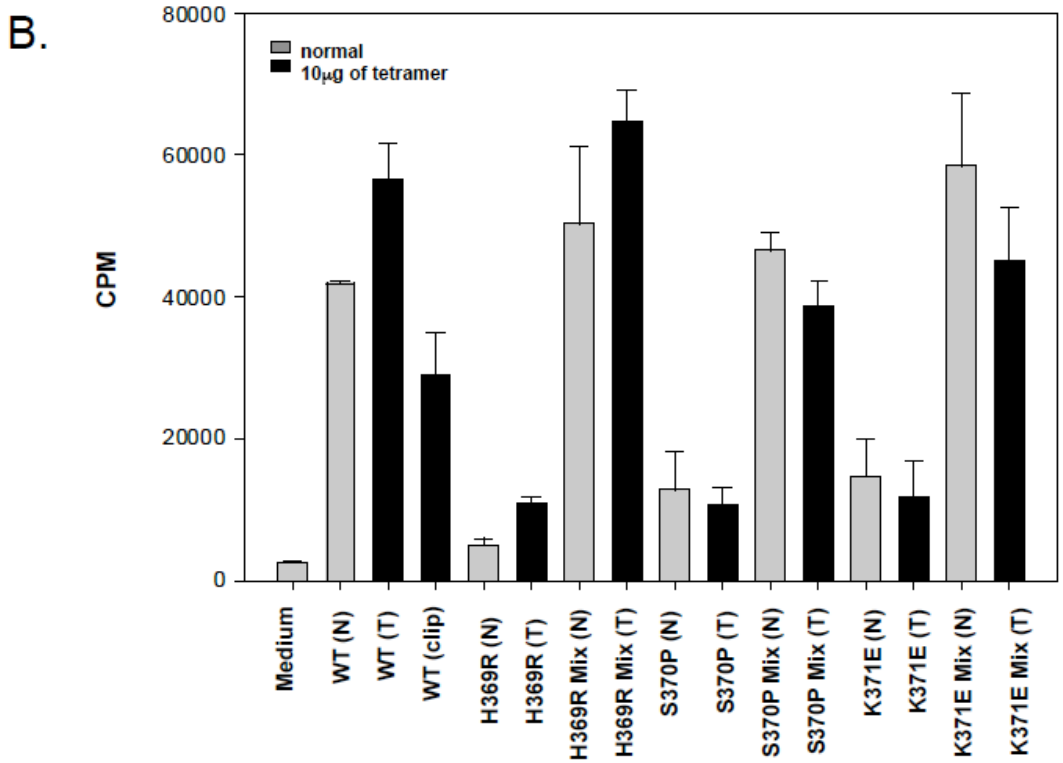
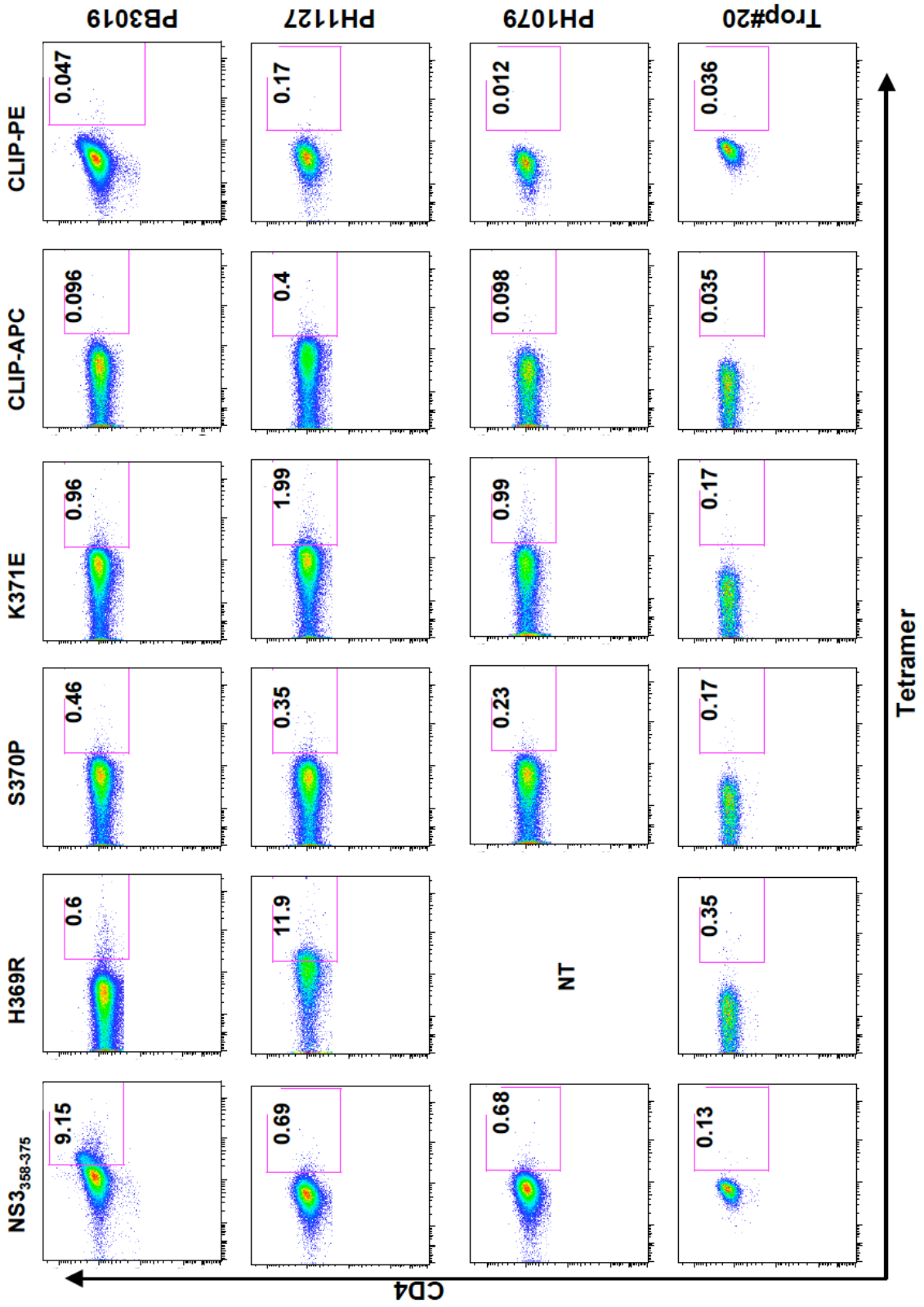


Figure 2.9 continued

Figure 2.10. MHC class II tetramers are able to stain $NS3_{358-375}$ antigen-specific $CD4^+$ T cells. PBMC from PB3019, PH1127, and PH1079 were prestained with CFSE and stimulated with $NS3_{358-375}$ synthetic peptide for 7 days. The $CFSE^{low}$ cells were sorted and then stained with each tetramer. The cells are >99% $CD4^+$ as determined by flow cytometry (data not shown). Trop#20 are $CFSE^{low}$ tropomyosin-specific $CD4^+$ T cells expanded by the same procedure as $NS3_{358-375}$. Trop #20 was used to control for tetramer specificity (first column). Nonspecific tetramer (CLIP) was used for each experiment (bottom 2 panels). $NS3_{358}$ and variant MHC class II tetramers stain PB3019, PH1127, and PH1079 $CD4^+$ $CFSE^{low}$ T cells. Not Tested (NT). Tetramer used is labeled on the right.


PB3019
PH1127
PH1079
Trop#20
CLIP-PE
CLIP-APC
K371E
S370P
H369R
NS3358-375
0.047
0.17
0.012
0.036
0.096
0.4
0.098
0.035
0.96
1.99
0.99
0.17
0.46
0.35
0.23
0.17
0.6
11.9
NT
0.35
9.15
0.69
0.68
0.13
CD4
Tetramer

CHAPTER 3

NATURALLY OCCURRING $CD4^+$ T CELL EPITOPE VARIANTS ACT AS ALTERED PEPTIDE LIGANDS LEADING TO IMPAIRED HELPER T CELL RESPONSES IN HEPATITIS C VIRUS INFECTION

Abstract

Hepatitis C virus (HCV) has a high rate of replication and lacks RNA-proofreading capabilities, thereby leading to variant or mutant viruses circulating within the host as quasispecies. Previous work in our laboratory identified viral variants that emerged in a class-II immunodominant epitope $NS3_{358-375}$ of the nonstructural-3 (NS3) protein region of HCV, the sequence of which is based on genotype 1A, the most prevalent genotype in the U.S. population. Further work suggested that positive immune selection pressure was driving viral variation. Paradoxically, viral variants account for only a small percentage of the circulating virus in humans and chimpanzees, suggesting that passive evasion is not the only means of escape by HCV. This observation suggests a unique pathogenesis for HCV as it persists in the host. In the current study, we hypothesize that viral variants are acting as altered peptide ligands (APLs). To test this hypothesis, we used cloned T cells specific for $NS3_{358-375}$ peptide, which demonstrated attenuated T cell and IFNγ responses to individual variant peptides, when compared to the $NS3_{358-375}$ stimulated T cell clones. Furthermore, such variants could act as APLs, based on their ability to antagonize the IFNγ proliferative responses of clones specific for $NS3_{358-375}$. In addition, MHC class II tetramer staining demonstrated that variant MHC complexes were able to specifically bind to $NS3_{358-375}$ T cell clones. Taken together, the results suggest that viral variants may act as APL to effectively blunt the T cell response to an important HCV epitope.

Introduction

T cell activation occurs when a T cell receptor (TCR) binds to cognate or specific peptide bound to major histocompatibility complex (pMHC) molecules on the surface of antigen presenting cells (APCs) (1). The engagement of the TCR to pMHC is necessary for the activation of $CD4^+$ and $CD8^+$ T cells, thereby leading to an effective adaptive immune response against an invading pathogen (2). Single amino acid substitution in the cognate peptide, termed altered peptide ligands (APLs), can elicit a gradient of effector function changes in a specific T cell (3).

The recognition of these segments of a pathogen or epitopes presented by the pMHC to T cells is critical for the clearance of viruses (4). As in the case of Hepatitis C virus (HCV) infection, clearance of the virus has been found to be dependent upon the quality of the $CD4^+$ T cell response in up to 30% of individuals infected (5-8). Mueller et al. (9) have detected virus-specific $CD4^+$ T cells in chronic HCV patients, suggesting a novel mechanism of viral persistence (10-14).

Previous work in our laboratory identified viral variants that emerged in an MHC class-II immunodominant epitope $NS3_{358-375}$ of the nonstructural-3 (NS3) protein region of HCV (15). Further work suggested that positive immune selection pressure was driving viral variation (16). In contradiction to viral escape models, HCV variants account for only a small percentage of the circulating virus in humans and chimpanzees (17, 18). Also, HCV is able to modulate the immune response specifically towards viral persistence, indicating that HCV may be able to exploit a different mechanism in place of a more passive evasion strategy, leading to nondetection of the HCV pathogen by the immune system (16).

One such mechanism that HCV may be able to exploit for persistence includes APLs, which have been observed within the hypervariable region 1 (19, 20), and in other pathogens, such as HBV (4, 21), HIV (22, 23) and *plasmodium falciparium* (24). Our previous studies identified and tracked viral variants arising in a single HCV chronic individual and further determined the effect these variants had on the *in vitro* immune response (15, 16, 18). Although HCV circulates in the host as a quasispecies, it appears that such variation does not lead to viral escape, but rather that some variants are able to suppress the immune response in an antigen-specific manner.

To test the hypothesis that viral variants could act as APLs, we used cloned T cells specific for the MHC class II epitope $NS3_{358-375}$ peptide. The APL peptides alone had low T cell and cytokine responses in comparison to cognate peptide, all of which has been documented elsewhere (16). Prepulsing clones with variant peptides revealed that variants were able to antagonize proliferative and IFNγ responses to wild type $NS3_{358-375}$ peptide. Further, MHC class II tetramers staining, loaded with variant peptides, are able to bind specifically to $NS3_{358-375}$ T cell clones. Thus, viral epitope variants are able to blunt the responses of the very T cells that should help with the elimination of virus. Our results also help to explain observations that $CD4^+$ T cells responses to HCV antigens seem attenuated or missing in chronically infected patients; they also bear on the findings that $CD8^+$ killer T cells are ineffective at eliminating HCV infected target cells (25, 26).

Materials and Methods

Blood Samples

These studies have been reviewed and approved by the University of Utah and the Medical College of Wisconsin Institutional Review Boards. Isolation of lymphocytes and subsequent HLA typing was previously described (27).

Synthetic Peptides

In vitro PBMC and T cell clones were stimulated with synthetic peptides representing one human leukocyte antigen DRB1*1501 restricted-epitope surrounding HCV NS3 amino acids 358-375 (aa 1384-1401 of the HCV polyprotein) as previously described (28, 29). The three single amino acid variants were identified in a chronic HCV patient (P.B3019) (28, 29). Peptide sequences were as follows: wild type 358-375 ($NS3_{358-375}$), VIKGGRHLIFCHSKKKCD; variant H369R, VIKGGRHLIFCRSKKKCD; variant S370P, VIKGGRHLIFCHPKKKCD; variant K371E, VIKGGRHLIFCHSEKKCD.

T Cell Proliferation Assay

To measure proliferative responses of PBMC and T cell clones following stimulation with wild type peptide $NS3_{358-375}$ and variants peptides, cells were plated at 1 x 10^5/ well in round-bottom 96 well plates and incubated at 37ºC, 5% CO_2 at indicated times and peptide doses. The cells were pulsed overnight with 1 μCi/well of titrated thymidine (^{3}H-TdR) (Perkin Elmer, Waltham, Massachusetts) and harvested onto glass

fiber filters (Perkin Elmer) for measurement of radiolabel incorporation by scintillation counter (Perkin Elmer).

T Cell Clones

Specific helper T cell clones, towards HCV NS3 peptide 358-375 ($NS3_{358}$), were generated from a resolved DRB1*1501/1301 resolved hemophiliac individual (PH1127). PH1127 PBMC were re-suspended and plated into 96 well flat bottom plates (200μl/well) at 2 x 10^6 cells/ml. Cells were stimulated with $NS3_{358-375}$ at 5μM/ml at 37°C, 5% CO_2 incubator. Cells were provided 10 U/ml rhIL-2 (BD bioscience). On day 14, cells were tested for responsiveness to $NS3_{358-375}$. Functional cells were further subcloned by limiting dilution at cell densities of 10, 3, 1, and 0.3 cells/well in 96 flat bottom plates, which contained 10^5 irradiated (3000 rad) autologous PBMC. T cells from 0.3cells/well were moved to 24 well plates and cultured with 10μM/ml $NS3_{358-375}$ peptide and 10U/ml of rhIL-2 in complete media. Culture of PBMC and T cell clones were cultured in complete media: RPMI 1640 tissue culture medium (BioWhittaker, Walkersville, ME) 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, Herndon, VA), 10 U/ml heparin sodium (Fisher Scientific, Pittsburgh, PA) and 10% pure human serum (Atlanta Biologicals, Lawrenceville, GA). T cell clones were tested for responsiveness to $NS3_{358-375}$ and variant peptides. Cells were cultured in a 37ºC, 5% CO_2 incubator at indicated times.

Cytokine ELISA

Cell culture supernatants IFNγ levels were determined by a commercial ELISA kit from Pharmingen, Inc. (San Diego, CA, USA) according to the manufacturer's instructions. Experimental values were determined by comparing the optical densities with a standard curve derived from recombinant IFNγ. Negative controls consisted of background levels of APC cultured with T cell clones and complete media. Each experiment was done in triplicate.

Antagonism Assay

Antigen presenting cells (APCs) were prepulsed 2 hrs. with wild type at 1μM, washed (3x) in complete media + 10%PHS and irradiated at 300 Rad. APCs were split (2 x 10^5 cells/well) into 96 well trays contain variant peptides at indicated concentrations and T cells were added at 1 x 10^4 cells/well. Cells were incubated at 37°C for 48 hrs. Then 100μl of supernatant was removed for ELISA screening and ^{3}[H]-thymidine was added for 18 hrs. before harvesting and measuring ^{3}[H]-thymidine incorporation.

Anergy Assay

APCs were prepulsed for 3 hrs. with variant peptides at 1μM, washed, and irradiated (3000 rad). The APCs were split into wells containing $NS3_{358-375}$ peptide at 0.1, 1, or 10μM concentrations. T cells were subsequently added at 1 x 10^4 cells/well and incubated at 37°C for 48 hrs. 100μl of supernatant was saved for ELISA screening and ^{3}[H]-thymidine was added for 18 hrs. before harvesting and measuring ^{3}[H]-thymidine incorporation.

Tetramer Staining

All tetramers were obtained from the NIH tetramer facility at Emory University. T cell clones were stained either with 358-375- Phycoerythrin (PE), or variant H369R-Allophycocyanin (APC); variant S370P-APC; variant K371E –APC tetramers for 1hr at 37°C. 7-AAD (BD bioscience), cell viability probe, CD4-pacific blue (BD bioscience), CD3-Amcyan (BD bioscience), CD8-FITC (eBioscience) were added for 15 min. at 4°C, washed with flow stain buffer (BD bioscience) and analyzed on a BD FACSCanto II. Negative controls consisted of staining cells with nonspecific peptide, CLIP-DR15 tetramer, labeled with either –PE label or –APC respectively. Further, T cell clone $HA_{306-318}$ was stained with each tetramer (data not shown). Results were compared using Student's *t*-test and considered significant if the $p<0.05$. Flow cytometry data analysis was performed using Flow Jo software (Tree Star).

Results

Epitope Variants Have an Attenuated T Cell Proliferative Response

To determine if naturally occurring epitope variants are acting as APL and thus antagonizing or anergizing potential helper T cell responses, we used T cell clones specific for $NS3_{358-375}$. The T cell clones specific for $NS3_{358-375}$ were derived from a resolved HCV patient PH1127 (HLA-DR15) that recognizes a previously characterized immunodominant $NS3_{358-375}$ epitope (Figure 3.1A, B) (30, 31). Furthermore, previous work has demonstrated that the variants are able to bind to HLA-DR15 molecules (16, 32). T cell clones derived from PH1127 are labeled as T.358 and these T cell clones have an attenuated T cell proliferative and IFNγ response to the variant peptides (Figure 3.1C,

D). The inability of variant peptides to activate T cell proliferation and IFNγ secretion when incubated with variant peptides led us to investigate if these variants were antagonizing the cognate T cell response.

Characterization of Variant Peptides as Antagonists

To determine if the variants could serve as antagonists, cells were prepulsed with 1μM $NS3_{358-375}$ (open circle) and co-cultured in a dose-dependent manner with variant peptides (Figure 3.2). The variants effectively antagonize the T cell response in both the polyclonal and T cell clone assays in a dose-dependent manner (Figure 3.2). Furthermore, the IFNγ cytokine levels are attenuated in a similar fashion as the proliferative response (Figure 3.2B,D). Taken together, the viral variants act as an antagonist due to the lack of both proliferative and IFNγ response, when both variant and cognate peptides are present.

To assess if the variant peptides are anergizing the cognate T cell response, APCs were prepulsed with 1μM variant peptides for 3 hrs., washed, irradiated, and cultured with T cell clones and cognate $NS3_{358-375}$ (Figure 3.3). H369R blunts both T cell proliferative and IFNγ response, but variants S370P and K371E act as partial-antagonists (Figure 3.3). These results suggest that one variant is able to anergize T cells and variants S370P and K371E act as partial-antagonists.

Viral Variants Engage $NS3_{358-375}$ T Cell Clones

To establish viral variants capacity to bind to $NS3_{358-375}$ T cells, T cell clones were stained with MHC class II tetramers (Figure 3.4). Tetramers loaded with each individual peptide were used to stain T358.3. All three variant tetramers were able to

bind to T358.3 clones (Figure 3.4, red histogram). The ability of these MHC class II tetramers to bind to the same T cell clones as the $NS3_{358-375}$ suggests that these variants are able to not only bind to the same TCR, but also to antagonize antigen-specific $CD4^+$ T cells.

Discussion

We demonstrate that variant epitopes modulate *in vitro* immune responses to the cognate peptide. Our results suggest that naturally occurring variants within a protective immunodominant epitope may act as APLs, leading to changes in the quality of T cell responses, which could allow for viral persistence.

This viral persistence seems relevant considering that HCV is able to modulate the immune response in an antigen-specific manner. This was shown by the fact that the variants were able to attenuate the response to $NS3_{358-375}$ in antagonism assays. Each variant showed unique effects on the wild type response that ranged from anergy by H369R to partial-antagonism by S370P and K371E. By comparison, work by Scotta et al. (20) studied the effects of hypervariable region 1 variants in a $CD4^+$ T cell epitope and found that the antagonists were able to induce apoptosis. We observed no evidence of induced programmed cell death, as Annexin-V/PI staining in polyclonal assays showed no difference in cultures treated with variants alone or in combination with wild type $NS3_{358-375}$ (Chapter 4). Interestingly, our lab had shown that variant S370P was found to be stable in a chronic HCV patient for over 2 years, consistent with selection and fixation of this variant (15). In the current study, S370P was still able to induce a proliferative and IFNγ response, albeit at lower levels than the wild type peptide, and are able to bind

specifically to T cell clones specific for cognate peptide. It seems that S370P is able to act on the *in vitro* response to HCV through varied suppressive mechanisms (33, 34). One such mechanism of suppression is Tregs, in that, preincubating PBMC with S370P increases phenotypic markers for Tregs (Chapter 2). Expanding these findings to a larger cohort of subjects, the Treg markers are found at significantly higher levels in PBMC from both resolved and chronically infected patients; we were able to show the antigen-specific induction of Tregs (Chapter 2).

A defining feature of APLs is the ability of the antagonist peptide, coupled to pMHC, to bind to the TCR. In the current study, we used MHC class II tetramers to determine whether variant peptides coupled to MHC were able to bind to T cell clones specific for $NS3_{358-375}$ and possess the same TCR. The variants were able to bind to the T cell clones, suggesting that this has an effect on the biological outcome of the T cell. These results do not clearly demonstrate the kinetics of the pMHC-TCR and is currently being investigated. Although tetramer staining addresses the ability of the variants to bind to the same TCR as the wild type tetramer, further investigation needs to be performed to determine if markers of antagonism are upregulated, such as the phosphatase SHP-1 (35).

Using cloned T cells specific for the MHC class II epitope $NS3_{358-375}$ peptide and previously identified naturally occurring APLs within a protective immunodominant epitope, suggests that changes in TCR activation may be used by HCV to deviate or blunt an HCV-specific T cell response (16). These results provide an explanation for the attenuated or missing $CD4^+$ T cells in chronically infected patients (25, 26). Although these results suggest that HCV is exploiting TCR signaling for viral persistence, further

investigation into APLs effect on the differentiation of these antigen-specific $CD4^+$ T cells is necessary to understand HCV pathogenesis.

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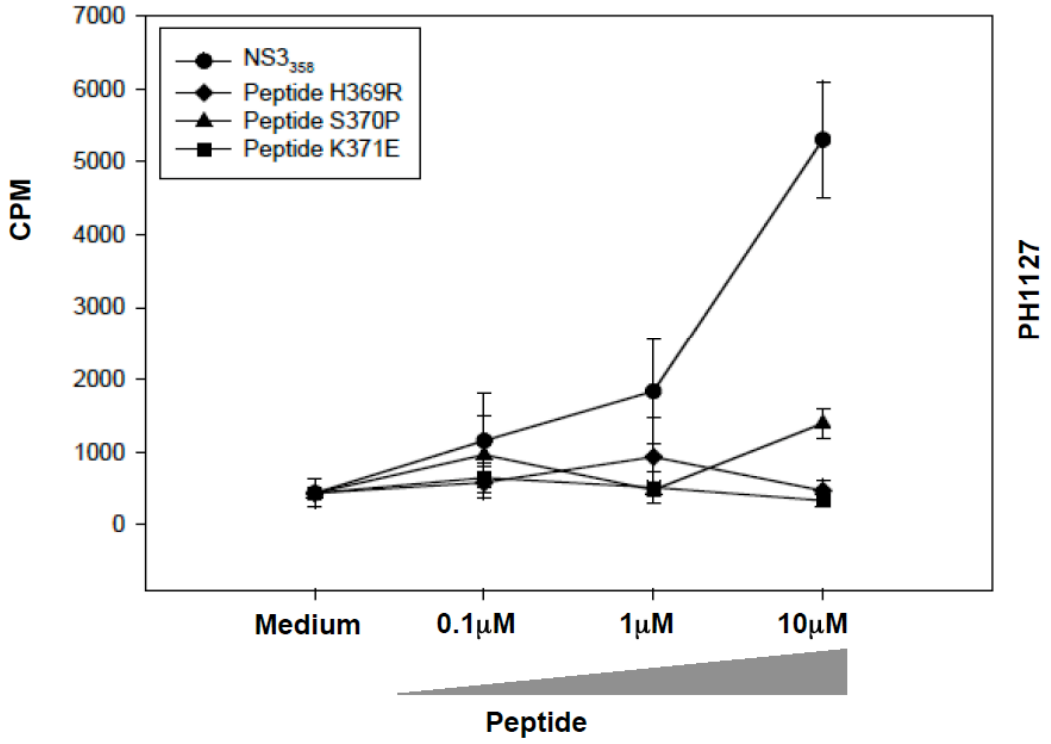
Figure 3.1. Viral variants H369R, S370P, and K371E have an attenuated T cell proliferative and IFNγ response in both polyclonal and T cell clone assays, when compared to cognate $NS3_{358-375}$ stimulated cells. A,C. Cognate peptide has a dose-dependent T cell response and the variant peptides have an attenuated T cell response. Medium alone was used as a negative control. Results are shown in mean counts per minute (CPM) +/- standard error of triplicate cultures. B,D. Corresponding IFNγ response for polyclonal and T cell clones. Results are shown pg/ml- medium +/- standard error of triplicate cultures. D. Medium was subtracted from the experimental for each sample. PBMC from PH1127 were cultured for 5 days and T cell clones for 72 hrs. at indicated peptide concentrations. *, $p<0.05$ as determined by student's *t*-test. D. Results are representative of 3 independent experiments.

A.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6000
5000
4000
3000
2000
1000
0
CPM
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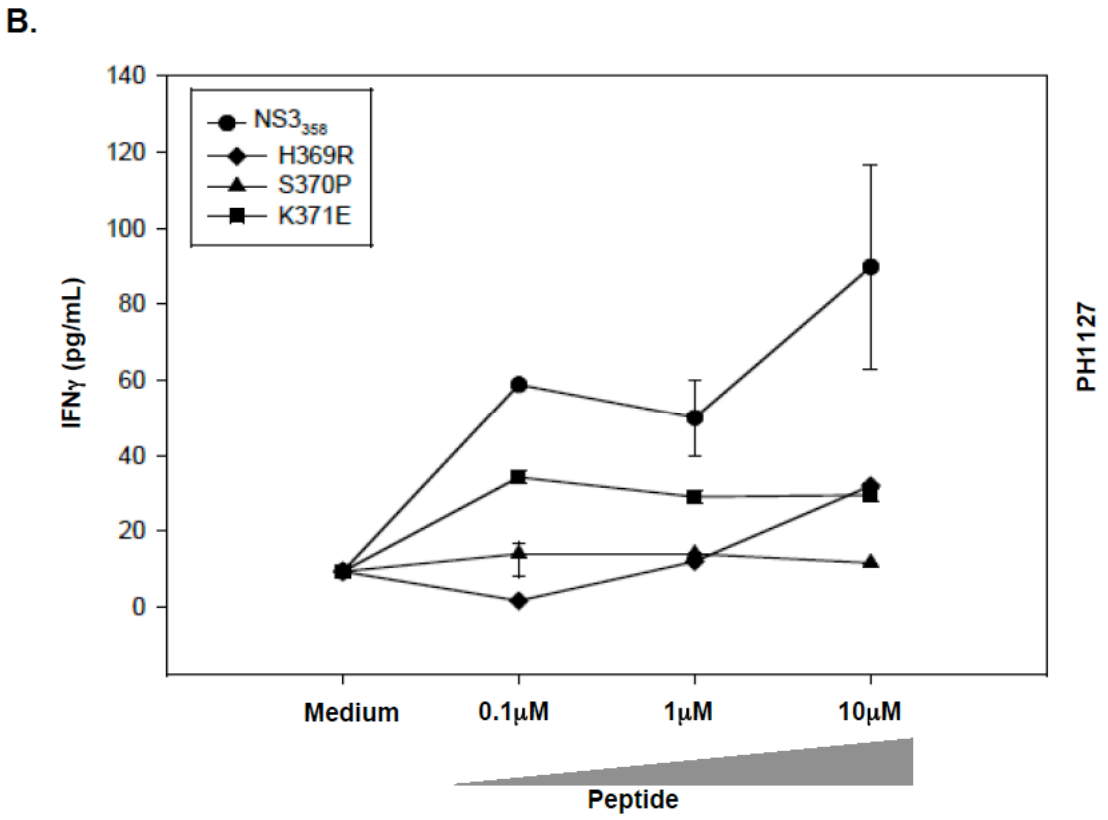
Peptide S370P
Peptide K371E
PH1127
Medium
0.1μM
1μM
10μM
Peptide



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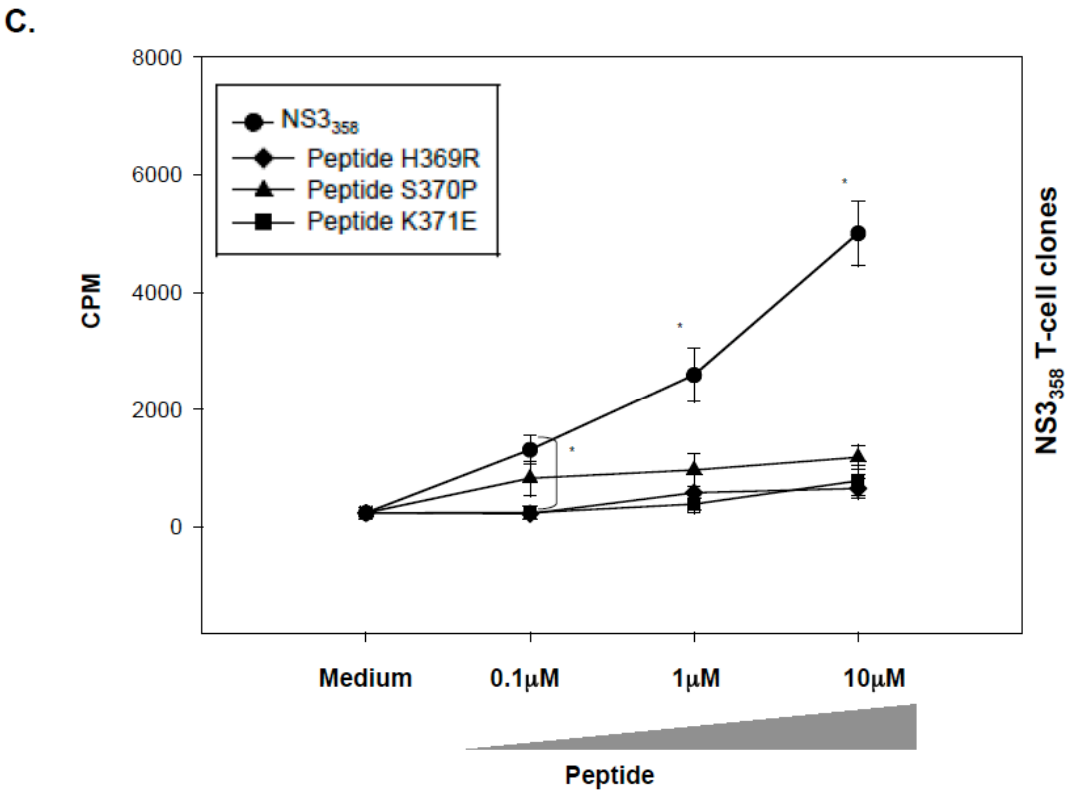

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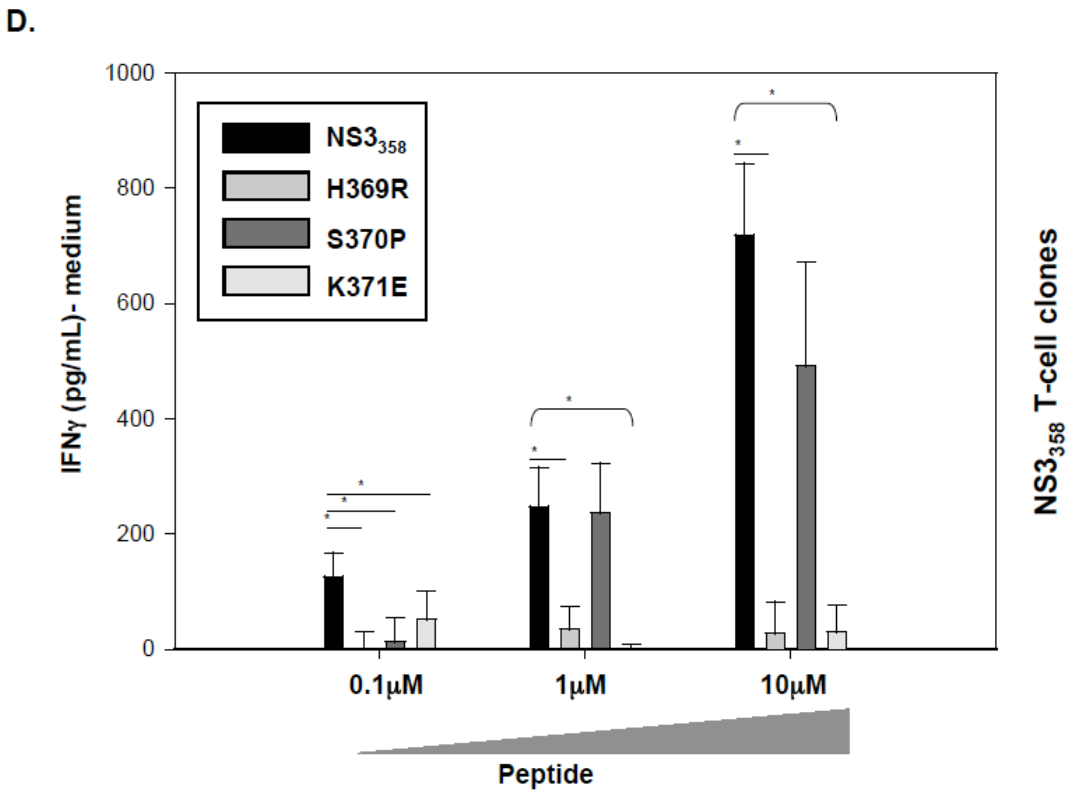
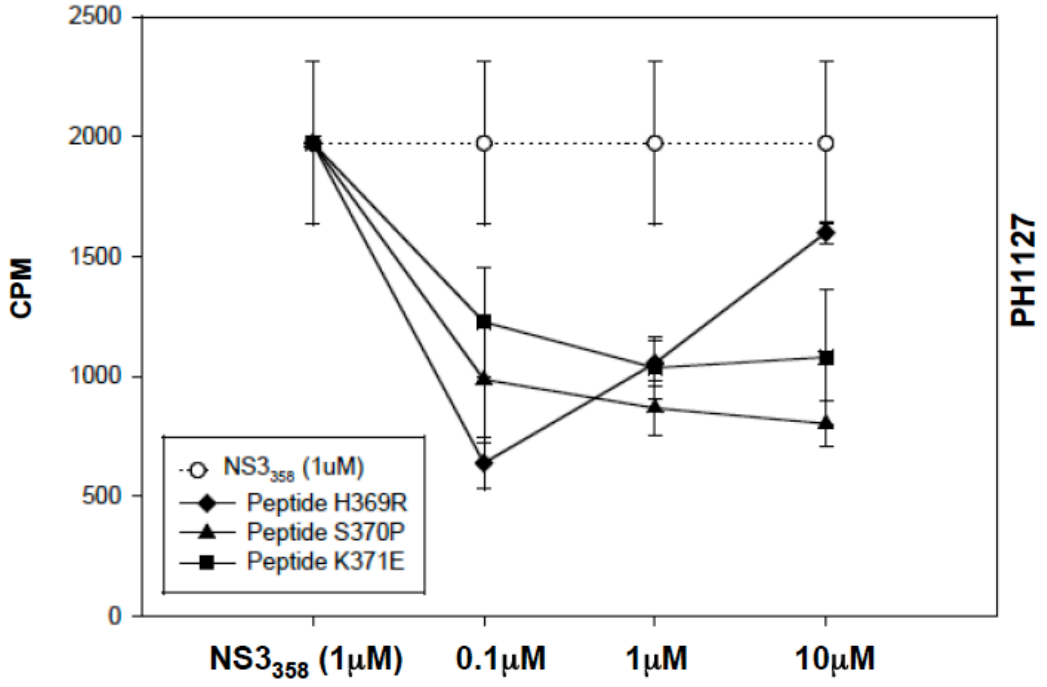


Figure 3.1 continued

Figure 3.2. Variant peptides act as antagonists. A. PBMC were incubated with 1μM $NS3_{358-375}$ for 3 hrs. and then washed. $NS3_{358-375}$ variant peptides were added to the culture at indicated doses and incubated for 5 days. B. Supernatants were collected at 48 hrs. from the cell culture and IFNγ levels were measured. C,D. Antigen-presenting cells were prepulsed with $NS3_{358-375}$ peptide for 3 hrs., washed, irradiated and cultured with T cell clones with variant peptide for 72 hrs. The dotted line with open circle represents proliferation by $NS3_{358-375}$ peptide prepulse alone. D. The supernatants from T358 were collected at 48 hrs. and IFNγ levels were measured. Each experiment was performed in triplicate.

A.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1500
1000
500
0
CPM
PH1127
NS3358 (1uM)
Peptide H369R
Peptide S370P
Peptide K371E
NS3358 (1μM)
0.1μM
1μM
10μM

B.

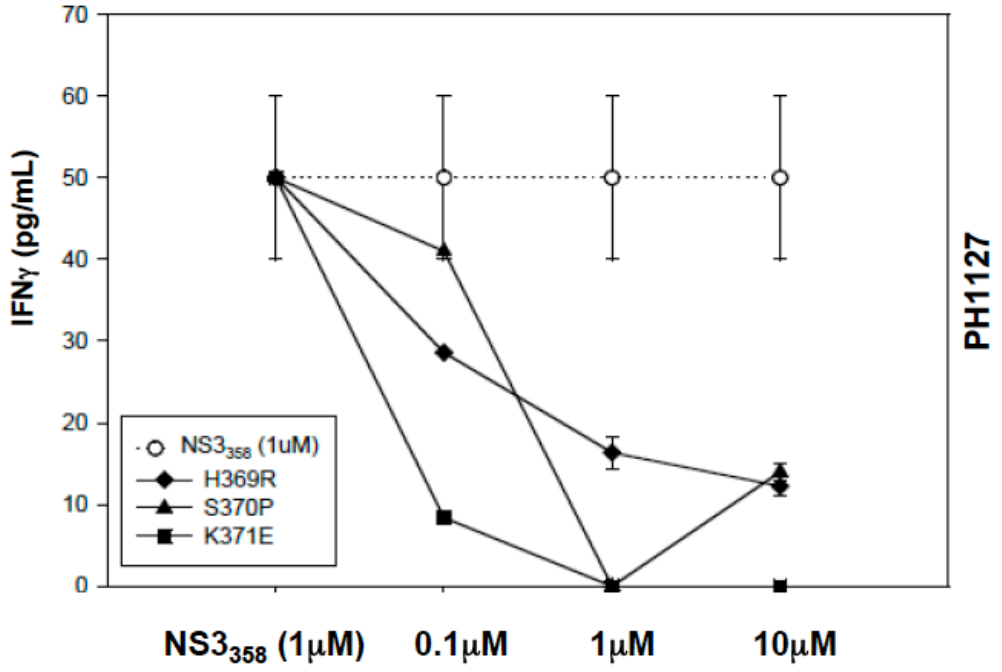
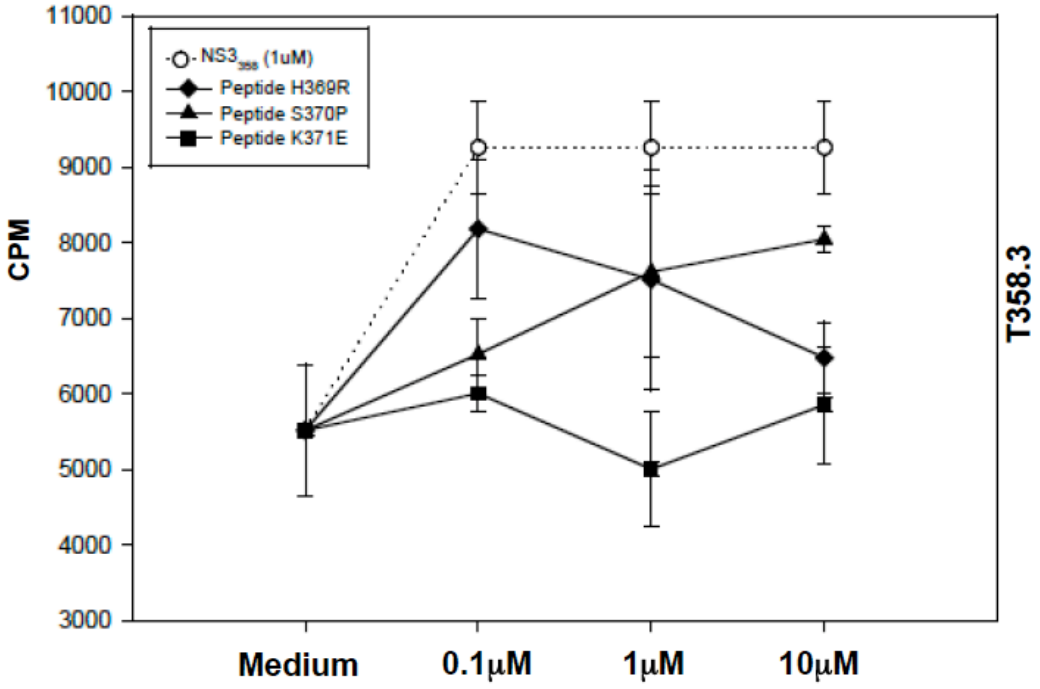


Figure 3.2 continued

C.



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D.

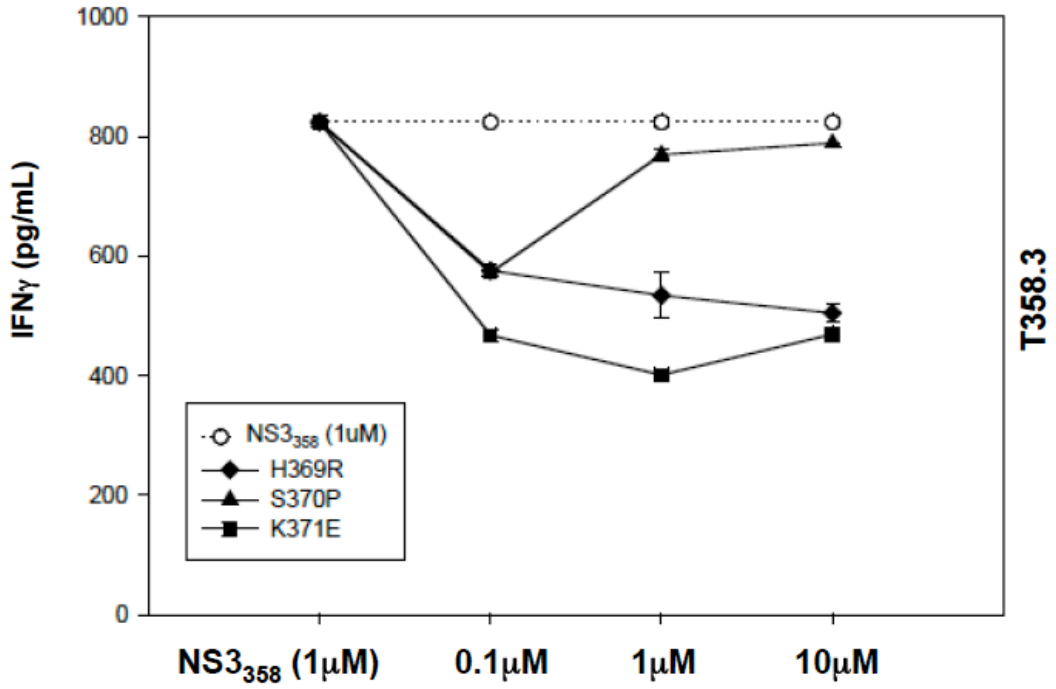
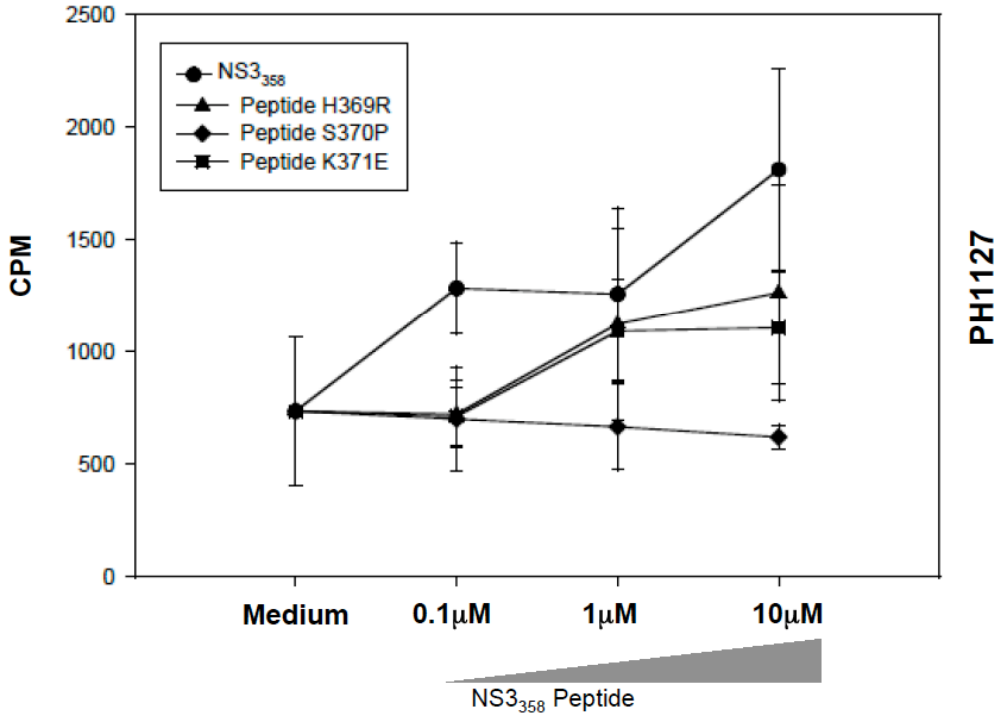


Figure 3.2 continued

Figure 3.3. H369R variant anergizes $NS3_{358-375}$ specific T cells. A-B. PBMC were prepulsed with indicated variant peptide and subsequently stimulated with $NS3_{358-375}$ peptide in a dose dependent manner. C-D. APC cells were incubated with variant peptide for 3 hrs., washed, irradiated and added to T cells with varying concentration of the cognate peptide. B,D. Supernatants were collected at 48 hrs. and the IFNγ levels were measured. Each experiment was performed in triplicate.

A.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2000
1500
1000
500
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Peptide S370P
Peptide K371E
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1μM
10μM
NS3358 Peptide
PH1127

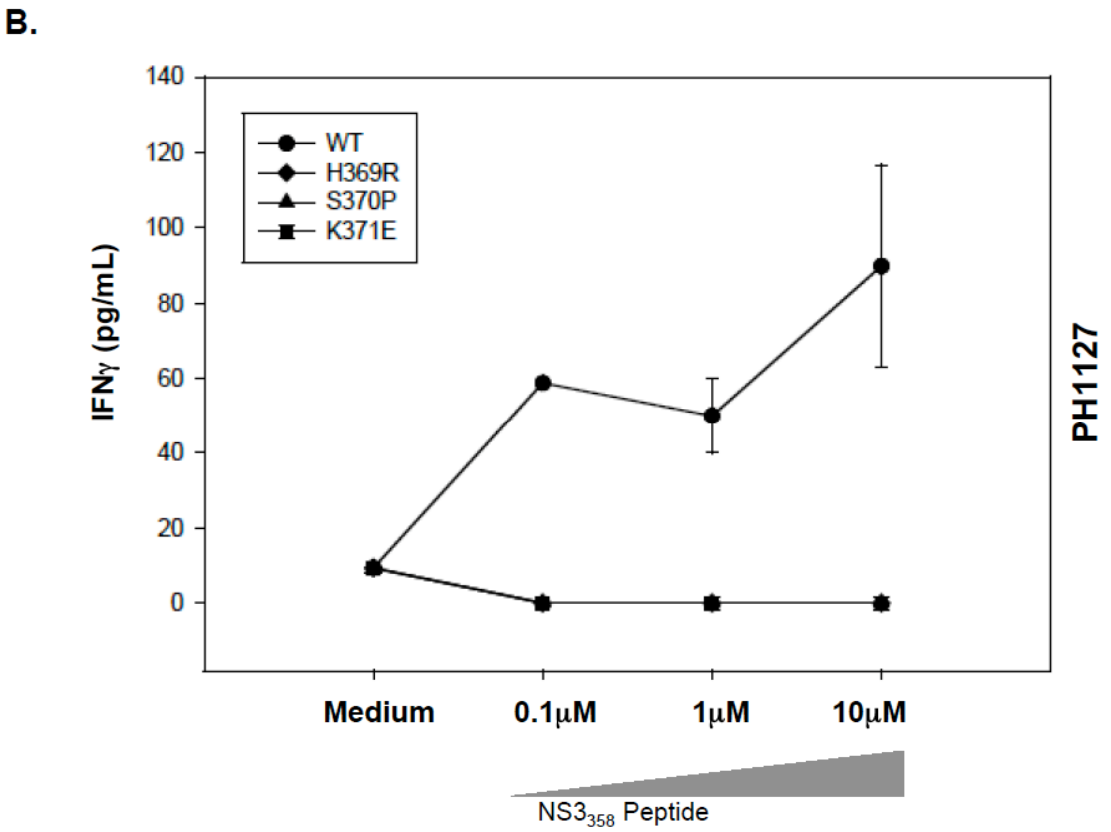
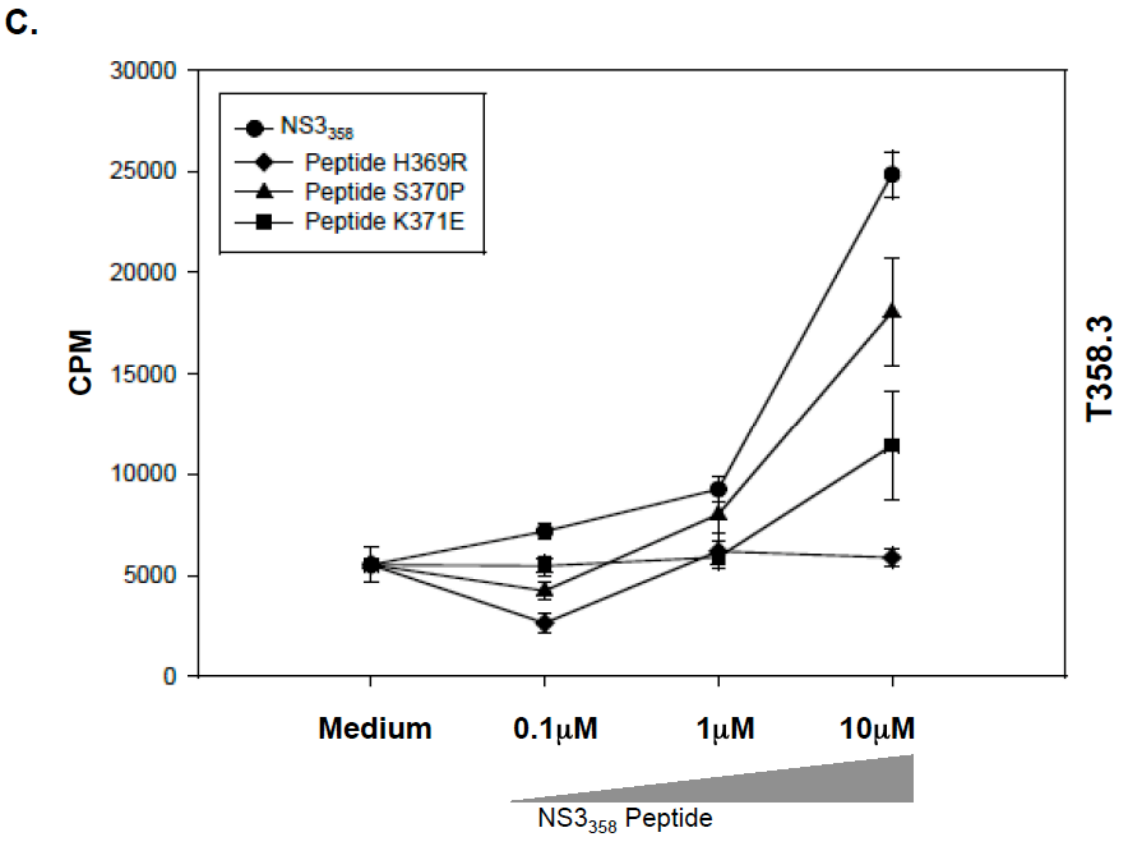


Figure 3.3 continued



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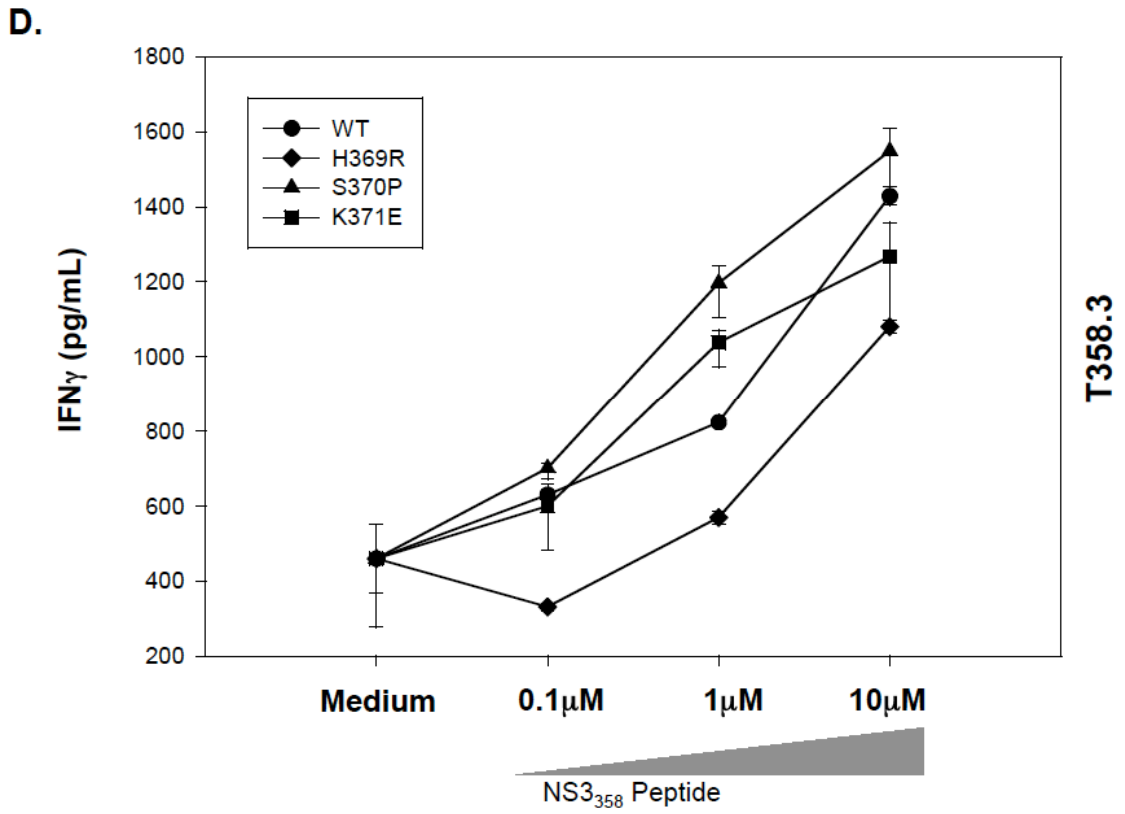

Figure 3.3 continued

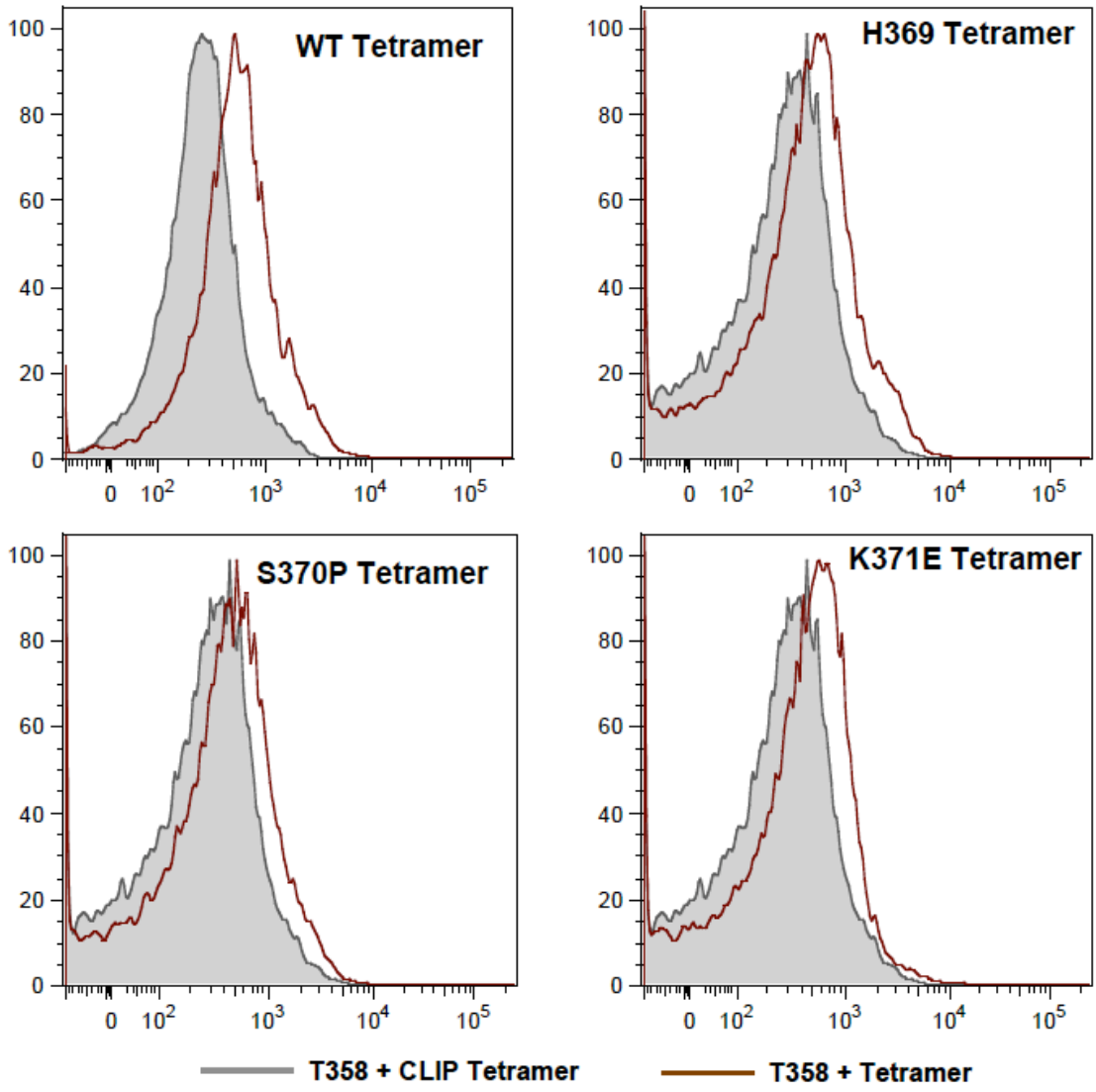

Figure 3.4. Variant MHC class II tetramers bind to $NS3_{358}$ T cell clones. Histograms of MHC class II tetramer (red) staining. CLIP loaded DR15 tetramer was used as a control (gray). The control for the variant tetramer was performed at the same time for the variant stains.

CHAPTER 4

CO-RECOGNITION OF WILD TYPE AND VARIANT HEPATITIS C VIRUS EPITOPES ANTAGONIZES $CD4^+$ HELPER T CELLS

Abstract

A wide breadth of work has demonstrated a correlation between Hepatitis C Virus (HCV) persistence and an increase in regulatory T cells (Tregs). The relationship suggests that HCV could modulate $CD4^+$ T cells in an antigen-specific manner, thereby leading to a symbiotic relationship between the host and the virus. The mechanism responsible for the increase of Tregs is unknown. One possibility for the up regulation of Tregs is through naturally occurring viral variants. Viral variants have been found to act as altered peptide ligands (APLs) in HCV infection, leading to modulated T cell responses. Further, we have demonstrated that one viral variant was able to induce Treg phenotypic markers. Taken together, we hypothesized that one possible mechanism of induction of Tregs is through viral variants antagonizing HCV-specific T cells. We performed *in vitro* assays using two subjects that respond to the wild type peptide. Our results indicate that both the wild type and variant peptides are necessary for the suppression of a T cell response. Using MHC class II tetramers to compare the avidity of the wild type to variant, the variant tetramer had a lower avidity for the antigen-specific $CD4^+$ T cells. Further, one viral variant, S370P, induced an up regulation of Foxp3 in MHC class II tetramer wild type positive cells. Lastly, confocal microscopy portrays variant and wild type tetramers binding to the same T cell. 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.

Introduction

Hepatitis C Virus (HCV) is a positive-stranded RNA virus that circulates the host as a heterogeneous population. The most frequently represented genome is referred to as the wild type sequence; the viral variants arising in the viral genome are called quasispecies (1). The majority of circulating viruses in chronic HCV subjects appear to be the wild type population (2-4). The role of these quasispecies or viral variants in HCV pathogenesis can range from simple escape to deviation of the immune response, possibly leading to viral persistence (5, 6). In opposition to simple escape models, HCV variants have been found to account for only a small percentage of the circulating virus in humans and chimpanzees (3, 4, 6, 7). This paradox of wild type viral genomes persisting in the presence of T cells implies that there may be another level of immuno-regulation that is modulated by HCV (8-12).

The recognition of HCV epitopes or segments of the virus presented by the peptide-major histocompatibility complex (pMHC) to T cells is necessary for HCV clearance (13-16). Previous studies have demonstrated that naturally occurring viral variants arising in $CD4^+$ "helper" T cell epitopes are able to modulate T cell signaling (6, 17). These results suggest one of the mechanisms that HCV may be able to exploit for persistence is altered peptide ligands (APLs) (17). APLs are capable of modulating the T cell response by having an amino acid substitution in the cognate peptide (18). A number of pathogens that have been shown to exploit APLs for persistence including HCV (17, 19), HBV (20, 21), HIV (22, 23), and *plasmodium falciparium* (24). Previous work in our laboratory identified naturally occurring viral variants arising in an immunodominant $CD4^+$ T cell epitope (6). One viral variant, S370P, was found to be stable for over 2

years, suggesting "selection and fixation" of this HCV viral isolate, which indicates a role for this variant in HCV pathogenesis (6, 25). Recently, we have found that viral variant S370P is able induce Tregs to suppress the antiviral T cell response in an antigen-specific manner (Chapter 2).

Tregs can be classified into two groups. One kind are Tregs induced in the periphery have been termed inducible Tregs, defined by secretion of IL-10, TGFβ, and IL-4 and natural Tregs (nTregs), which are selected for in the thymus and are characterized by the extra cellular surface markers $CD4^+$ $CD25^+$ (26, 27). Although there are other extra cellular surface markers suggestive of Tregs, the most accepted marker is the expression of the transcription factor Forkhead Box P3 (Foxp3) (28). The expression of Foxp3 is critical in the development of Tregs (29-33). An increase in Treg markers has been shown in cohorts of chronic HCV subjects when compared to resolved and noninfected individuals, suggesting that HCV is inducing Tregs for viral persistence (3, 34-39).

Recent work has demonstrated that HCV is able to exploit naturally occurring viral variants to modulate $CD4^+$ T cell responses (17, 19). 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 respond to the wild type peptide. Both the wild type and variant peptide are necessary for the 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 the avidity was different in comparison to the wild type tetramer. Further, one viral variant, S370P, induced an up regulation of Foxp3 in MHC class II tetramer wild

type positive cells. Lastly, fluorescent microscopy clearly shows that both the variant and the wild type tetramers stain the same T cell. 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.

Materials and Methods

Lymphocytes

These studies have been reviewed and approved by University of Utah and Medical College of Wisconsin Institutional Review Boards. Peripheral blood mononuclear cells (PBMC) were isolated isolation over Lymphocyte Separation Medium (GE-Healthcare) and preserved in liquid nitrogen, as previously described (40). Human leukocyte antigen (HLA) class II typing had been performed on each patient, as previously described. PB3019 is DRB1*0701/1501 and PH1127 is DRB1*1501/1301. PB3019 is a chronic HCV subjects infected with genotype 1a, as previously described (2). Hemophiliac individual PH1127 is a resolved HCV subject with detectable antibody for HCV (5).

Synthetic Peptides

Synthetic peptides representing one DRB1*1501 restricted-epitope surrounding HCV NS3 amino acids 358-375 (aa 1384-1401 of the HCV polyprotein) were synthesized as previously described (5, 41). The three single amino acid variants were identified in a chronic HCV patient (P.B3019) (5, 41). Peptide sequences were as follows: wild type 358-375 ($NS3_{358-375}$), VIKGGRHLIFCHSKKKCD; variant H369R,

VIKGGRHLIFCRSKKKCD; variant S370P, VIKGGRHLIFCHPKKKCD; variant K371E, VIKGGRHLIFCHSEKKCD.

Cell Culture and Media

Culture of PBMC was in RPMI 1640 tissue culture medium (BioWhittaker, Walkersville, ME) 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, Herndon, VA), 10 U/ml heparin sodium (Fisher Scientific, Pittsburgh, PA) and 10% human serum (Atlanta Biologicals, Lawrenceville, GA). Cells were cultured in a 37ºC, 5% CO_2 incubator.

T cell Proliferation Assay

To measure proliferative responses of PBMC following stimulation with indicated peptides, cells were plated at indicated cell concentrations in round-bottom 96 well plates (Costar) and incubated at 37ºC, 5% CO_2 at indicated times and peptide doses. The cells were pulsed for the last 16-18 hrs. of incubation with 1 μCi/well of titrated thymidine (^{3}H-TdR) (Perkin Elmer, Waltham, Massachusetts) and harvested onto glass fiber filters (Perkin Elmer) for measurement of radiolabel incorporation by scintillation counter (Perkin Elmer).

$CD4^+$ T Cell Line

Antigen-specific $NS3_{358-375}$ $CD4^+$ CFSE T cells were derived from PH1127 and PB3019. PBMC from PH1127 and PB3018 were labeled with 0.5μM

Carboxyfluorescein succinimidyl ester (CFSE) and stimulated with $NS3_{358-375}$ synthetic peptide. On day 7, the cells were stained with CD4-APC (Invitrogen, Carlsbad, CA) and cell sorted at the University of Utah cell sorting core facility. The $CD4^+$ T cells were sorted by either $CFSE^{high}$ (non-proliferating) or $CFSE^{low}$ (proliferating). The cells were washed and expanded by CD3/CD28 dynabeads (Invitrogen) with 10U/ml of human rIL-2 ((BD Pharmingen, San Diego, CA). Prior to testing the CFSE cells for responsiveness to $NS3_{358-375}$, the CD3/CD28 beads were removed, washed, and rested for 3 days. $CD4^+$ CFSE cells (2 x 10^4 cells/well) were stimulated with increasing concentrations of $NS3_{358-375}$ peptide by a mixture of chronic and resolved HLA-DR15 APCs treated with 10μg/ml Mitomycin C (Sigma Aldrich, USA) at 1 x 10^5 cells/well for 3 hrs. prior to incubation.

Antagonism Assay

APCs were prepulsed for 2 hrs. with indicated peptide concentration, washed (3x) in complete media + 10%PHS and then treated with Mitomycin C at 5μg/ml for 1hr and washed (3x) in complete media + 10% PHS. APCs were split (1 x 10^5 cells/well) into 96 well trays contain variant peptides at indicated concentrations and T cells were added at 2 x 10^4 cells/well. Cells were incubated at 37°C for 48 hrs. Then 100μl of supernatant was removed for ELISA screening and ^{3}[H]-thymidine was added for 16-18 hrs. before harvesting and measuring ^{3}[H]-thymidine incorporation.

Tetramer Staining

T cells were stained with tetramers (NIH tetramer facility at Emory University) 358-375- Phycoerythrin (PE), or variant H369R-Allophycocyanin (APC); variant S370P-

APC; variant K371E –APC tetramers for 1hr at 37°C. 7-AAD (BD bioscience), CD4-pacific blue (BD bioscience), CD3-Amcyan (BD bioscience), CD8-FITC (eBioscience) were added for 15 min. at 4°C, washed with flow stain buffer (BD bioscience) and analyzed on a BD FACSCanto II (BD Biosciences). Negative controls consisted of staining cells with nonspecific peptide, CLIP-DR15 tetramer, labeled with either –PE label or –APC respectively. Further, $CD4^+$ $CFSE^{low}$ T cells specific for Tropomyosin were stained with each tetramer. Flow cytometry data analysis was performed using Flow Jo software (Tree Star).

Foxp3 Staining

Cultures were then stained with 358-375- Phycoerythrin (PE) tetramer for 1hr, CD4-Pacific Blue, CD3-Amcyan, CD25-APC, and 7-AAD were added for the last 20 min. at 4˚C and then washed 2x with stain buffer (BD Pharmingen). Using eBioscience Foxp3 staining kit, the cells were fixed and permeabilized for 1hr at 4˚C, washed 2x in permeabilization buffer. Normal rat serum was added (2µl/100µl) for 15 min. and then stained with Foxp3-FITC (eBioscience) for 1hr at 4˚C, washed 2x with stain buffer and analyzed on a BD FACS Canto II. To account for fluorescence spill over and nonspecific staining, we performed fluorescence-minus-one (FMO) with isotype control. These FMO controls contain all of the antibody conjugates used in the experiment except Foxp3-FITC, with the addition of FITC- isotype control. This was performed for each culture condition. Flow cytometry data analysis was performed using Flow Jo software (Tree Star).

Fluorescent Microscopy

Images were obtained at the University of Utah School of Medicine Cell Imaging Facility. The $CD4^+$ T cells were fixed in BD cytofix for 1hr at 4°C. The cells were washed 1x with BD pharmingen's Stain Buffer [Dulbecco's Phosphate Buffered Saline (DPBS), pH 7.4, 0.2% Bovine Serum Albumin, containing 0.09% Sodium azide]. 1 x 10^5 cells/50μl $CD4^+$ T cells were stained with 10μg/ml of MHC class II tetramers for 2 hrs. at 4°C, washed 2x in Stain Buffer, and imaged. Fluorescent images were obtained using a Nikon AR1 system with a 60x oil objective. The Emissions wavelength (E_mW) and Excitation wavelength (E_xW) for -PE (E_mW/ E_xW) was 595/561.2 and –APC (E_mW/ E_xW) was 700/635.8.

Results

Both $NS3_{358}$ and Variant S370P Peptides are Necessary for T Cell Suppression

Previous work demonstrated that variant S370P was able to attenuate the T cell responses to $NS3_{358}$ peptide. To determine if viral variant S370P was able to suppress a T cell response to a nonspecific antigen, PBMC from PB3019 were preincubated with either an individual peptide or both peptides when the cells were stimulated with 2μg/ml PHA (Figure 4.1). PBMC preincubated for 3 hrs. with either $NS3_{358}$ or S370P had no effect on the T cell response to PHA, whereas PBMC preincubated with S370P (1μM) for 3 hrs. with the addition of $NS3_{358}$ (1μM) and PHA (2μg/ml) suppressed the T cell response (Figure 4.1, black bar). These results suggest that the suppressive phenotype was dependent on the presence of both the viral variant and cognate peptide.

Creation of a $NS3_{358}$ Antigen-Specific T Cell Line

To amplify $NS3_{358}$ antigen-specific $CD4^+$ T cells from PH1127 (resolved) and PB3019 (chronic) HCV subjects, PBMC were prelabeled with CFSE and subsequently cell-sorted by $CD4^+$ $CFSE^{high/low}$ (Figure 4.2A-B). After expanding the sorted $CD4^+$ T cells (>99%, data not shown) with CD3/CD28 beads, the $CD4^+$ T cells (2×10^4) were tested for responsiveness to $NS3_{358}$ peptide by using APCs (1×10^6 cells/ml) from DR15-subjects that were treated with Mit-C, after the cells were rested for three days (Figure 4.2C-D). The $CFSE^{low}$ cells are specific for $NS3_{358}$ peptide in a dose-dependent manner and the $CFSE^{high}$ T cells had no T cell response (Figure 4.2C-D). The development of $NS3_{358-375}$-specific $CD4^+$ T cell lines led us to investigate if variant S370P was able to antagonize these $NS3_{358}$-specific T cells.

Variant S370P Acts as an Antagonist

To establish variant S370P as an antagonist, $CD4^+$ $CFSE^{low}$ T cells were stimulated with either $NS3_{358}$ or S370P peptides in a dose-dependent manner (Figure 4.3A). Variant S370P had an attenuated T cell response, which is consistent with antagonism. When the APCs were prepulsed with variant S370P (1μM) and the cells were cultured in an increasing concentration of $NS3_{358}$ peptide, S370P was able to attenuate the T cell response in an antigen-specific manner (Figure 4.3B).

Variant Tetramers Have Lower Avidities in Comparison to $NS3_{358}$ Tetramer

To determine if the viral variants could bind to the TCR of $NS3_{358}$ $CD4^+$ T cells, MHC class II tetramer dilution assays were performed (Figure 4.4). Nonspecific CLIP-

tetramer at 12μg/ml was performed for each experiment as a control and tetramer staining dilutions were performed by a dilution factor of three for each tetramer (Figure 4.4A). The frequency of tetramer positive cells is similar between H369R and $NS3_{358}$ but S370P and K371E variants have a lower frequency (Figure 4.4B). The median fluorescence intensity (MFI) had a similar trend as the frequency of tetramer positive cells, in that wild type and H369R are similar and variants S370P and K371E had a four fold lower MFI (Figure 4.5C). The $CD4^+$ T cell line was stained with each variant and $NS3_{358}$ tetramer, thereby competing each variant against the wild type tetramer. Variant H369R has a much higher avidity at 2μg/ml then $NS3_{358}$ tetramer, indicating that variant is anergizing these T cells (Table 2.1). Taken together, the variant peptides have different avidities for the same TCR, which suggests that the variants are able to modulate $NS3_{358\text{-}375}$-specific $CD4^+$ T cells.

Variant S370P Induces Foxp3 in $NS3_{358}$ $CD4^+$ T Cells

To address if variant S370P was antagonizing $NS3_{358}$ $CD4^+$ T cells to differentiate into Tregs, Foxp3 cell frequency was determined in $NS3_{358}$ MHC class II tetramer positive cells. Using $NS3_{358}$ $CD4^+$ T cell lines derived from both PH1127 and PB3019, stimulated with either the individual peptides or both S370P and $NS3_{358}$ peptides at 1μM were cultured with Mit-C treated APCs and peptides for 72 hrs. Subsequently, the cells were stained with the $NS3_{358}$ tetramer in combination with the indicated fluorochromes (Figure 4.5). CLIP-DRB1*1501-PE tetramer was used as a control and fluorescence minus one (FMO); the isotype control for Foxp3 antibody was also used for each experiment (Figure 4.5). The histograms represent the 7-AAD^- $CD8^-$ $CD3^+$ $CD4^+$

$NS3_{358}$-DRB1*1501^{+} cells that are Foxp3^{+} (Figure 4.5). The S370P stimulated T cells have a higher frequency of Foxp3 (Figure 4.5, red histogram). Taken together, S370P antagonizes the CD4 T cell lines to differentiate in Tregs. To our knowledge, this is the first demonstration of a naturally occurring variant inducing T_H1-cells to differentiate into a Treg.

MHC II S370P Tetramer Stains the Same Cell as MHC II $NS3_{358}$ Tetramer

To solidify if variant S370P was binding to the same T cells as the wild type tetramer, we performed MHC class II tetramer staining on $NS3_{358}$-specific CD4^{+} T cells. $NS3_{358}$ and S370P tetramers were added to the indicated CD4^{+} T cell line in tandem (Figure 4.6). The $NS3_{358}$ tetramer appears to stain the majority of T cells but the S370P tetramer does not individually stain the cells. Instead the variant co-localizes with the $NS3_{358}$ tetramer (Figure 4. 6). CD4^{+} Tropomyosin specific T cells were used as a control for the tetramers (Figure 4.6, last column). The CD4^{+} Tropomyosin-specific T cells were stained at the same time and with the same tetramers as PH1127 and PB3019 T cells (Figure 4.6, third row). These results show that variant S370P is able to bind to the same TCR as the cognate peptide, albeit at a lower avidity in comparison to the wild type tetramer.

Discussion

We demonstrate that naturally occurring APLs induce antigen-specific CD4^{+} T cells to differentiate into Tregs *in vitro*. Furthermore, the wild type and variant peptide is necessary for the suppression of a 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 T cell differentiation of a T_H1 $CD4^+$ T cell to a Treg cell.

The maintenance of an abundant population of wild type HCV sequences has been observed in infected humans and chimpanzees even years into an ongoing infection (2-4, 42). Viral variation accounts for < 20% of the circulating viral sequence in the chronically infected person used in this study (2, 6). From these identified viral variants arising in the $NS3_{358-375}$ epitope, there was a subset of variants able to attenuate the T cell proliferative responses to the cognate peptide (2). Interestingly, the viral variant S70P, previously shown to induce Treg phenotypic markers, was unable to suppress mitogen activated T cells, but if S370P was presented in conjunction with the cognate peptide, the T cell response to the mitogen was inhibited. Taken together, the presence of the wild type in conjunction with the variant is necessary for the suppressive effect of these T cells, but the induction is antigen-specific.

The quality of $CD4^+$ 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 (7, 13-16). To circumvent the issue of $CD4^+$ T cell expansion from a chronic HCV subject, we developed an $NS3_{358-375}$-specific $CD4^+$ T cell line. Variant S370P was able to antagonize $NS3_{358-375}$-specific $CD4^+$ T cells. In support of these results, work by Frasca et al. (17) has shown that APLs were able to antagonize the HVR1-specific T cell clones and suppress the T cell response when both the APL and cognate peptides were presented on the same APCs. Further work by Scotta et al. (19) ascertained that certain APLs were able to induce cell death, but the viral variants used in the current study did not induce

programmed cell death in comparison to the wild type $NS3_{358-375}$ peptide (Figure 4.7 and 4.8). Recently, Mueller et al. (43) had detected virus-specific $CD4^+$ T cells in chronic HCV patients, indicating the presence of HCV-specific $CD4^+$ T cells in chronic individuals, indicating that naturally occurring APLs could be exploiting other mechanisms for persistence, such as T cell anergy or rendering the T cells unresponsive.

A defining feature of APLs is the ability of the antagonist peptide coupled to MHC (pMHC) to bind to the TCR. Tetramer avidity tests using MHC class II tetramers clearly demonstrate a difference in avidity between the wild type and variants with the exception of H369R. It appears that H369R has a similar avidity to the wild type tetramer. Functional data indicate that the H369R variant is blunting the $NS3_{358-375}$- T cell proliferation, which is indicative of T cell anergy but needs further investigation. Similar results to the tetramer avidity tests were found when each variant was added into culture with the wild type tetramer, in that S370P and K371E were double positive, but H369R had a higher avidity for the T cells (Figures 4.9- 4.11). Also, these tetramer competition assays indicate that both the variant and the wild type tetramer are 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. 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 an HCV-specific T cell response.

We have previously demonstrated that variant S370P induced Foxp3 expression in an antigen-specific manner and had a dose-dependent suppressive effect *in vitro*, reflective of Tregs (Chapter 2). Chronic HCV infected individuals have an increase in

Treg markers when compared to noninfected individuals (3, 34-39). This induction of Tregs in chronic HCV subjects appears to be antigen-specific due to the identification of $Foxp3^+$ MHC class II tetramer positive cells from PBMC of HCV patients (44). Heeg et al. (39) has tracked MHC class II tetramer positive cells that were HCV-specific $CD4^+$, and expressed $Foxp3^+$ T cells during the course of HCV infection in a cohort of patients. Further, they observed an attenuated antigen-specific T cell proliferative response and lowered IFNγ secretion in MHC class II tetramer positive cells (39). Also, MacDonald et al. (3) had identified inducible Tregs that recognized the same HCV core epitopes as T_H1 IFNγ-secreting T cells. Even more compelling, evidence suggesting that Tregs are induced, possibly leading to viral persistence, was demonstrated in acute infection (45). These studies support HCV-induction of Tregs but do not explore the mechanism of induction of Tregs (39, 44). In our study, naturally occurring APLs alter the TCR signaling as suggested by our T cell proliferation data and these variants induce a Tregs phenotype. Taken together, we determined that variant S370P was able to induce Foxp3 expression in $NS3_{358-375}$-MHC class II tetramers positive cells that are antigen-specific in two individuals. This mechanism of Treg induction is HCV antigen-specific, and the phenomenon of Treg induction is not exclusive to one HCV chronic subject.

The most widely accepted Treg marker, Foxp3, is dependent on IL-10 signaling in $CD4^+$ T cells (46). Further, it has been demonstrated that IL-10 serum levels are significantly higher in chronic vs resolved HCV subjects, thereby providing an environment that is conducive to Treg differentiation and maintenance (3). Although the source of the endogenous IL-10 has been found higher in the serum of chronic subjects, the cell type(s) responsible for the IL-10 secretion has not been clearly defined and needs

further investigation. Similarly, the viral-variant S370P was able to activate and maintain Foxp3 expression in comparison to the $NS3_{358-375}$ peptide when in the presence of IL-10, whether produced endogenously in a chronic patient or provided exogenously to cells in a resolved subject (data not shown). 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, we found the T cell response was not significantly lower when compared to resolved HCV subjects (Chapter 2).

Although the cohort of chronic HCV subjects had an intact memory T cell response, the chronic subject used in this study had an attenuated T cell response to influenza antigens, suggesting that there immune system was dampened in this one chronic HCV subject and this could be due to being chronically infected. To circumvent the issue of IL-10 in our assay system, we used a mixture of chronic and resolved APCs. Herein, we show that Foxp3 is induced in an antigen-specific manner.

In conclusion, we demonstrate that naturally occurring viral variants are acting as APLs that work synergistically with the cognate peptide to cause antigen-specific differentiation in T cells. Such T cells are thence able to suppress nonspecific T cell responses reminiscent of Tregs. Thus, by tolerating mutation in specific T cell epitopes, HCV may be able to shift the immune response towards conditions that are conducive to viral persistence. Further work should determine if priming strong CD4+ T cell responses could provide protective immunity against HCV or lead to viral elimination in chronically infected patients.

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Table 4.1. Variant and $NS3_{358}$ tetramer competition for $PB3019^{low}$ and $PH1127^{low}$ $CD4^{+}$ T cells. H369R variant had the highest avidity in comparison to $NS3_{358}$ tetramer. Each variant tetramer was added to $CD4^{+}$ $CFSE^{low}$ $NS3_{358}$ antigen-specific T cells at the same time as the $NS3_{358}$ tetramer. Flow plots are shown in Figures 4.9- 4.11.

Tetramers	Patients	
	PB3019	PH1127
$NS3_{358}$ /H369R		
$NS3_{358}$	0.076	0.11
H369R	4.56	1.69
$NS3_{358}$ + H369R	0.2	0.085
$NS3_{358}$ /S370P		
$NS3_{358}$	0.059	0.078
S370P	0.31	0.52
$NS3_{358}$ + S370P	0.074	0.1
$NS3_{358}$ /K371E		
$NS3_{358}$	0.18	0.53
K371E	0.5	0.65
$NS3_{358}$ + K371E	0.059	0.23

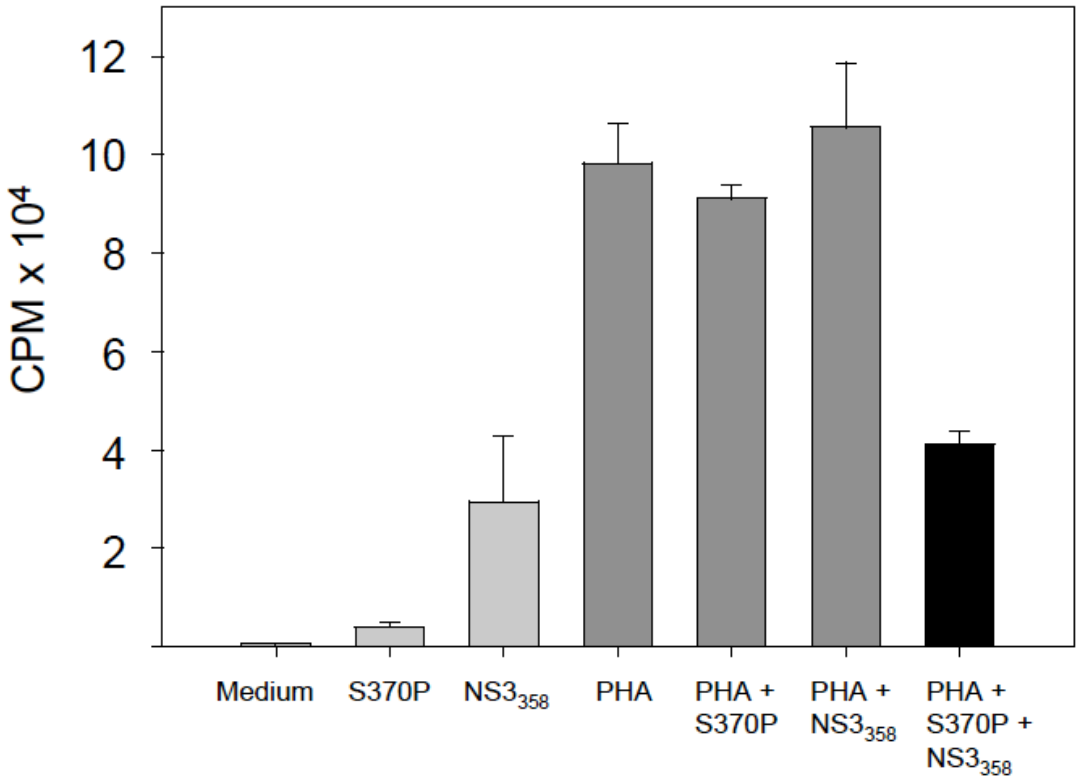
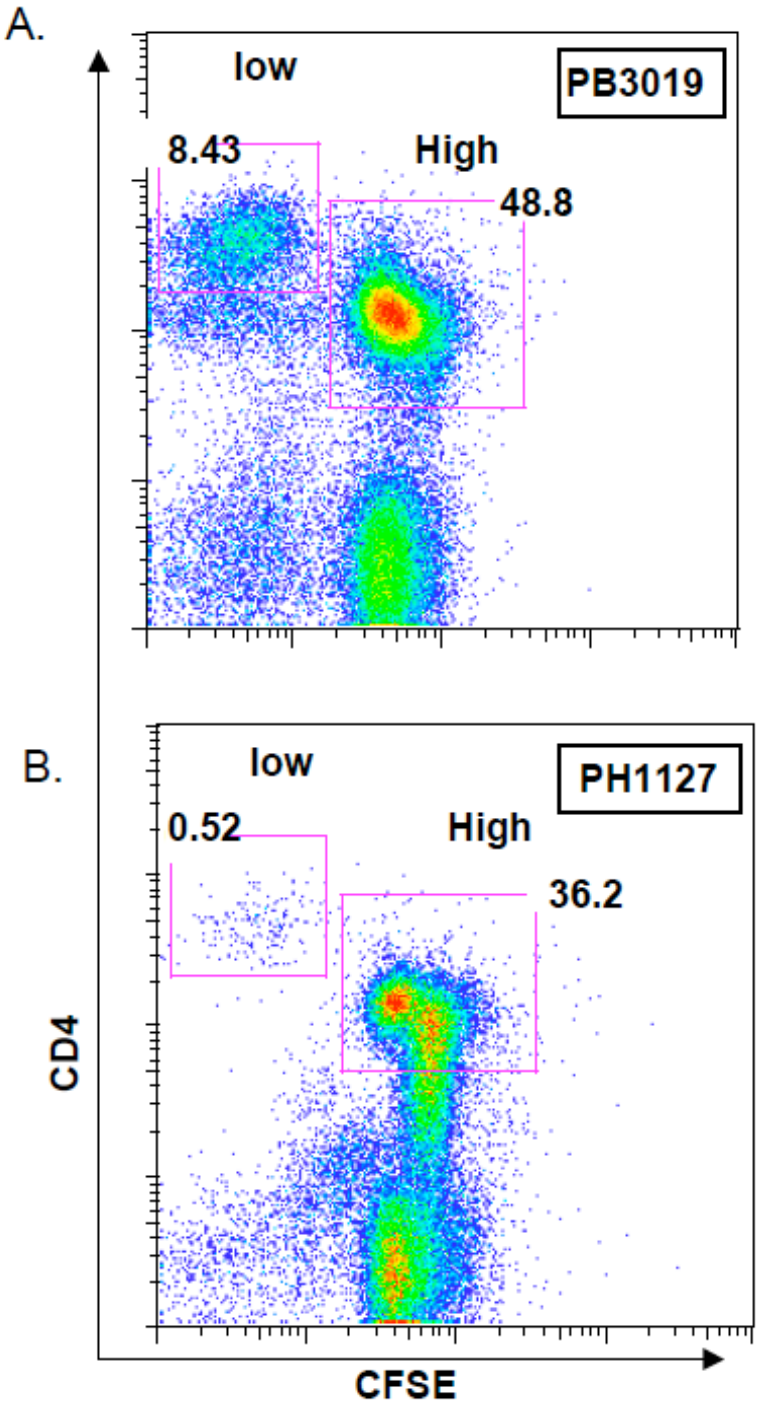


Figure 4.1. Both cognate and variant peptides are necessary for the suppression of T cell proliferation. P.B3019 PBMC was stimulated with individual peptides (light gray bars). The PBMC were preincubated with either S370P or $NS3_{358}$ 3 hrs. prior to the addition of PHA (1μg/ml) (dark gray bars). PBMC were preincubated with S370P for 3 hrs. and $NS3_{358}$ and PHA (1μg/ml) were added (black bar). ^{3}H-thymidine was added at day 6 and ^{3}H-thymidine uptake was measured 16-18 hrs. later.

Figure 4.2. $CD4^+$ $CFSE^{low}$ T cells stimulated with $NS3_{358}$ peptide are antigen specific T cells. A-B. PBMC from PB3019 and PH1127 were prelabeled with CFSE. The cells were stimulated with 1μM of $NS3_{358}$ peptide for 7 days. Cells were sorted by proliferating cells (low) and nonproliferating cells (high). Cells were expanded with CD3/CD28 beads and rIL-2 (10U/ml). Prior to stimulation with cognate peptide, the beads were removed and rested for 3 days. C-D. APCs (1×10^5 cells/well) were added with increasing concentrations of $NS3_{358}$ peptide. $CD4^+$ T cells were added at 2×10^4 cells/well and incubated for 72 hrs. ^{3}H-thymidine was added to the cultures 16-18 hrs. prior to measuring the T cell response. The experiment was performed in triplicate.


A.
low
PB3019
8.43
High
48.8
B.
low
PH1127
0.52
High
36.2
CD4
CFSE

C.

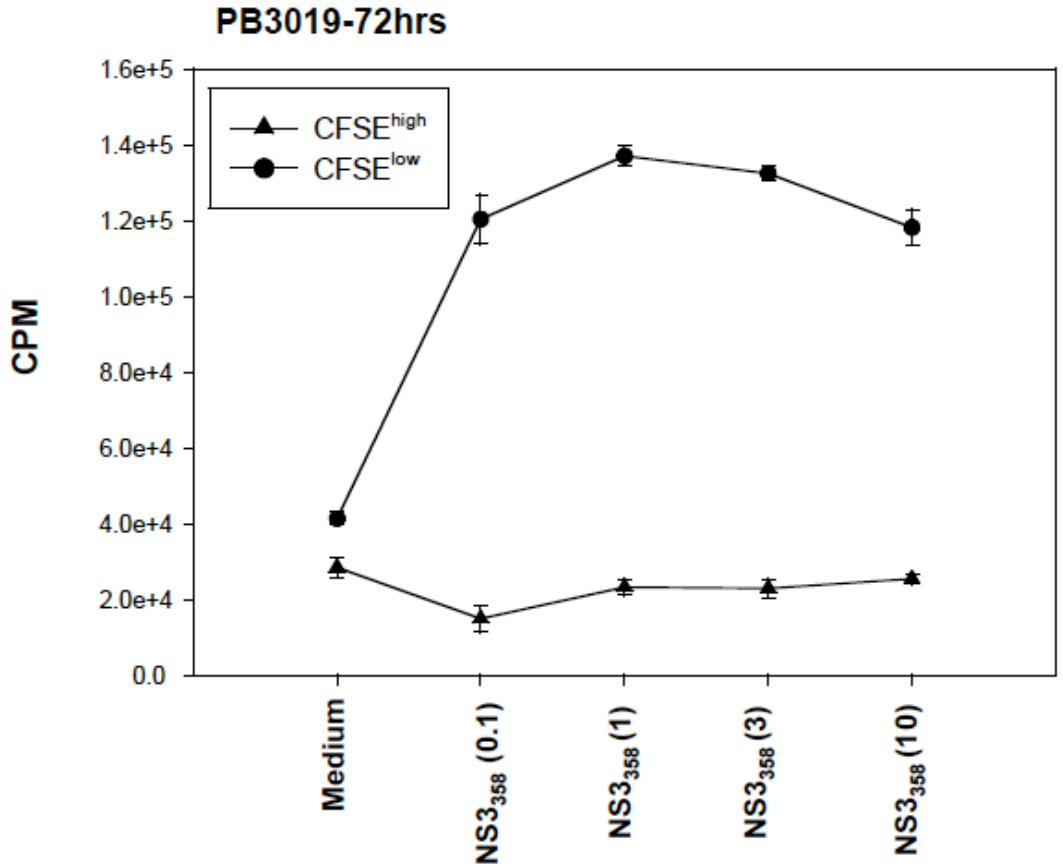


Figure 4.2 continued

D.

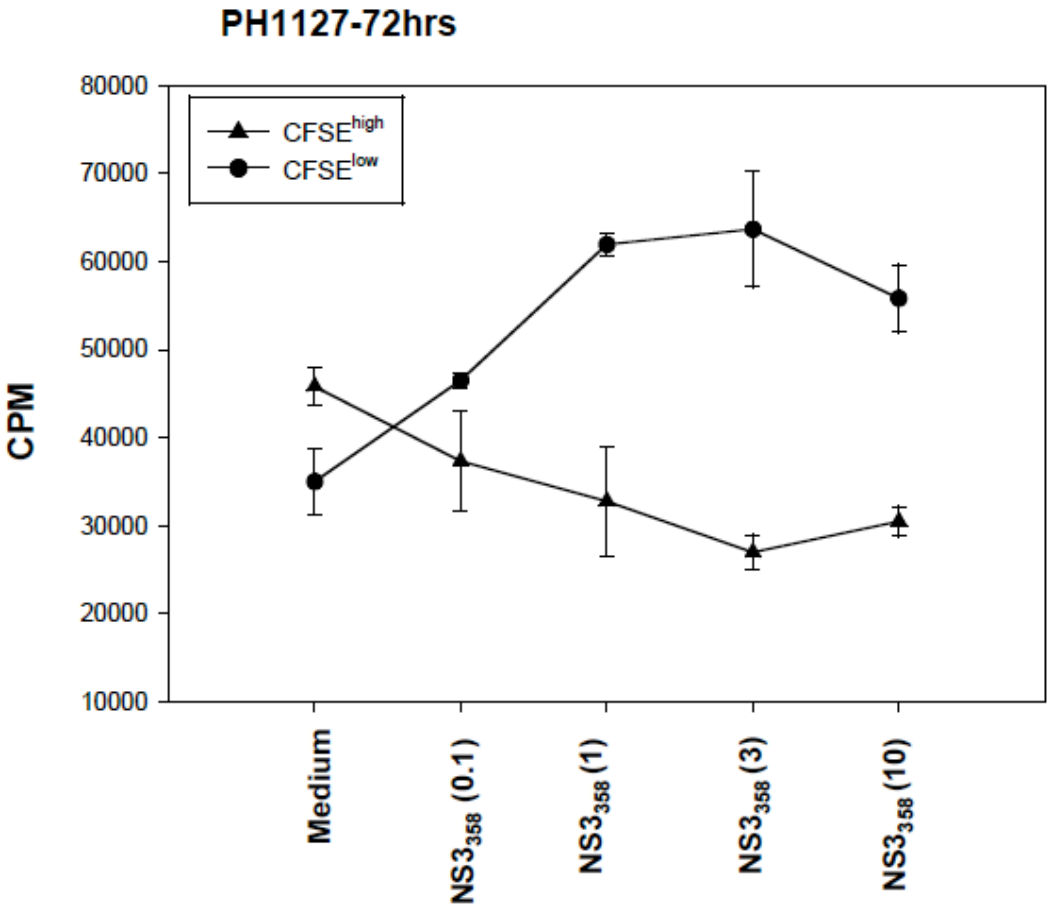
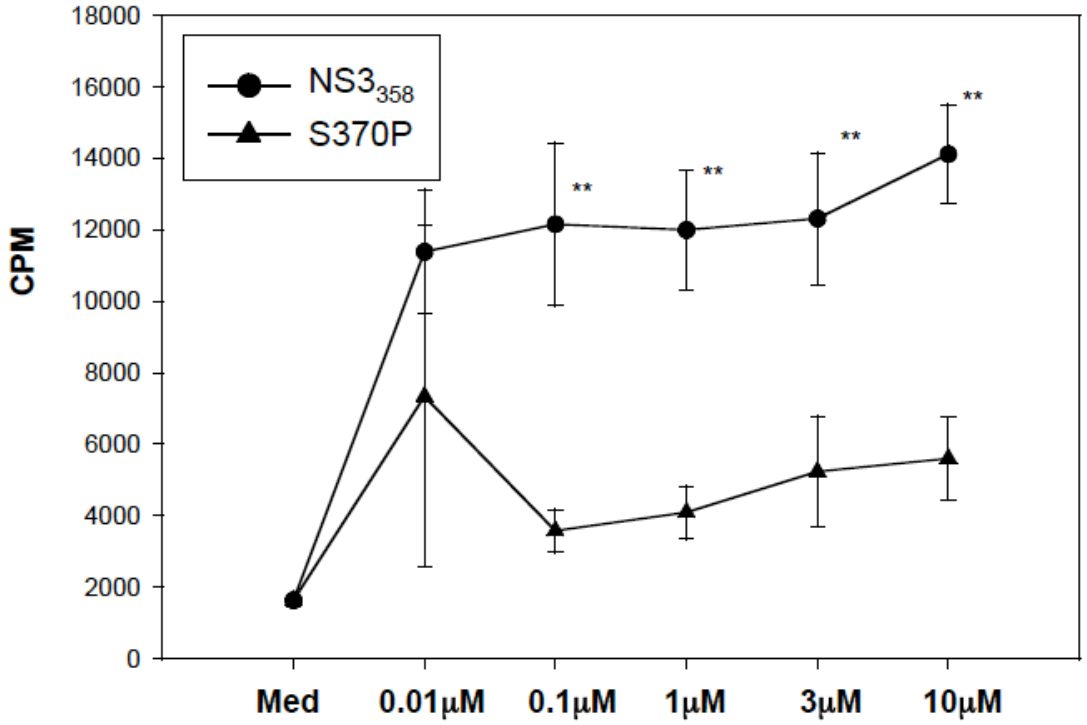


Figure 4.2 continued

Figure 4.3. Variant S370P antagonizes $NS3_{358}$ antigen-specific T cells. A. T cell proliferation assay with individual peptides added in a dose dependent manner to $CD4^+$ $CFSE^{low}$ T cells (2 x 10^4 cells/well) from both PH1127 and PB3019 patients with 1 x 10^5 APCs/well and incubated for 72 hrs. B. APCs (1 x 10^5 cells/well) were prepulsed with S370P for 3 hrs., washed, treated with Mitomycin C and added to $CFSE^{low}$ T cells (2 x 10^4 cells/well) for 72 hrs. in the presence of $NS3_{358}$ peptide. ^{3}H-thymidine was added to the cultures 16-18 hrs. prior to measuring ^{3}H-thymidine uptake. Assays were performed in triplicate and data is representative of 2 independent experiments for both PH1127 and PB3019.

A.


18000
16000
14000
12000
10000
8000
6000
4000
2000
0
CPM
NS3358
S370P
**
**
**
**
Med
0.01μM
0.1μM
1μM
3μM
10μM

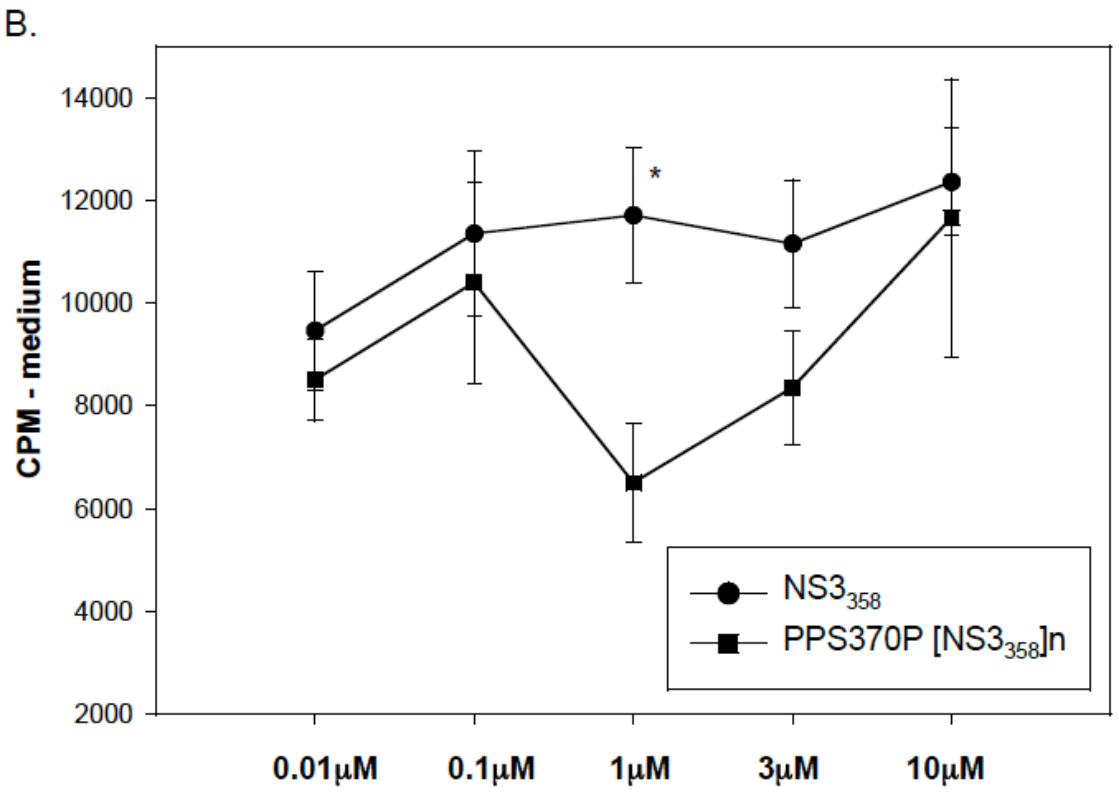
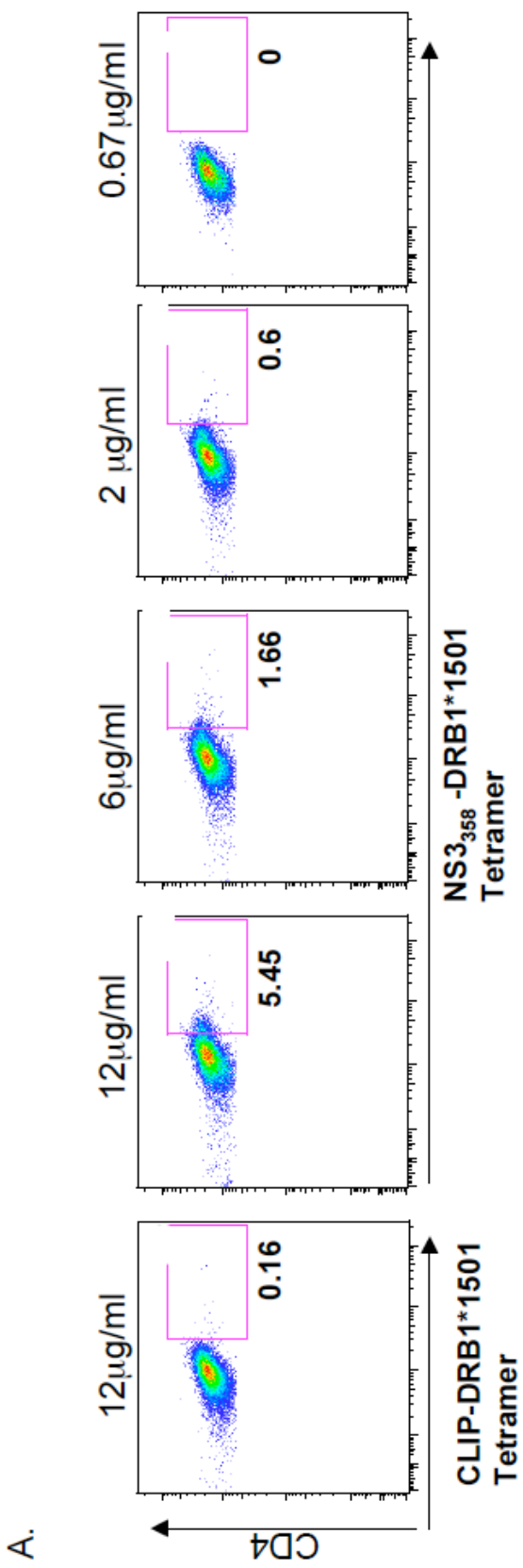


Figure 4.3 continued

Figure 4.4. $NS3_{358}$ and variant tetramers have different TCR avidities. A. Representative flow plots of $NS3_{358}$- MHC class II staining of $CD4^+$ $CFSE^{low}$ T cells was performed in decreasing concentrations of individual tetramers. B. Frequency of tetramer positive cells for each tetramer. C. Median Fluorescence Intensity (MFI) for each tetramer is shown.


A.
CD4
12μg/ml
0.16
CLIP-DRB1*1501
Tetramer
12μg/ml
5.45
6μg/ml
1.66
2 μg/ml
0.6
0.67μg/ml
0
NS3358 -DRB1*1501
Tetramer

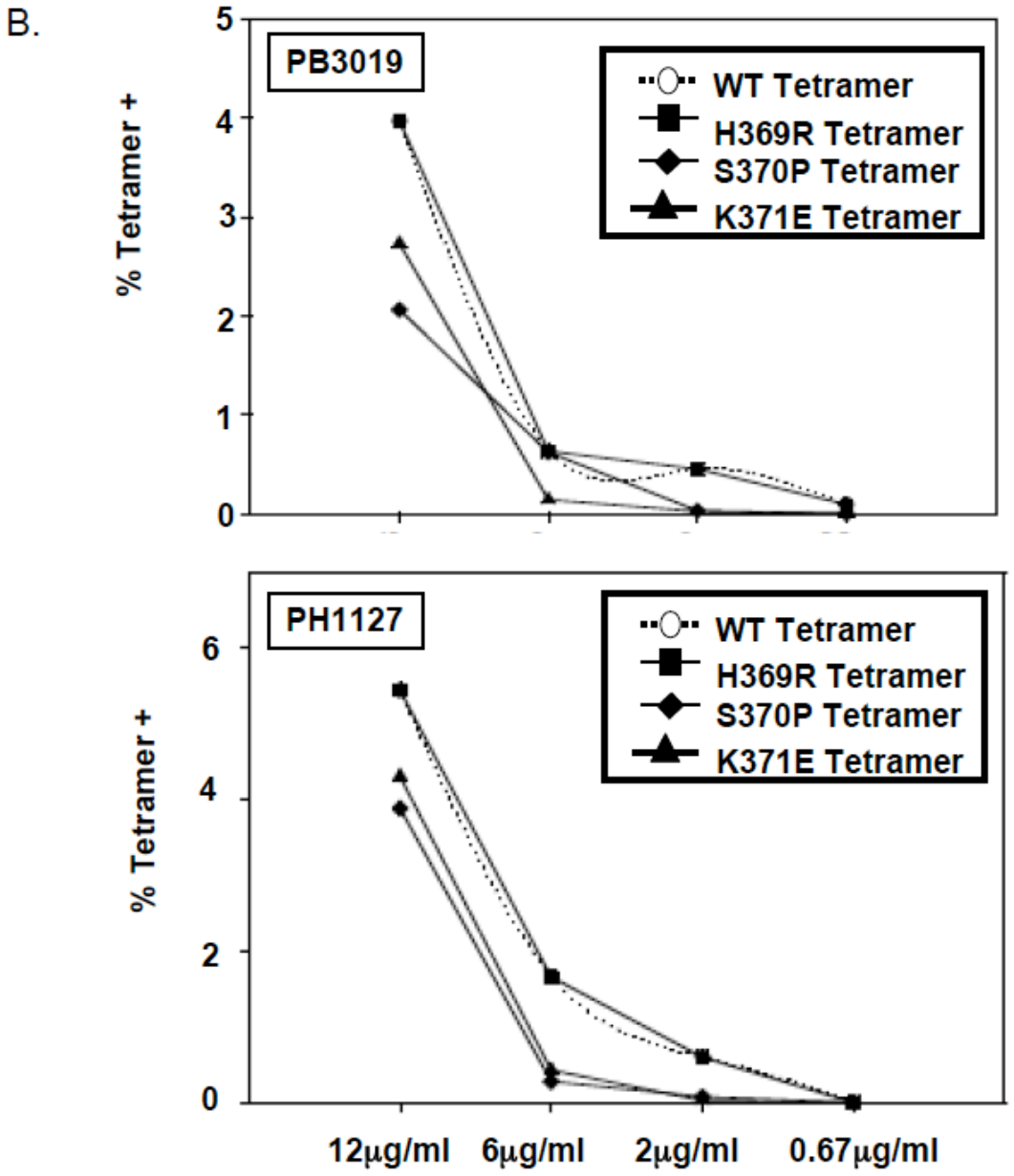


Figure 4.4 continued

C.

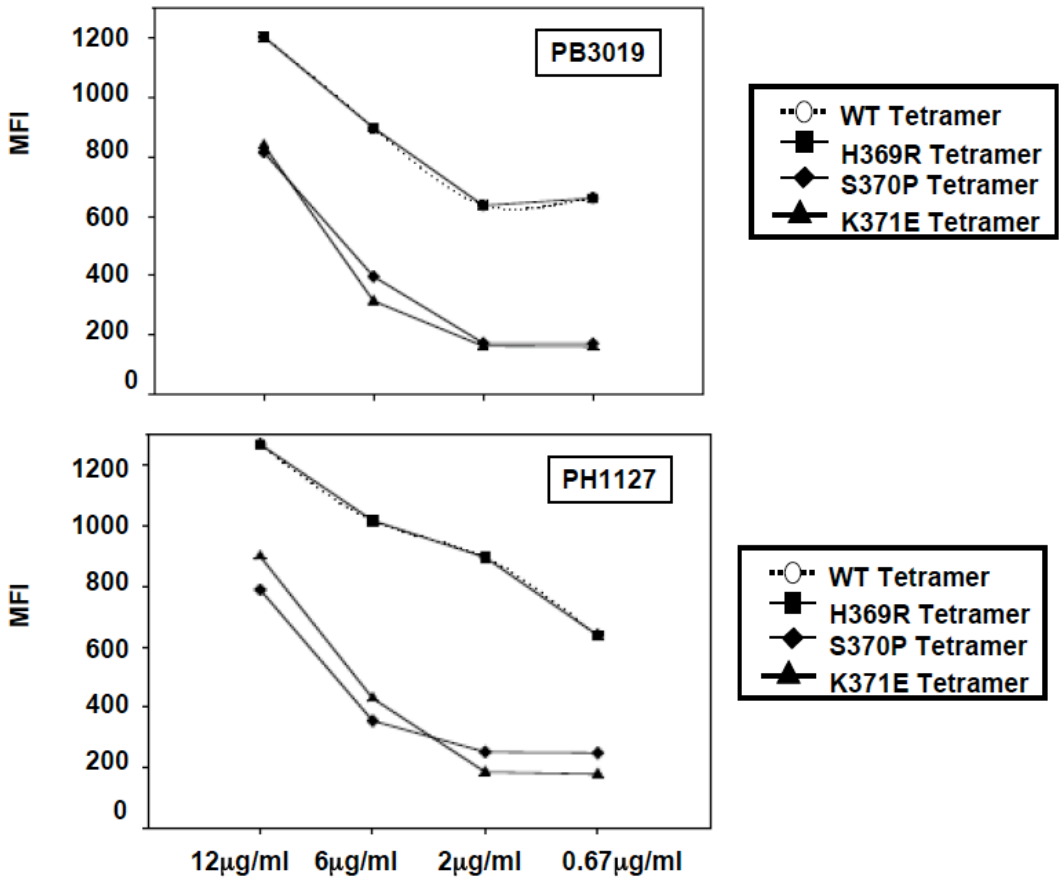
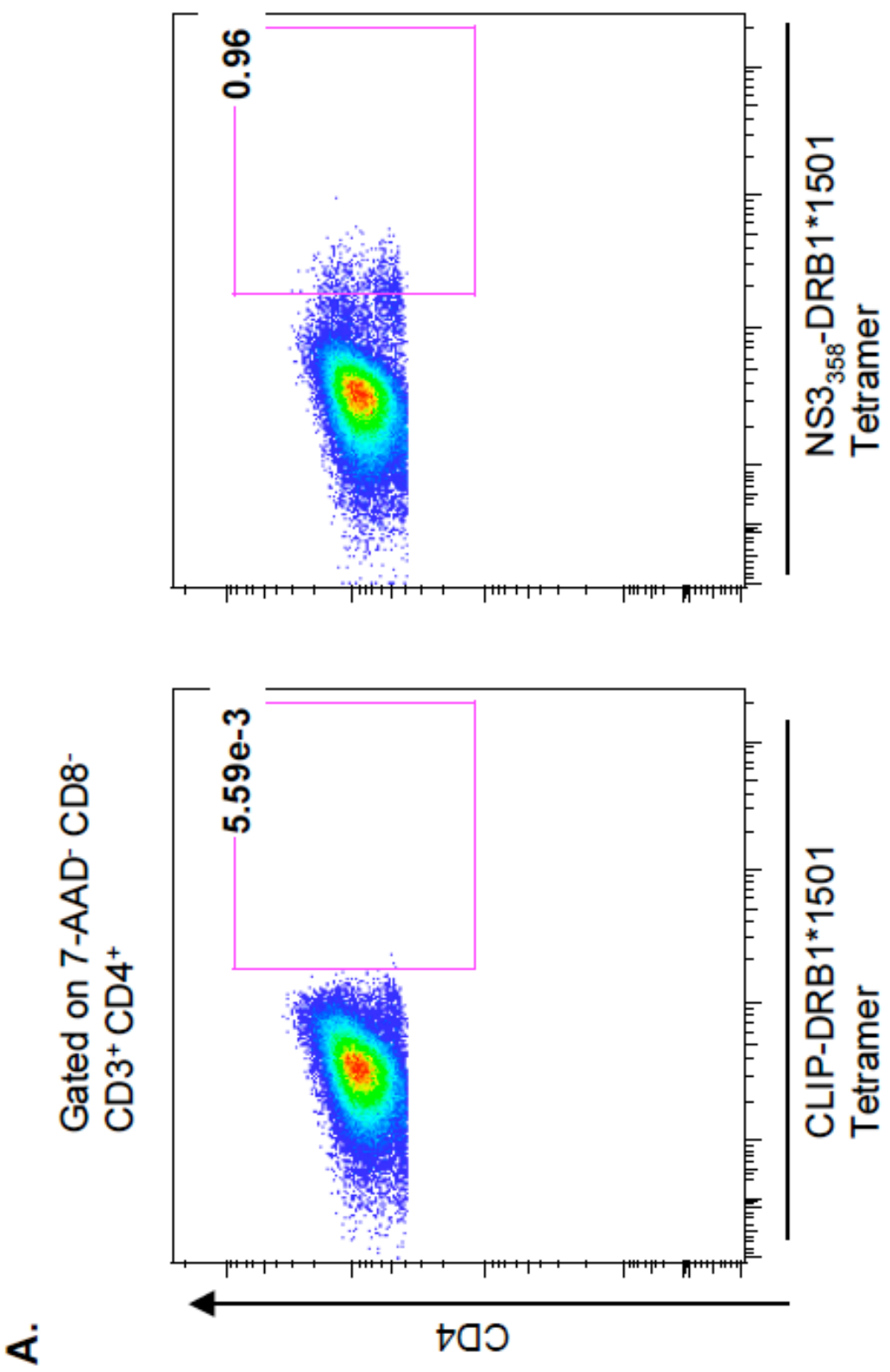


Figure 4.4 continued

Figure 4.5. Viral variant S370P is able to induce Foxp3 expression in an antigen specific manner. $CD4^+$ $CFSE^{low}$ T cells from PH1127 and PB3019 were stimulated with either individual peptides (1μM/ml) or prepulsed with S370P (1μM) and cultured for 72 hrs. The cells were stained with $NS3_{358}$ MHC class II tetramer. Tetramer positive cells were stained for Foxp3 (histogram). Gating for MHC class II tetramer staining was determined by control tetramer CLIP-DRB1*1501 tetramer. Dark gray histogram is $NS3_{358}$ stimulated T cells and red histogram is the S370P stimulated $CFSE^{low}$ cells that are MHC class II tetramer positive.


A.
Gated on 7-AAD- CD8-
CD3+ CD4+
5.59e-3
0.96
CD4
CLIP-DRB1*1501
Tetramer
NS3358-DRB1*1501
Tetramer

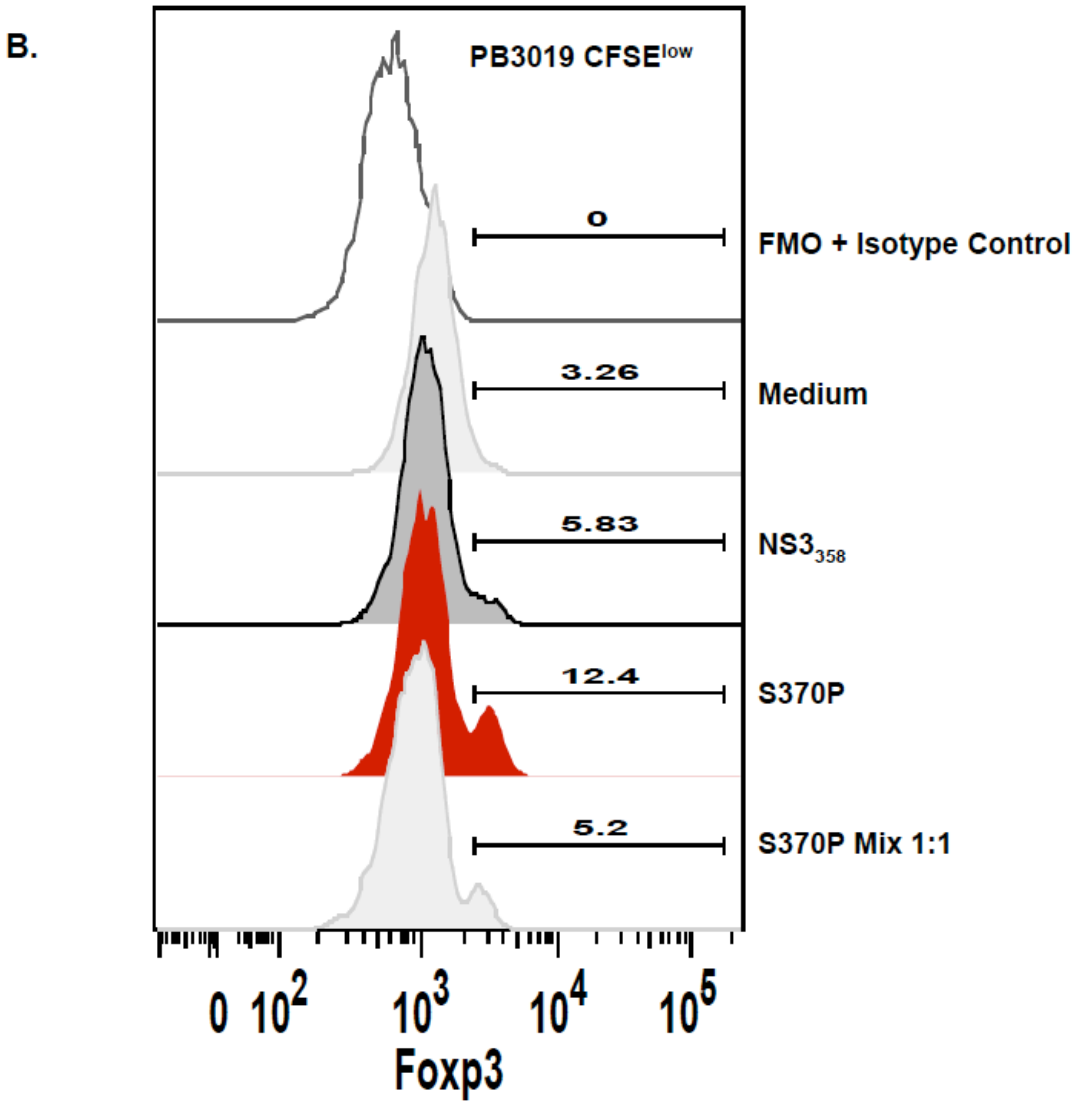


Figure 4.5 continued

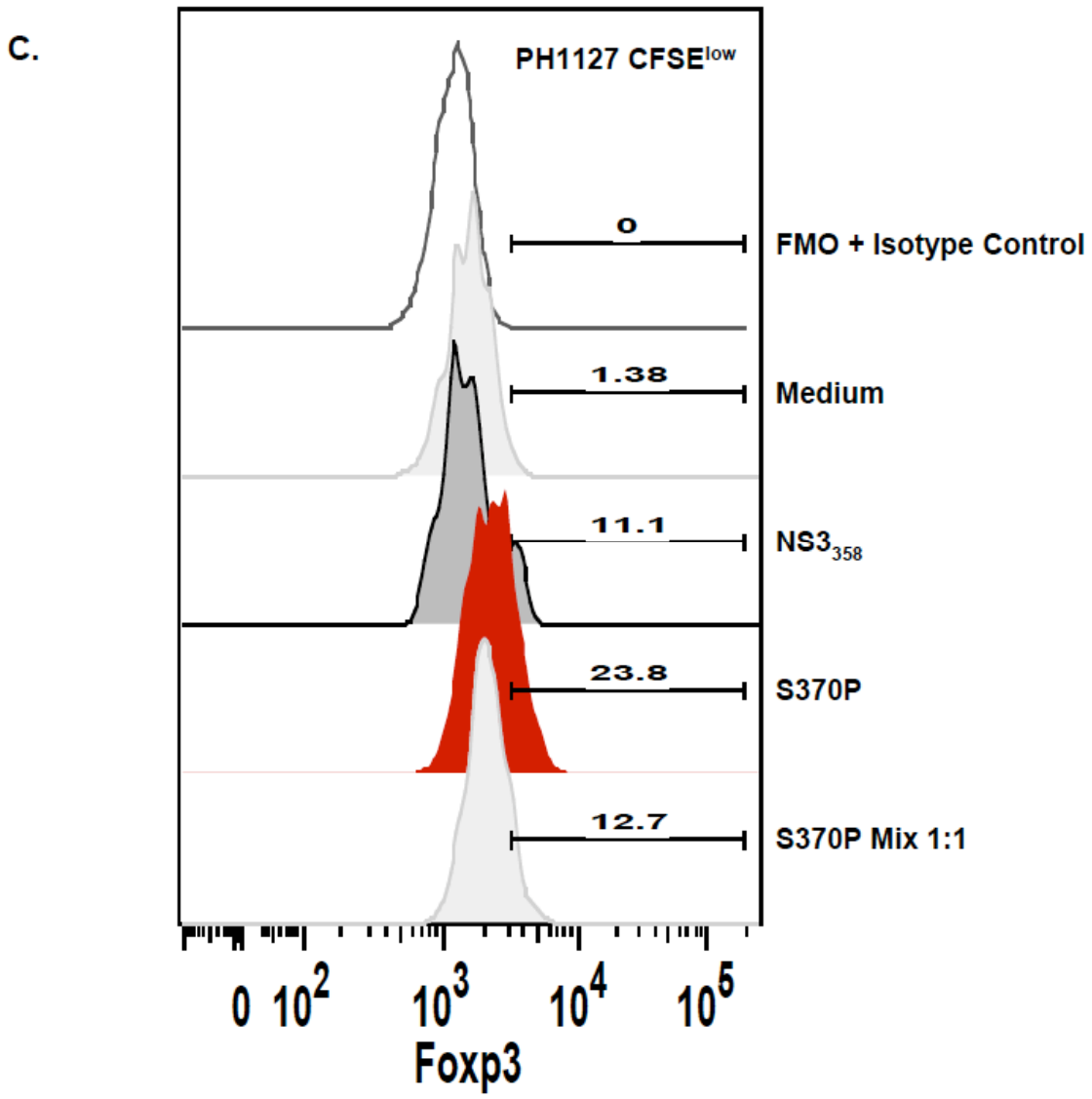


Figure 4.5 continued

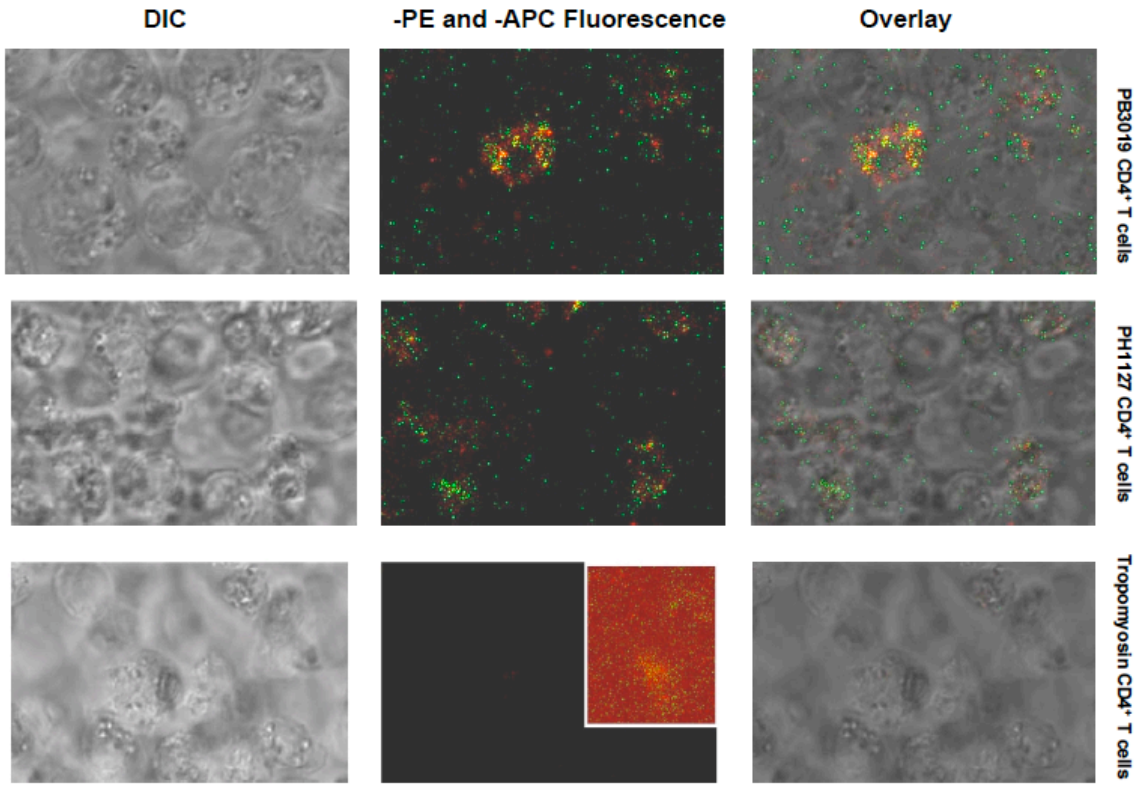


Figure 4.6. $NS3_{358}$ and S370P MHC class II tetramers are able to bind to $CD4^+$ $NS3_{358}$ T cells. The fluorescent images were all taken using identical confocal settings including laser power, emission filters, pinhole, and scan speed. The cells were stained with both $NS3_{358}$-PE (green) and S370P-APC (red) MHC class II tetramers at 10μg/ml. $PB3019^{low}$ and $PH1127^{low}$ $CD4^+$ T cells are specific for $NS3_{358}$ (rows one and two). $CD4^+$ Tropomyosin specific T cells were used as a control (third row). The inset (column 3, panel 2) has been multiplied by three to reveal the level of background for comparison to the experimental panels shown in the same column.

Figure 4.7. 48 hour staining of Annexin V and PI.

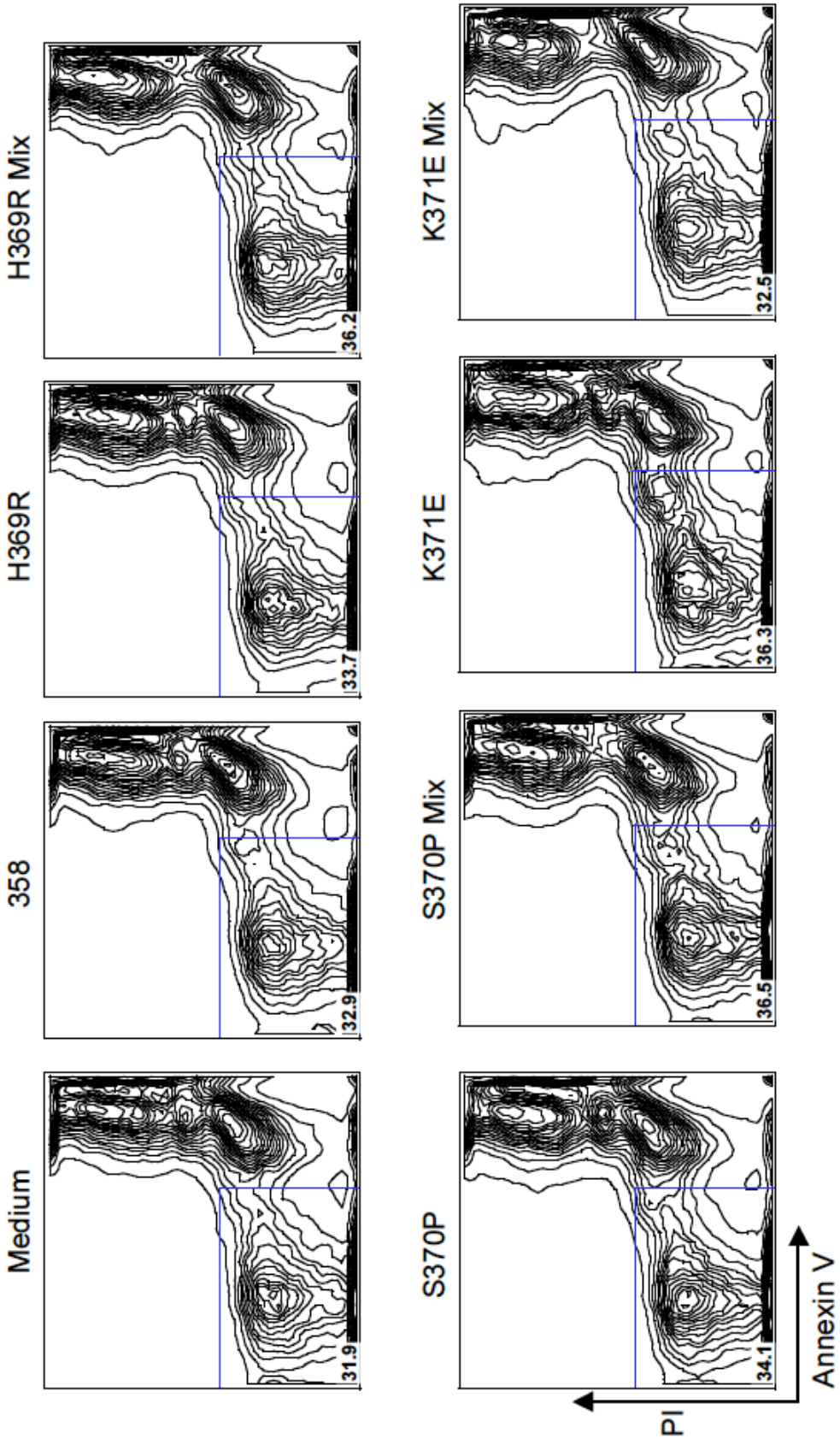
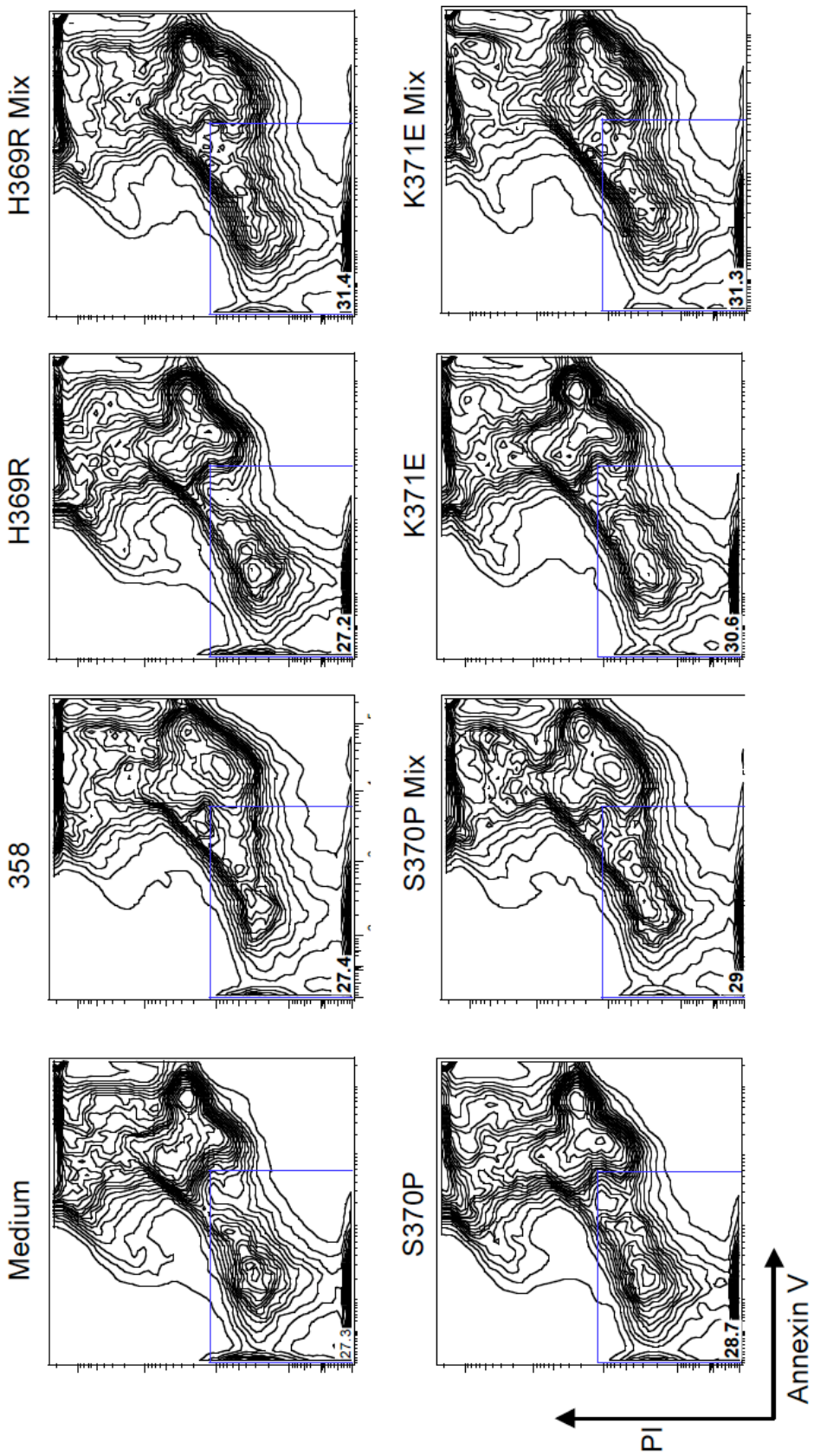

Medium
358
H369R
H369R Mix
S370P
S370P Mix
K371E
K371E Mix
31.9
32.9
33.7
36.2
34.1
36.5
36.3
32.5
PI
Annexin V

Figure 4.8. 8 days poststimulation of Annexin V and PI.


Medium
358
H369R
H369R Mix
S370P
S370P Mix
K371E
K371E Mix
27.3
27.4
27.2
31.4
28.7
29
30.6
31.3
PI
Annexin V

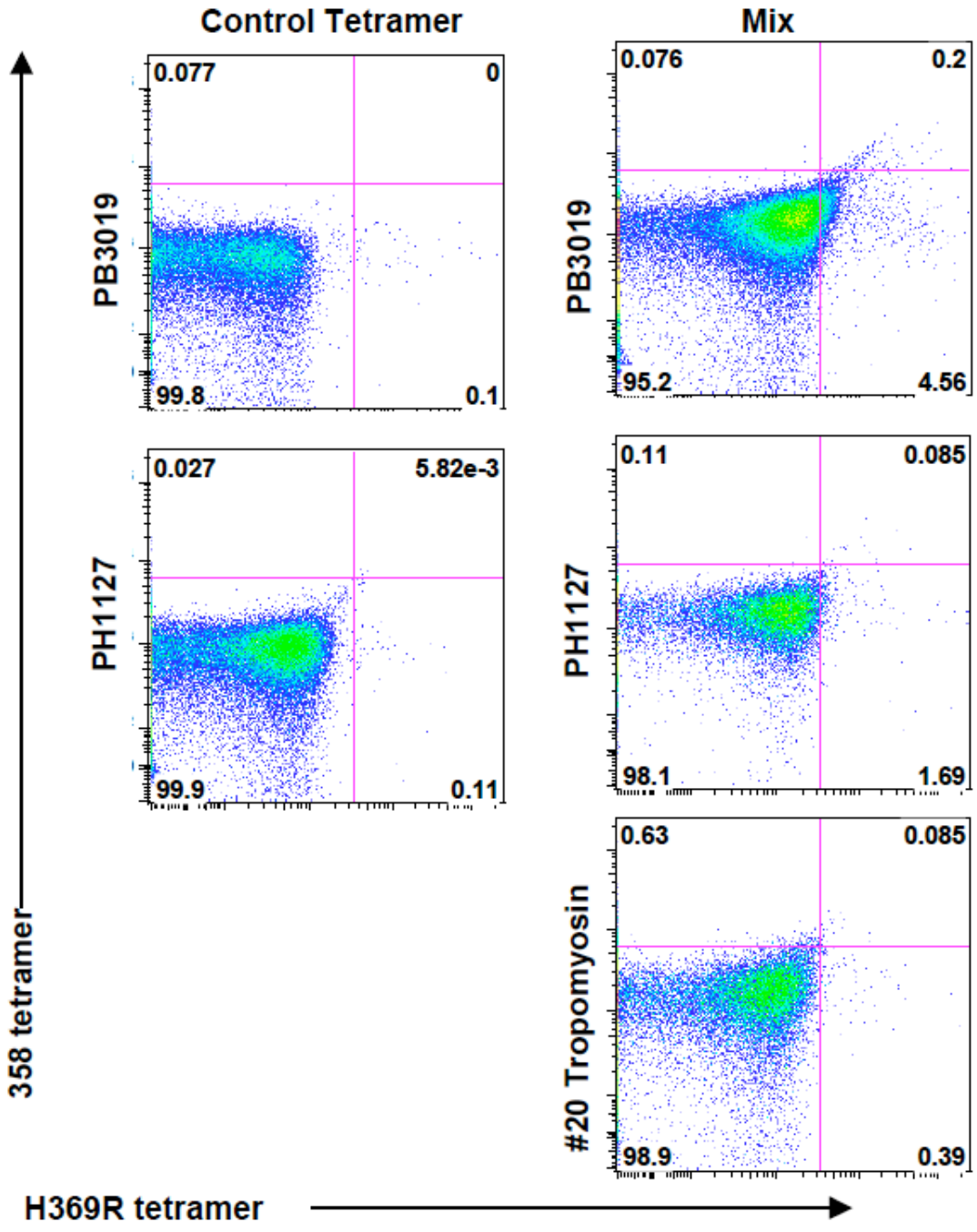


Figure 4.9. H369R and $NS3_{358}$ tetramer competition assay. Both H369R and $NS3_{358}$ tetramers (10μg/ml) were added to the indicated $CD4^+$ T cell lines at the same time. Patient #20 tropomyosin was used as a control for the tetramers and CLIP loaded tetramers were used to test for nonspecific staining.

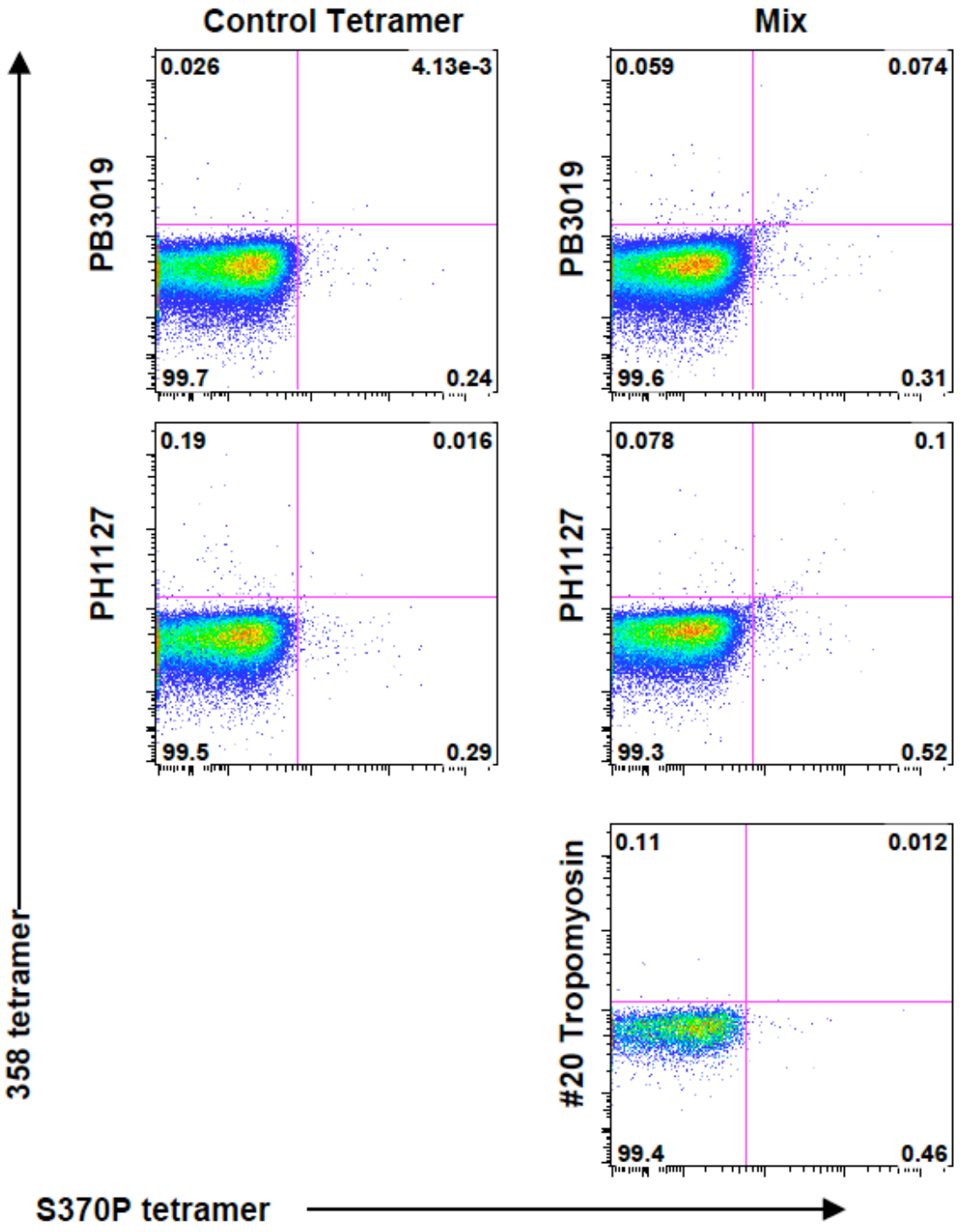


Figure 4.10. S370P and $NS3_{358}$ tetramer competition assay. Both S370P and $NS3_{358}$ tetramers (10μg/ml) were added to the indicated $CD4^+$ T cell lines at the same time. Patient #20 tropomyosin was used as a control for the tetramers and CLIP loaded tetramers were used to test for nonspecific staining.

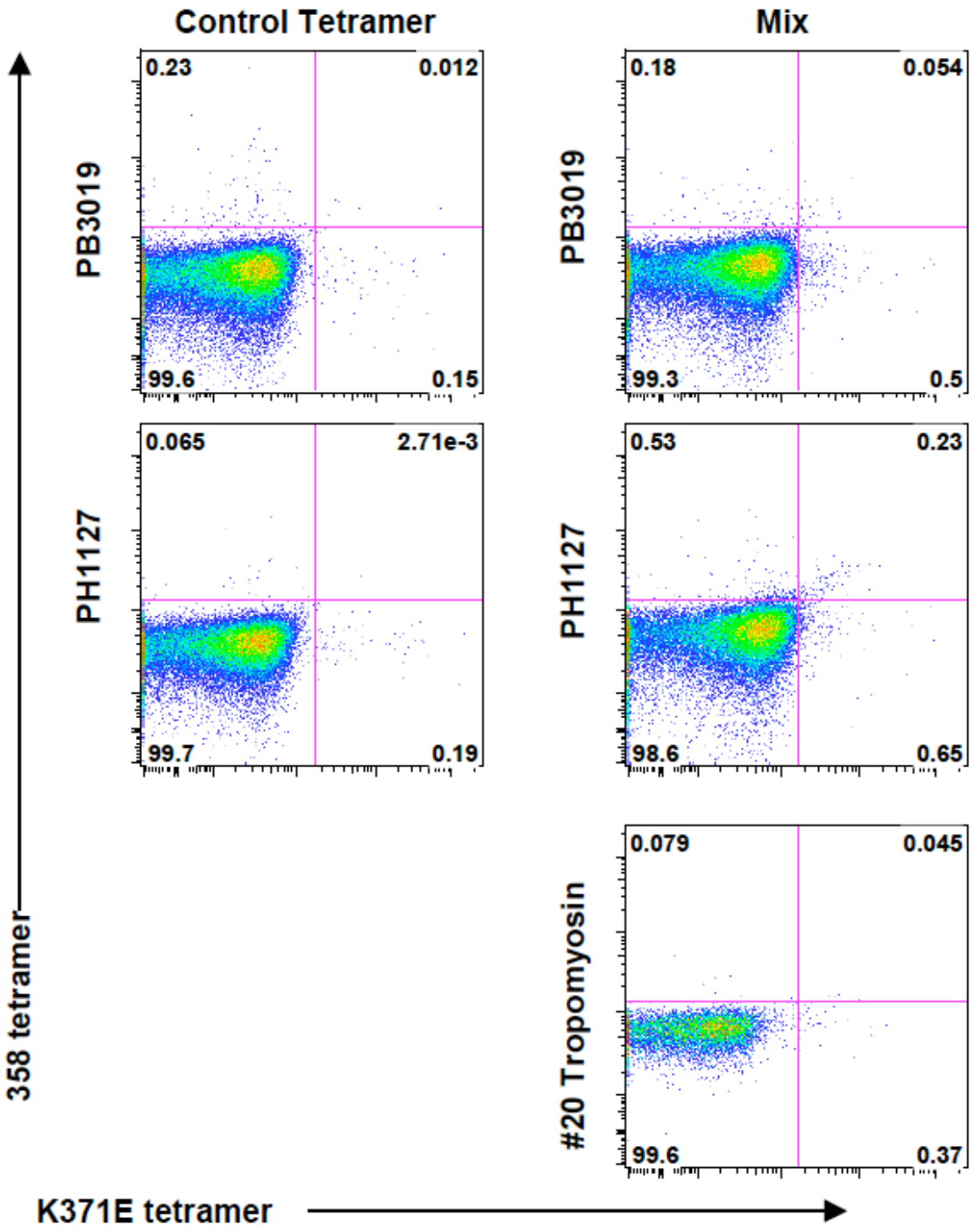


Figure 4.11. K371E and $NS3_{358}$ tetramer competition assay. Both K371E and $NS3_{358}$ tetramers were (10μg/ml) added to the indicated $CD4^+$ T cell lines at the same time. Patient #20 tropomyosin was used as a control for the tetramers and CLIP loaded tetramers were used to test for nonspecific staining.

CHAPTER 5

DISCUSSION

This dissertation describes the methodology and potential influence of the *in vitro* induction of Tregs capable of suppressing protective antigen-specific T cell responses to an immunodominant epitope of HCV. I hypothesized that previously defined viral variants in this T_H1 immunodominant epitope could be responsible for the induction of Tregs, a postulation based upon the cytokine shift and attenuated T cell response (1). These findings offer new insight into the occurrence of chronically infected HCV subjects exhibiting significantly lower T cell responses in comparison to resolved subjects. These attenuated T cell responses correlate with the induction of Treg lineage-specific markers in proliferating T cells specific for rNS3. Additional investigation of this process suggests that naturally occurring variants induce Tregs and may act as APLs, leading to changes in the quality of the T cell responses. The latter part of this dissertation blends the two previously described concepts by demonstrating that naturally occurring APLs serve as the mechanism of Treg induction, leading us to term this mechanism of viral-specific induction of Tregs as "convergent suppression" (Figure 1.5).

Naturally occurring escape variants of HCV have been identified but very little work has been done to characterize the possible impact that viral variants could have on T cell responses to the wild type HCV epitope. The work performed in this dissertation demonstrates that viral variants attenuate T cell responses to wild type peptide, but not unrelated peptide, thereby suggesting that APLs serve as a mechanism by which HCV may be able to deviate the immune response and persist. Furthermore, the specific variant, S370P, induced Foxp3 in an antigen-specific manner in a chronic HCV patient. In an effort to generalize our finding from one chronic subject, we were able to detect variant-specific T cells in multiple HLA-matched chronic and resolved subjects. The

ability to detect the wild type specific T cells, along with variant-specific T cells, suggests that this mechanism of Treg induction by variants is not exclusive to one chronic HCV subject. Future studies into mechanisms of Treg induction would use more HLA-matched individuals, both chronic and resolved, in functional assays to further demonstrate APL induction of Tregs as described in this dissertation.

Compelling evidence for the existence of HCV-specific Tregs *in vivo* was presented by Ebinuma et al. (2) who identified $CD4^+$ $CD25^+$ $Foxp3^+$ MHC class II tetramer positive T cells in peripheral blood of HCV patients, supporting our results obtained from one patient. Furthermore, Heeg et al. (3) performed a longitudinal study using MHC class II tetramer staining to track HCV-specific $CD4^+$ $Foxp3^+$ T cells during the course of HCV infection in a cohort of patients. Although Heeg's (3) study did not find a correlation between an increase in Foxp3 expression and viremia, they did observe an attenuated antigen-specific T cell proliferative response and decreased IFNγ secretion in MHC class II tetramer positive T cells. These studies did not identify viral variants arising in the epitopes analyzed, thereby giving no indication whether viral variants could have an effect on the wild type T cell response (2, 3). By depleting HCV-specific T cells that bound to MHC class II tetramers specific for the individual variants, we found an enhanced T cell proliferative response to the $NS3_{358\text{-}375}$ peptide and a restoration of suppression when the variant-specific T cells were added back, suggestive of Tregs.

Consistent with the concept of variants acting as antagonists and leading to a suppressed T cell response, the T cell response was higher when $CD4^+$ $CD25^+$ T cells and T cells specific for variant K371E were removed, indicating that this variant might affect yet a different subset of T cells. Furthermore, adding pooled variant tetramer positive T

cells back into $NS3_{358-375}$-stimulated cultures had a dose-dependent suppressive effect, suggestive of Tregs. Combining these results suggests that antigen-specific Tregs are responsible for suppression of an effector T cell response. These studies did not address if the variants are able to induce a subpopulation of $CD4^+$ T cells and/or if these variants are causing the wild type-specific T cells to differentiate into Tregs, which needs further investigation. The second part of this dissertation demonstrates that these viral variants are able to antagonize clonal T cells specific for $NS3_{358-375}$, and that these variants have different avidities, possibly modulating the T cell activation, leading to the induction of Treg phenotype. Further evidence for antigen-specific induction of Tregs by MacDonald et al. (4) demonstrates that inducible Tregs are activated in the same HCV core-specific T cell clones, thereby providing supporting evidence that the antigen-specific induction of Tregs is not a phenomenon isolated to a singular chronic HCV subject or to these particular NS3 viral variants.

The conundrum is that the wild type genome is the dominant species circulating infected host (4-7). Interestingly, the viral variant S370P, previously shown to induce Treg phenotypic markers, was unable to suppress mitogen activated T cells when added alone, but if S370P was presented in conjunction with the wild type peptide; the T cell response to the mitogen was inhibited. These findings indicate that the presence of the wild type peptide in conjunction with the variant peptide is necessary for the suppressive effect of these T cells, but the induction is antigen-specific, thus suggesting that the maintenance of the wild type genome is necessary for suppression of the immune response.

Another mechanism that HCV can exploit for deviation of the immune response is APLs. In order for a viral variant to be considered an APL, the variant must be able to bind to the same TCR as the wild type or immunogenic peptide and have a lower potency in comparison to wild type peptide-stimulated T cells (Figure 1.2). In this dissertation, MHC class II tetramers were used to determine whether variants coupled to MHC were able to bind to T cell clones expressing a TCR specific for $NS3_{358-375}$. The variants were able to bind to the wild type-specific T cell clones, suggesting that this has an effect on the biological outcome of the T cell. In Chapter 4, tetramer avidity tests using MHC class II tetramers clearly demonstrate a difference in avidity between wild type and variant tetramer staining, with the exception of the H369R variant. This finding implies that the H369R variant tetramer has a similar or higher avidity when compared to the wild type tetramer. Functional data suggest that the H369R variant is blunting the $NS3_{358-375}$-specific T cell proliferation, which is indicative of T cell anergy, but this blunting/ anergy phenomenon requires further investigation. If the addition of IL-2 to PBMC cultures stimulated with both the H369R and wild type peptides causes a restoration of T cell proliferation to the wild type peptide, then the H369R variant is anergizing the T cells. Further, tetramer competition assays were used to detect both the variant and wild type tetramers binding to the same T cell, thereby providing more evidence that both the variant and wild type peptides are working in-concert on the same T cell. These naturally occurring APLs within an immunodominant epitope could possibly have differential effects on T cell activation and may be used by HCV to deviate or blunt an HCV-specific T cell response.

Further work testing if these variants deviate the T cell response demonstrates variant S370P inducing Foxp3 expression in an antigen-specific manner in polyclonal *in vitro* assays, and likewise variant S370P has a dose-dependent suppressive effect *in vitro*, reflective of Tregs. As previously noted, chronic HCV-infected individuals have an increase in Treg markers when compared to noninfected individuals (3, 4, 8-12). These studies support HCV induction of Tregs but do not explore the mechanism of induction of Tregs (2, 3). In this dissertation, naturally occurring APLs alter the TCR signaling, as suggested by our T cell proliferation data, and these variants induce a Tregs phenotype. In combining these findings, we determined that variant S370P was able to induce Foxp3 expression in $NS3_{358\text{-}375}$-MHC class II tetramer positive cells that are antigen-specific in two individuals. We also determined that this mechanism of Treg induction is HCV antigen-specific, suggesting that this phenomenon of Treg induction is not exclusive to one HCV chronic subject. This is the first demonstration that APLs are able to induce Tregs, therefore defining how HCV is able to maintain a wild type genome even in the presence of an intact immune system.

T Cell Signaling Induced by Viral Variants

Although these results suggest that HCV is exploiting TCR signaling for viral persistence, further investigation into APL's effect on the differentiation of these antigen-specific $CD4^+$ T cells is necessary to understand HCV pathogenesis. Also, examining the role of downstream signaling molecules would solidify the conclusion that these viral variants are able to either antagonize or anergize these antigen-specific T cells. In this aspect, the tetramer staining addresses the ability of the variant tetramers to bind to the

same TCR as the wild type tetramer on the same T cell, but further investigation needs to be performed to determine if markers of antagonism, such as protein tyrosine phosphatase (SHP-1) or zeta-chain-associated protein kinase 70 (ZAP-70) phosphorylation, are up regulated. Such information would further clarify if these variants are acting as APLs (13). Studies have demonstrated that SHP-1 is a cytosolic protein tyrosine phosphatase that has been shown to negatively regulate T cell activation (14), and that ZAP-70 is a protein tyrosine kinase that plays a crucial role in T cell signaling (15). Although the wild type's presence is necessary for the variants to have a suppressive effect, this dissertation does not address whether or not these two antigens need to be present on the same APC. Our microscopy data demonstrates the variant and wild type tetramers co-localize which suggests that they need to be presented on the same APC. This determination can be accomplished by prepulsing the APCs with either individual peptides and added into culture with a T cell clone or prepulse the APCs with both the wild type and variant peptides, so as to test the effect that these antigens have on T cell proliferation. Furthermore, screening for these T cell proteins would give an indication as to whether or not these variants are antagonizing or anergizing specific T cells.

The Role of IL-10 in the Induction of Tregs

An important question that arises when studying $CD4^+$ T cells is the impact that the extra-cellular milieu has on the fate of these T cells. One mechanism by which Tregs mediate suppression is through the secretion of IL-10 (16). Stimulating PBMC from a chronic subject with the S370P peptide increases the median fluorescence intensity of

$CD4^+$ $IL\text{-}10^+$ T cells, indicative of IL-10 secretion (data not shown). However, the addition of an anti-IL-10 antibody to cultures did not significantly restore the proliferative capacity of T the cells (data not shown). This finding suggests that suppression by Tregs requires contact, a finding that supports numerous other studies demonstrating Treg suppression through contact-mediated mechanisms (10, 17). Although our results do not define a single mechanism of suppression, these findings suggest that secretion of IL-10 by Tregs could be important for the maintenance of the antigen-specific Tregs (18). For example, a recent study by Murai et al. (18) in a murine colitis model showed that IL-10 was essential for the maintenance of Foxp3 expression and suppressive function of Tregs. Therefore the presence of IL-10 should be necessary for the maintenance of Foxp3 in our *in vitro* model system. The viral variant S370P was able to activate and maintain Foxp3 expression in comparison to the $NS3_{358\text{-}375}$ peptide when in the presence of IL-10, whether the IL-10 produced endogenously in a chronic patient or provided exogenously to PBMC in a resolved subject. Therefore, Foxp3 could be induced in an antigen-specific manner, and the maintenance of Foxp3 expression was dependent on IL-10 (Figure 5.1).

The most widely accepted Treg marker, Foxp3, is dependent on IL-10 signaling in $CD4^+$ T cells (18). Furthermore, it has been demonstrated that IL-10 serum levels are significantly higher in chronic patients in comparison to resolved HCV subjects, providing an environment that is conducive to Treg differentiation and maintenance (4). Although the level of endogenous IL-10 has been found to be high in the serum, the cell type(s) responsible for the IL-10 secretion has not been clearly defined and needs further investigation. The suppressive nature of IL-10 is nonspecific, and presumably the

immune system in chronic HCV subjects would be dampened by this suppressive cytokine.

Results from this dissertation indicate that Foxp3 is induced in an antigen-specific manner while previous work has demonstrated that the maintenance of Foxp3 expression is dependent on IL-10. To circumvent the issue of IL-10 in our assay system, we used a mixture of chronic and resolved APCs. Further clarification of this issue would be to the effect of this immunodominant epitope when added into the suppressive milieu of a chronically infected HCV patient. Preliminary data (Figure 5.1) suggest that when this wild type epitope is delivered into culture with high levels of IL-10, Treg phenotypic markers are not increased. This information leads us to hypothesize that this epitope is a plausible candidate for a vaccine for use in individuals who were unable to initially clear the virus. Performing studies that use infected, resolved, and noninfected APCs to test the impact of the extracellular milieu on antigen-specific T cells give a "snap-shot" of what could happen *in vivo* when these viral antigens are presented to T cells in the context of an infection and the potential impact that the extracellular milieu has on T cell signaling.

Alternative Therapy for HCV Clearance

Although the goal of these experiments was to define *in vitro* correlates of HCV pathogenesis leading to a better understanding as to how vaccines could be used to prime the immune system, so that one could clear the virus, the results of these studies indicate that there are alternative approaches to clearing the virus other than using a vaccine. One such alternative to vaccination is to transplant an individual's T cells back into the

chronically infected patient after selecting HCV-specific $CD4^+$ T cells. In this scenario, HCV-specific T cells that drive viral clearance would be added back into chronically infected patients. Our work and that of others have characterized epitopes that are conducive for viral clearance and work in this dissertation clearly describes a technique for selecting, expanding, and characterized antigen-specific $CD4^+$ T_H1 cells. The premise of this approach would be to identify $CD4^+$ T_H1 cells in a chronically infected individual, use CFSE-labeled PBMC from this individual stimulated with the T_H1 epitopes by peptide stimulation and sort the epitope-specific $CD4^+$ T_H1 cells. These $CD4^+$ T cells would then be cultured with CD3/CD28 beads leading to nonspecific expansion of these T cells. The antigen-specific T cells could then be adoptively transferred back into these chronically infected individuals in large numbers, driving the immune system towards viral clearance by $CD4^+$ T_H1 T cells, and possibly leading to viral clearance in a chronically infected patient.

In conclusion, the work presented in this dissertation shows that naturally occurring viral variants are acting as APLs that work synergistically with the wild type peptides to differentiate T cells, in an antigen-specific manner, as well as suppress T cell responses nonspecifically a mechanism which we have termed “convergent suppression” (Figure 5.2). This information reveals that HCV may be able to shift the immune response towards viral persistence suggesting that priming the immune system with a viral clearing $CD4^+$ T cell response could provide protective immunity against HCV.

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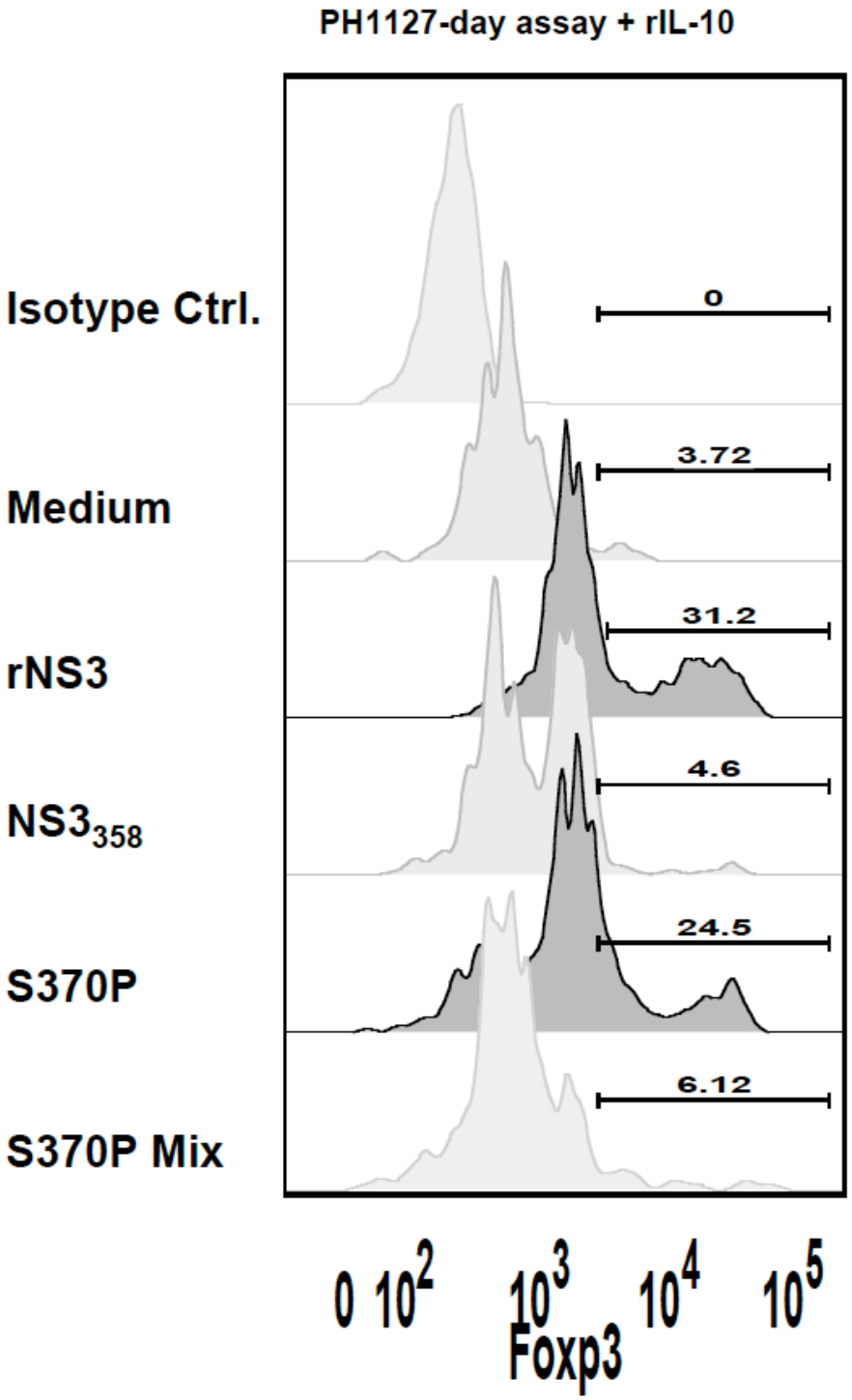
Figure 5.1. Foxp3 expression is induced in an antigen-specific manner with the addition of IL-10. (A) Histogram of $Foxp3^+$ cells in $CD4^+$ $CD127^-$ $CFSE^{low}$ lymphocyte population, stimulated with indicated peptides, and cultured in 2ng/ml of rhIL-10 (gating scheme Fig.3A). (B) PBMC from PH1127 were cultured under normal conditions (gray bars) or with rIL-10 (2ng/ml) (black bars) and PBMC were stimulated with 1μM of synthetic peptide for 7 days and analyzed by flow cytometry. Under normal conditions (-rIL-10), Foxp3 is transiently expressed in proliferating $CD4^+$ $CD127^-$ T cells but S370P stimulated cells with rIL-10 have a significantly higher percentage of $Foxp3^+$ cells in comparison $NS3_{358-375}$-stimulated cells. The normal and rIL-10 were run in tandem (n=2) and the data were normalized to medium. **, $P<0.005$. The standard deviation is shown. Results are representative of 2 experiments.

A.


PH1127-day assay + rIL-10
Isotype Ctrl.
Medium
rNS3
NS3358
S370P
S370P Mix
0
3.72
31.2
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Foxp3

B.

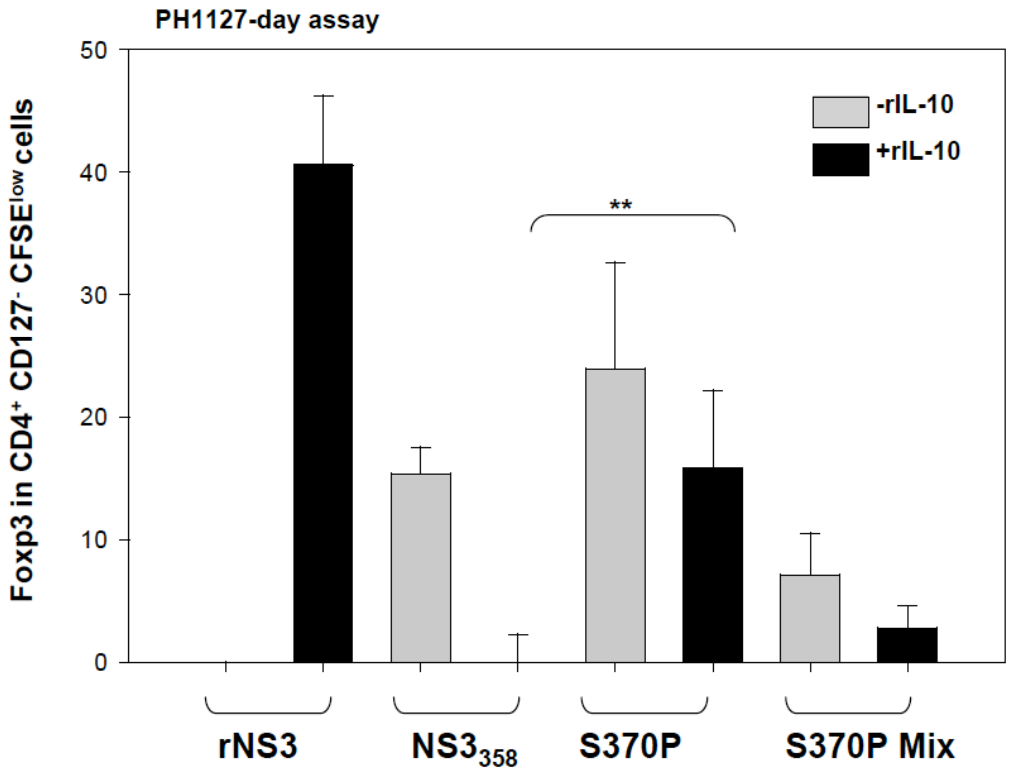

Figure 5.1 continued

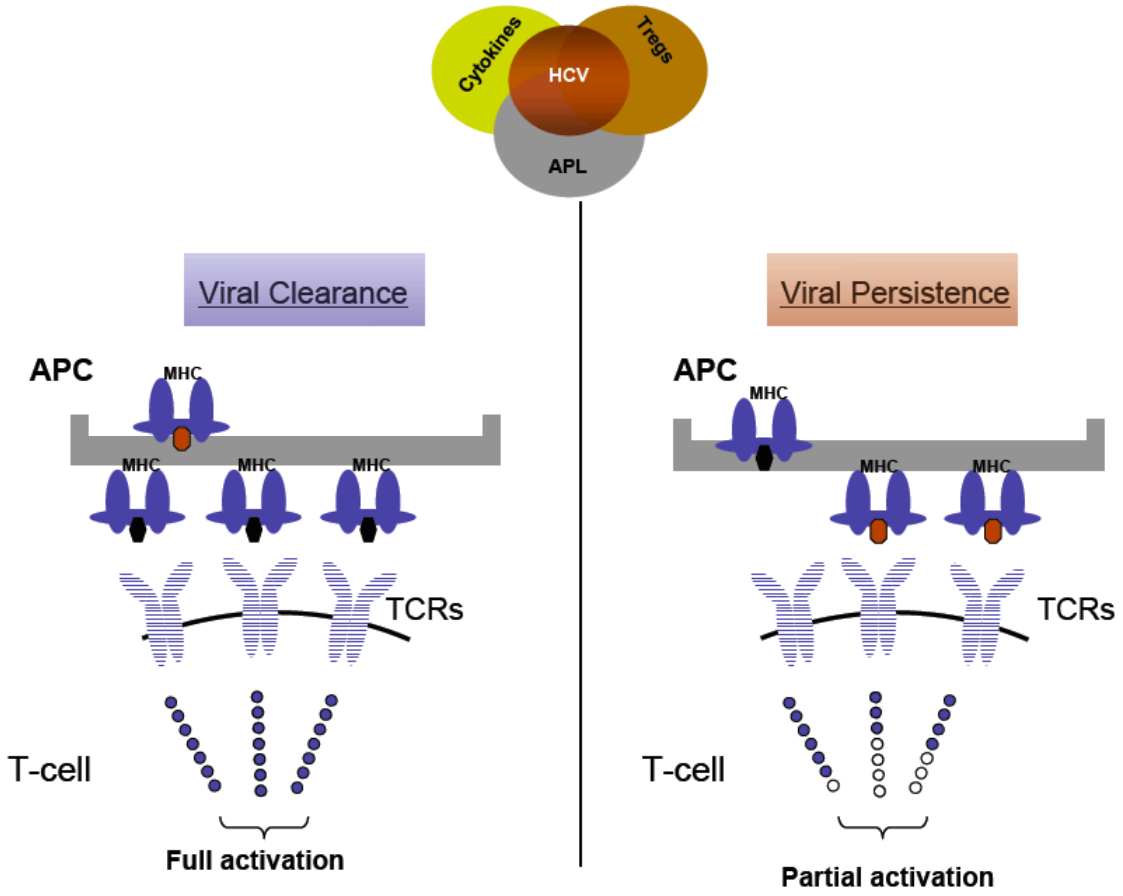

Figure 5.2. Model of convergent suppression in HCV infection. If the wild type peptide (black) is presented to the $CD4^+$ T cell prior to the variant variant peptide (red), this could lead to viral clearance. If the viral variant (red) is presented to the wild type-specific $CD4^+$ T cell before the wild type peptide, this could lead to viral persistence.

APPENDIX

IN VITRO RESPONSES TO AVIAN INFLUENZA H5 BY

HUMAN CD4 T CELLS

The Journal of Immunology

In Vitro Responses to Avian Influenza H5 by Human CD4 T Cells[1]

Matthew F. Cusick,[2] Shuping Wang,[2] and David D. Eckels[3]

To address the question of whether human T cells are capable of recognizing novel isolates of influenza virus, in vitro responses to recombinant Ags and synthetic peptides derived from the sequences of H1, H3, and H5 were examined in a cohort of 64 individuals selected from a healthy blood donor population. Humans respond in vitro to H1 and H3 following exposure through natural infection and vaccination. Responses to H5 were well correlated with those to H1 or H3, and thus, a significant repertoire of H5-responsive T cells is present in many individuals; clear nonresponders to H1, H3, and H5, however, do exist. Differences were observed in the cytokine responses to H1, H3, and H5, whereas both IL-2 and IFN-γ production characteristic of memory responses were observed for H1 and H3, and H5-specific responses elicited primarily IL-2 and little or no IFN-γ, consistent with a naive T cell phenotype. Responses to all influenza HA were restricted by HLA-DR molecules. To address the structural basis for T cell recognition of H1 and H5, overlapping synthetic peptides were used to identify epitopes and to determine whether recognition of H5 was limited to homologous sequences in H1, the most closely related HA phylogenetically. Although responses were generally correlated, no complete structural overlap was observed. These results suggest that helper T cell cross reactivity between different influenza strains may impart cross-protection to H5N1 strain of influenza. ***The Journal of Immunology,*** **2009, 183: 6432–6441.**

The potential risks surrounding a pandemic outbreak of influenza would apply to any new variant of the influenza virus, although, there is a significant clinical difference in the immunopathology of certain influenza strains when humans have been infected (1–5). In the case of H5N1, vaccine makers are scrambling to develop recombinant vaccine materials because H5N1 is lethal to eggs, thus rendering conventional approaches inadequate (6–16); even ramping up production for a more conventional vaccine such as that for the new "swine flu" can be problematic. Little attention, however, has been given to the intrinsic ability of humans to mount an immune response against an emerging strain of influenza. Furthermore, despite the availability of recombinant influenza viruses and corresponding sequence information, potential in vitro correlates of protective levels of responsiveness to influenza strains have not been established. We have examined in vitro T cell responses to H5 Ag and compared them to those against H1 and H3 to ascertain whether normal healthy humans might be able to mount an immune response against newer isolate(s) of influenza in the absence of prior immunization.

Strain A/H5N1 was first identified in domestic chicken stocks in the Far East (5, 17–22). Only sporadic evidence of human infection was available early on, but there has been a steady increase in the incidence and severity of small human outbreaks as well as an increasing possibility of a pandemic (5, 23). Although early vaccine trials are underway, whether or not current Ag constructs will prove to be protective under field conditions remains an open question. There has been additional speculation on the nature of the mutations that would be required for human to human spread (20, 24–34). Although anywhere from a few to several different changes must occur in order for a human-adapted strain to emerge (30, 34, 35), reassortment in animal species and high viral mutability make such events likely. Assessment of responsiveness to naturally occurring variants or mutant viruses is important.

Significant overlap exists among sequences of H1, H3, and H5 influenza viral isolates (36–39). Whereas protective neutralizing Abs are generally focused upon three major membrane distal epitopes of hemagglutinin (HA),[4] there is evidence that Abs can recognize conserved areas of HA as well as neuraminidase (40–44). The extent to which Abs against conserved epitopes are able to confer protection against infection is unknown. Protection against infection, however, is not the only measure of an effective immune response against a pathogen; immune recognition of conserved epitopes by Abs and, in particular, T cells might be able to protect an individual from death due to infection.

Previous work has shown that both helper and cytotoxic T cells are not only able to recognize conserved regions of the surface glycoproteins of influenza virus but also internal, largely invariant viral proteins such as the matrix protein and ribonucleoprotein (42, 45–47). The extent to which cellular immune processes contribute to resistance to influenza viral infection has been examined extensively in mice, but their importance in human disease is less well understood compared with the significance of neutralizing Abs. Using H5 as an archetypal example of an emerging pathogen, we reason that because Ab production and killer T cell differentiation both depend on strong T cell helper responses, the demonstration

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[4] Abbreviations used in this paper: HA, hemagglutinin; Io, ionomycin; SFC, spot-forming cell; SI, stimulation index.

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of helper T cell responses to H5 would form a solid basis for effective vaccination protocols. Activation of CD4 T cells would drive the appropriate production of Abs by B cells and killing of virus-infected cells by CTL. To find those regions of the virus that T cells recognize, we used recombinant HA Ags and synthetic peptides and show that responsiveness to H5 is well correlated with a subject's ability to respond to other influenza HA Ags such as H1 or H3. Human in vitro responses to H5 are qualitatively different, however, probably due to the lack of expansion of H5-specific CD4 memory T cells that would occur following environmental or vaccine exposure. The fact that a viable H5-specific T cell repertoire seems to exist at all in unexposed humans should provide some optimism for preventive vaccination strategies (48–50).

Materials and Methods

Subjects

A group of 64 healthy blood donors between the ages of 18 and 65 years provided repeat samples and follow-up histories. To mitigate immune response differences due to genetic heterogeneity, we arbitrarily used only patients positive for HLA-DR1 and/or DR4 in the study. Patients were excluded from the study if they had traveled out of the United States within 1 year of donation. Peripheral blood samples were obtained during routine donation, and a leukocyte-enriched fraction was provided for testing. Samples were diluted 1/4 in RPMI 1640, layered over leukocyte separation medium, and centrifuged at 1200 × *g* for 30 min to obtain PBMC. PBMC were suspended at 10^7/ml in RPMI 1640 containing 20% FCS and 7.5% DMSO as a cryoprotectant and frozen in a control-rate liquid nitrogen freezer. All experiments were performed on thawed samples. This study was approved by the Blood Center of Wisconsin and University of Utah Institutional Review Board committees.

Ag preparations

Recombinant H1 (A/New Caledonia/1999), H3 (A/Phillipines/1992), and H5 (A/Vietnam/2004) were obtained from Protein Sciences. Ags were dissolved in a drop (~50 μl) of DMSO, then diluted to 1.0 mM in RPMI 1640 tissue culture medium. Synthetic peptides were obtained from Mimotopes in sets consisting of H1 and H5 sequences of 18-mer overlapping by 11 aa covering the HA1 chain of both molecules. Where necessary, two N-terminal lysines were added to promote solubility. In addition, 38 peptides corresponding to a limited set of known naturally occurring H5 variants from different isolates were synthesized; we have termed these "mutant" peptides throughout (Table I and supplemental Table I).[5] Assuming a scale of synthesis of ~4 mg/peptide, each was solubilized in a drop of DMSO and brought up to 10 mM in PBS. For comparison, the amino acid sequences of H1, H3, and H5 are provided in Fig. 1.

Proliferation assays

To measure proliferative responses, PBMC from subjects were resuspended at a concentration of 1×10^6/ml in RPMI 1640 tissue culture medium containing 25 mM HEPES, 2.0 mM L-glutamine, 1.0 mM sodium pyruvate, 10 U/ml heparin sodium, 100 U/ml penicillin, 100 mg/ml streptomycin, 5.0 mg/ml gentamicin, and 10% pooled human serum. To 96-well round-bottom plates, 100-μl aliquots of PBMC were added to Ags of influenza virus at indicated concentrations in 100 μl of 10% pooled human serum tissue culture medium. Controls consisted of medium without Ags. Cultures were incubated at 37°C in 5% humidified CO_2 for 6 days, pulsed overnight with 1.0 μCi/well tritiated thymidine ($[^3H]$-TdR), and harvested onto glass fiber filters. Radioactive label incorporation was measured by gas scintillation spectroscopy. Results are represented as the mean ± SEM of at least triplicate cultures.

Proliferation was also assessed by staining cells with CFSE and analyzing by flow cytometry. Cells were stimulated with Ags at indicated concentrations, stained with 0.5 μM CFSE as recommended by Quah et al. (50), and analyzed at 7 days. This approach enables simultaneous phenotyping of cells using fluorescently labeled mAbs for CD4, CD8, and CD45RO as compared with medium only controls or irrelevant Ag (HCV-NS3). Analysis was done using FlowJo software (Tree Star).

[5] The online version of this article contains supplemental material.

ELISPOT assays

PBMC were assayed for spot-forming cells (SFC) producing IFN-γ, IL-2, IL-4, IL-5, IL-6, and IL-10 in the presence of H1, H3, H5, and media. Ninety-six-well plates with a polvinylidene difluoride filter base (Millipore) were coated with 5 μg/ml capture Ab IFN-γ, IL-2, IL-4, IL-5, IL-10 (BD Biosciences), and 10 μg/ml IL-6 (R&D Systems) in sterile coating buffer (1× PBS) overnight at 4°C. The plates were washed three times for 5 min each with 200 μl PBS/well and blocked with 200 μl/well of RPMI 1640, 10% FBS, 25 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine for 2 h at room temperature. PBMC were plated at 1×10^5 cells/well with 1 μg/ml of recombinant Ag, in a total volume of 200 μl/well for 44–48 h at 37°C in 5% CO_2. The plates were washed with 0.05% Tween 20/PBS. A solution of 100 μl of biotinylated anti-human detection Ab specific for IFN-γ, IL-2, IL-4, IL-5, IL-6, or IL-10 was added at 2 μg/ml in PBS containing 10% FBS to each well for 2 h at room temperature. Enzyme conjugate (100 μl/well streptavidin-HRP diluted 1/1000) was added to each well and incubated at room temperature for 1 h and washed as above. Color substrate (3-amino-9-ethyl-carbazole) was added at 100 μl/well for 10 min to 1 h at room temperature; for IL-6, the assay was developed with 5-bromo-4-chloro-3-indolyl phosphate/NBT Chromagen (R&D Systems) for 30 min in the dark. Color development was stopped with water, and the membranes were dried overnight in the dark. SFC were counted on the Immunospot Analyzer (CTL Analyzers). Positive controls were specific for the individual cytokine tested and consisted of the following: for IFN-γ, PBMC were cultured at 5×10^3/well, stimulated with 5 ng/ml PMA (Sigma-Aldrich) and 500 ng/ml ionomycin (Io; Sigma-Aldrich) for 24 h. For IL-2, PBMC were cultured at 2.5×10^5/well, stimulated with 5 ng/ml PMA and 500 ng/ml Io for 24 h. For IL-4 and IL-5, PBMC were cultured at 1×10^4/well after prestimulation with immobilized anti-human CD3 Ab (10 μg/ml for plate coating), 2 μg/ml soluble anti-human CD28, 10 ng/ml recombinant human IL-2, and 50 ng/ml recombinant human IL-4 for 48 h. The cells were washed, then cultured in medium containing 10 ng/ml recombinant human IL-2 and 50 ng/ml recombinant human IL-4 for another 48 h. Finally, the cells were harvested, washed, and restimulated with 5 ng/ml PMA and 500 ng/ml Io for 15 h. For IL-10, PBMC were cultured at 1×10^5/well and stimulated with 1 μg/ml LPS (Sigma-Aldrich) for 24 h and enumerated as above.

Results

Initial screening with recombinant Ags

To compare human T cell responses to recombinant HA isolates, recall responses were measured in vitro. PBMC from a subset (n = 14) of healthy blood donors, all of whom shared either or both HLA-DR1 and DR4, were stimulated in proliferation assays with recombinant H1, H3, or H5 Ags at indicated doses (Fig. 2*A*). Indicative of cross-reactivity in responding subjects, T cell proliferative responses to H1 and H3 generally could be measured at lower Ag concentrations than those to H5 (Fig. 2*A*). Responses were quite variable and ranged from those not significantly above negative controls to more than 10-fold over background. Because of individual variation in assays with human PBMC, we transformed the data by designing an algorithm that could compensate for such wide ranging background proliferation while not distorting individual responsiveness. Typically, others have used the stimulation index (SI) to accomplish this: $SI = [\bar{X}_{exp} - \bar{X}_{bkg}]/\bar{X}_{bkg}$, where $\bar{X}$ is the mean triplicate value of experimental (exp) or background (bkg) cultures. Such a transformation normalizes the magnitude of individual differences such that an $\bar{X}_{exp}$ response of 1000 cpm over an $\bar{X}_{bkg}$ of 100 cpm yields an SI of 9, which is identical to another individual with an $\bar{X}_{exp}$ response of 100,000 cpm over an $\bar{X}_{bkg}$ of 10,000 cpm. Because the SI neglects differences in the magnitude of responses by different subjects, we used an alternative transformation to reduce the contribution of individual variability: $\log_{10}\Delta cpm = \log_{10}[\bar{X}_{exp} - \bar{X}_{bkg}]$, not to be confused with the geometric mean. Using the above example, the $\log_{10}\Delta cpm$ transformation yields values of 2.95 and 4.95, or 900 and 90,000 respectively, and preserves the linearity of the relationship between different subjects. This transformation is used for all pairwise comparisons of responsiveness to H1, H3, and H5 recombinant Ags

Table I. *18-Mer overlapping peptides spanning the influenza A HA protein*[a]

Peptides	H1 Sequence	H5 sequence	Peptides	Mutant sequence
A1	MKAKLLVLLCTFTATYAD	KKMEKIVLLFAIVSLVKSDQ	H10	KKMEKIVLLLAIVSLVKSDQ
B1	LLCTFTATYADTICIGYH	KKFAIVSLVKSDQICIGYHA		
C1	TYADTICIGYHANNSTDT	KSDQICIGYHANNSTEQV	A7	KSDQICIGYHANNWTEQV
D1	IGYHANNSTDTVDTVLEK	GYHANNSTEQVDTIMEKN	B7	GYHANNWTEQVDTIMEKN
E1	STDTVDTVLEKNVTVTHS	TEQVDTIMEKNVTVTHAQ		
F1	VLEKNVTVTHSVNLLEDS	MEKNVTVTHAQDILEKKH	C7	MEKNVTVTHAQDILEKTH
G1	VTHSVNLLEDSHNGKLCL	THAQDILEKKHNGKLCDL	D7	THAQDILEKTHNGKLCDL
H1	LEDSHNGKLCLLKGIAPL	EKKHNGKLCDLDGVKPLI	E7	EKTHNGKLCDLNGVKLIL
A2	KLCLLKGIAPLQLGNCSV	KKLCDLDGVKPLILRDCSVA	G10	KKLCDLNGVKPLILRDCSVA
B2	IAPLQLGNCSVAGWILGN	KKKPLILRDCSVAGWLLGNP		
C2	NCSVAGWILGNPECELLI	KKCSVAGWLLGNPMCDEFIN		
D2	ILGNPECELLISKESWSY	KKLGNPMCDEFINVPEWSYI		
E2	ELLISKESWSYIVETPNP	EFINVPEWSYIVEKANPV	F7	EFINVPEWSYIVEKANPA
F2	SWSYIVETPNPENGTCYP	WSYIVEKANPVNDLCYPG	G7	WSYIVEKANPANDLCYPG
G2	TPNPENGTCYPGYFADYE	ANPVNDLCYPGDFNDYEE	H7	ANPANDLCYPGDFNDYEE
H2	TCYPGYFADYEELREQLS	CYPGDFNDYEELKHLLSR		
A3	ADYEELREQLSSVSSFER	DYEELKHLLSRINHFEKI		
B3	EQLSSVSSFERFEIFPKE	LLSRINHFEKIQIIPKSS	A8	LLSRINHFEKIQIIPKNS
C3	SFERFEIFPKESSWPNHT	FEKIQIIPKSSWSSHEAS	B8	FEKIQIIPKNSWSSHEAS
D3	FPKESSWPNHTVTGVSAS	PKSSWSSHEASLGVSSAC	C8	PKNSWSSHEASLGVSSAC
			D8	PKSSWLSHEASLGVSSAC
			B11	PKSSWSSHEVSLGVSSAC
E3	PNHTVTGVSASCSHNGKS	HEASLGVSSACPYQGKSS	E8	HEASLGVSSVCPYQGKSS
			F8	HEASLGVSSACPYQRKSS
F3	VSASCSHNGKSSFYRNLL	SSACPYQGKSSFFRNVVW	G8	SSVCPYQGKSSFFRNVVW
			H8	SSACPYQRKSSFFRNVVW
G3	NGKSSFYRNLLWLTGKNG	GKSSFFRNVVWLIKKNST	A9	RKSSFFRNVVWLIKKNST
			B9	GKSSFFRNVVWLIKKNNA
H3	RNLLWLTGKNGLYPNLSK	NVVWLIKKNSTYPTIKRS	C9	NVVWLIKKNNAYPTIKRS
A4	GKNGLYPNLSKSYVNNKE	KNSTYPTIKRSYNNTNQE	D9	KNNAYPTIKRSYNNTNQE
B4	NLSKSYVNNKEKEVLVLW	IKRSYNNTNQEDLLVLWG		
C4	NNKEKEVLVLWGVHHPPN	TNQEDLLVLWGIHHPNDA		
D4	LVLWGVHHPPNIGDQRAL	VLWGIHHPNDAAEQTKLY	E9	VLWGIHHPNDAAEQTRLY
			D11	VLWGIQHPNDAAEQTKLY
E4	HPPNIGDQRALYHTENAY	PNDAAEQTKLYQNPTTYI	F9	PNDAAEQTKLYQNPTTYV
			E11	PNDAAEQTRLYQNPTTYI
F4	QRALYHTENAYVSVVSSH	TKLYQNPTTYISVGTSTL	G9	TKLYQNPTTYVSVGTSTL
G4	ENAYVSVVSSHYSRRFTP	TTYISVGTSTLNQRLVPR	H9	TTYVSVGTSTLNQRLVPR
H4	VSSHYSRRFTPEIAKRPK	TSTLNQRLVPRIATRSKV	A10	TSTLNQRLVPKIATRSKV
			F11	TSTLNQRSVPKIATRSKV
A5	RFTPEIAKRPKVRDQEGR	LVPRIATRSKVNGQSGRM	B10	LVPKIATRSKVNGQNGRM
B5	KRPKVRDQEGRINYYWTL	RSKVNGQSGRMEFFWTIL	C10	RSKVNGQNGRMEFFWTIL
C5	QEGRINYYWTLLEPGDTI	SGRMEFFWTILKPNDAIN	D10	NGRMEFFWTILKPNDAIN
			G11	SGRMEFFRTILKPNDAIN
D5	YWTLLEPGDTIIFEANGN	WTILKPNDAINFESNGNF		
E5	GDTIIFEANGNLIAPWYA	DAINFESNGNFIAPEYAY		
F5	ANGNLIAPWYAFALSRGF	NGNFIAPEYAYKIVKKGD		
G5	PWYAFALSRGFGSGIITS	EYAYKIVKKGDSTIMKSE	E10	EYAYKIVKKGDSAIMKSE
H5	SRGFGSGIITSNAPMDEC	KKGDSTIMKSELEYGNCN	F10	KKGDSAIMKSELEYGNCN
A6	IITSNAPMDECDAKCQTP	MKSELEYGNCNTKCQTPM		
B6	MDECDAKCQTPQGAINSS	GNCNTKCQTPMGAINSSM		
C6	CQTPQGAINSSLPFQNVH	QTPMGAINSSMPFHNIHP		
D6	INSSLPFQNVHPVTIGEC	NSSMPFHNIHPLTIGECP		
E6	QNVHPVTIGECPKYVRSA	NIHPLTIGECPKYVKSNR		
F6	IGECPKYVRSAKLRMVTG	GECPKYVKSNRLVLATGL		
G6	VRSAKLRMVTGLRNIPSI	KSNRLVLATGLRNSPQRE		
H6	MVTGLRNIPSIQSRGLFG	LATGLRNSPQRERRRKKR		

[a] Forty-eight peptides (18 aa overlapping by 11 aa) were synthesized according to the HA1 segments of the A/New Caledonia H1 and the A/Vietnam H5 HA. Underlined amino acids represent changes in amino acids for the H5 mutant sequences relative to the H5 peptides from the A/Vietnam reference strain. See supplemental Table I[5] for National Center for Biotechnology Information accession numbers. H1: A/New Caledonia/20/99. (H1N1) H5: A/Viet Nam/1203/2004. (H5N1).

shown herein. Responders were considered those producing responses greater than 2 SDs above $\bar{X}_{bkg}$. Fig. 2, *B* and *C*, show a pairwise comparison of responses to H1, H3, and H5 where the medium background was subtracted from the maximum triplicate response (based on an Ag dose titration) to give a Δ_{max} value plotted on a $\log_{10}$ scale. Although a cohort of subjects failed to respond to H1, H3, or H5 Ags (Fig. 2*B*, gray area), significant responses to the H5 Ag correlated with responses to either H1 or H3 recombinant Ags in a few high responders (Fig. 2*C*), that is, if a subject responded to H5 they also responded to H1 or H3; seven subjects responded to all three (red circles). The results suggest significant cross-reactivity may exist in the repertoire of any given individual.

Class II-restricted, helper T cell proliferation

To determine whether such proliferative responses were due to class II-restricted helper ($CD4^+$) T cell responses, four typical responders were selected for assays in which proliferation was

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                                                                  H1
H1          LLVLLCTFTATYADTICIGYHANNSTDTVDTVLEKNVTVTHSVNLLEDSHNGKLCL 56
H3                      -L-L-H--VPNGTL-K-ITNDQIE--NATE-VQS-ST-RI-D 42
H5       MEKIVL-FAIVSLVKS-QI----------EQ---IM--------AQDI--KK------D 59

                          C2/D2/E2
H1       LKGIAPLQLGNCSVAGWILGNPECELLISKESWSYIVETPNPENGTCYPGYFADYEELRE 116
H3       SP-HRI-DGK--TLIDAL--D-H-DGFQN--K-DLF--RSKAFSN----YDVP--AS--S 99
H5       -D-VK--I-RD------L----M-DEF-NVPE------KA--V-DL----D-N-----KH 119

                                   F3
H1       QLSSVSSFERFEIFPKESSWPTHTVT-GVSASCSHNGKSSFYRNLLWLTGKNGLYPNLSK 175
H3       LVA-SG---TL-FINEGFN-TGV-QS--G-YT-KRGSNN--FSR-N--YESESK--V-NV 155
H5       L--RINH--KIQ-I------SS-EASL---SA-PYQ--S--F--VV--IN--ST--TIKR 178


H1       SYVNNKEKEVLVLWGVHHPPNIGDQRALYHTENAYVSVVSSHYSRRFTPEIAKRPKVRDQ 235
H3       TMP--GKFDK-YI--I---STDKE-TN--IRASGR-T-STKRSQQTVI-N-GS--W--GL 215
H5       --N-TNQEDL-V---I---NDAAE-TK--QNPTT-I--GT-TLNQ-LV-R--T-S--NG- 238

                C5/ D5/ E5                                  B6/C6/D6
H1       EGRINYYWTLLEPGDTIIFEANGNLIAPWYAFALSRGFGSGIITSNAPMDECDAKCQTPQ 295
H3       SS--SI---IVK---ILLINST------RGY-KIRT--K-S-MR-D--IGT-SSE-I--N 274
H5       S--MEFF--I-K-N-A-N--S---F---E--YKIVKKGD-T-MK-ELEYGN-NT-----M 298

                          E6/F6
H1       GAINSSLPFQNVHPVTIGECPKYVRSAKLRMVTGLRNIPSIQSRGLFGAIAGFIEGGWTG 355
H3       -S-PNDK-----NKI-Y-A--R--KQNT-KLA--M--V-EK-T--I---------N--E- 334
H5       ------M--H-I--L---------K-NR-VLA-----S-QRET--------------Q- 358


H1       MVDGWYGYHHQNEQGSGYAADQKSTQNAINGITNKVNSVIEKMNTQFTAVGKEFNKLERR 415
H3       -I-----FR---SE-T-Q---L----A--DQ-NG-L-R----T-EK-HQIE---SEV-G- 394
H5       ----------S----------KE---K--D-V------I-D------E---R---N---- 418


H1       MENLNKKVDDGFLDIWTYNAELLVLLENERTLDFHDSNVKNLYEKVKSQLKNNAKEIGNG 475
H3       IQD-E-Y-E-TKI-L-S--------A---QH-I-LT--EMNK-F--TRK--RE--EDM--- 454
H5       I------ME-----V----------M-----------------D--RL--RD----L--- 478


H1       CFEFYHKCNNECMESVKNGTYDYPKYSEESKLNREKIDGVKLESMGVYQILAIYSTVASS 535
H3       --KI----D-A-IG-IR-----HDV-RD-ALN--FQ-K--E-K-GYKDW--W-SFAISCF 514
H5       --------D-------R-------Q----AR-K--E-S------I-I----S-------- 538

H1       LVLLVSLGAISF              547
H3       -LCVVL---FIMWACQKGNIRCNICI 539
H5       -A-AIMVAGL-LWMCSNGSLQCR--- 561
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FIGURE 1. Comparison of full-length HA (HA1 and HA2 chains) from isolates of H1, H3, and H5 strain A influenza viruses. Gray highlights represent sequences that stimulated T cell responses to both H1 and H5 peptides. Bold letters above the sequence represent the synthetic peptide label as indicated in Table I.

blocked with the mAb L243, specific for all human DR molecules (Fig. 3*A*). As a negative control, murine IgG was added in parallel cultures; each Ab was added at 2 μg/ml. In response to 2.5 μM recombinant H5 Ag, without exception, the presence of L243 reduced proliferation significantly. Flow cytometric analysis of proliferating T cells stained with CFSE revealed that the vast majority of dividing cells are derived from the class II-restricted $CD4^+$ population (Fig. 3*B* and Table II). The $CD4^+$ population consisted mainly of memory T cells (~90% $CD45RO^+$) as shown in one subject in Fig. 3*C* and generalized for six subjects in Table II). As with the results above, different subjects responded to H1, H3, or H5 to varying degrees; responses to an HCV recombinant NS3 control peptide were comparable to medium (data not shown).

Cytokine production

To address the qualitative nature of the helper T cell response to HA Ags, ELISPOT analysis was performed looking at cytokine production in response to stimulation by H1, H3, or H5 recombinant Ags. In general, responders produced IL-2, IL-4, IL-5, and IFN-γ in response to H1 and H3 strains of HA (data not shown). In contrast, H5 elicited little or no IL-4 or IL-5, whereas producing limited quantities of IL-2 or IFN-γ (Fig. 4). In this limited cohort (n = 26), only one H5-responder produced significant levels of IFN-γ SFC and five subjects produced moderate levels of IL-2 SFC, but essentially no IFN-γ.

Structural basis for H1 and H5 responsiveness

Because H5 is phylogenetically most closely related to H1, two sets of synthetic peptides were constructed (18-mer overlapping by 11 aa) based on the A/New Caledonia/1999 and A/Vietnam/2004

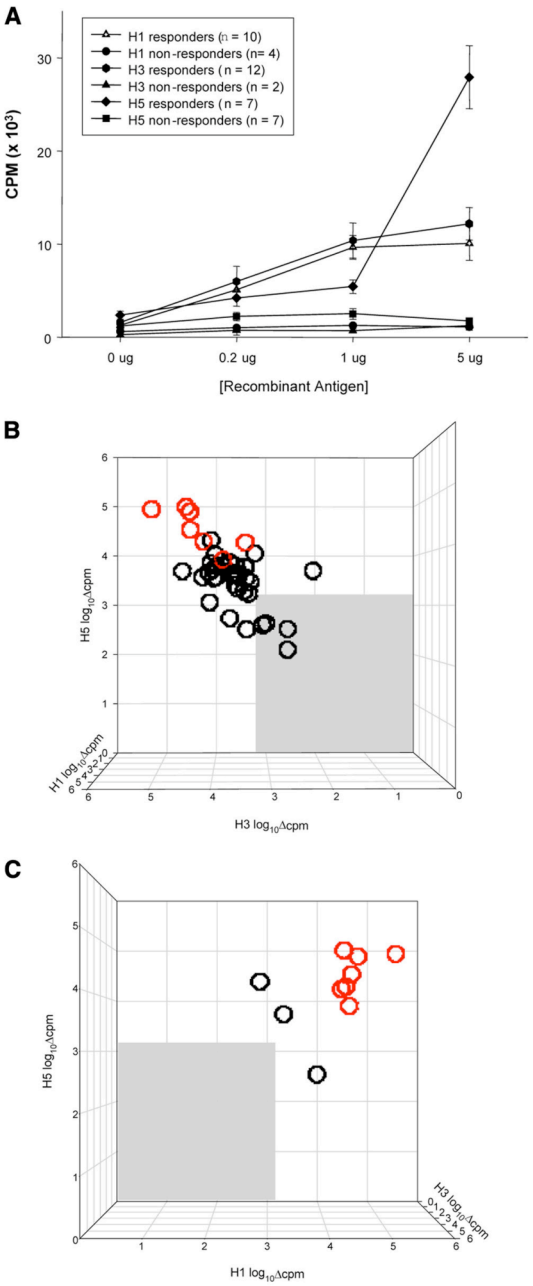

FIGURE 2. Comparison of proliferative T cell responses to recombinant H1, H3, and H5 proteins. Proliferation was determined following stimulation with recombinant H1, H3, and H5 at varying concentrations for 7 days by [^{3}H]TdR uptake as described in *Materials and Methods*. *A*, Dose response to recombinant H1, H3, and H5 HA. Responders to H1, H3, and H5 were compared with nonresponders for each protein as indicated in the graph. *B* and *C*, Proliferative responses to (1 μg/ml) recombinant protein Ags comparing individual responses to each protein. To account for individual variation, data were transformed as described in *Results* using the equation: $\log_{10}\Delta\text{cpm} = \log_{10}[\bar{X}_{exp} - \bar{X}_{bkg}]$. Subjects responding to all three Ags are represented by red circles.

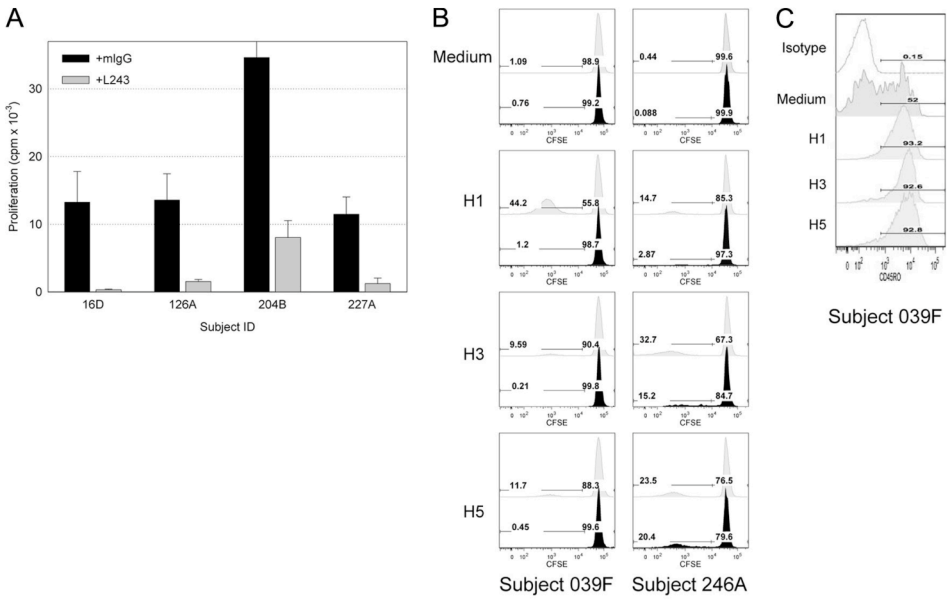

FIGURE 3. Responses to H5 are class II restricted. *A*, Proliferative responses in all cases could be blocked by the L243 mAb specific for the DRβ-chain of all human class II MHC molecules in four subjects (▤) in comparison to IgG control (■). *B*, Representative figures of CFSE staining and cytofluorometric phenotyping indicates the majority of proliferating cells were CD4 positive (▤) and >85% were CD45RO positive as shown in *C*. *B*, Limited proliferation was observed in the CD8 population of T cells for the majority of subjects tested (■).

sequences along with recent mutants consisting of H5 (Fig. 1 and Table I). Responses were evaluated in 24 subjects to address the possibility of a structural basis for H1/H5 cross-reactivity (Fig. 5). Every peptide was recognized by T cells from at least one subject; conversely, no peptide was recognized universally. Thus, responders and nonresponders were observed for each peptide, but the same subjects were not always responders or nonresponders to H1 and H5 peptides.

Responses to 48 homologous pairs of 18-mer peptides from the H1 and H5 sequences were compared (Fig. 6, *A–F*, and supplemental Fig. 1).[5] Linear regression (SigmaPlot 2000) was used to determine relatedness of each pair. Correlation coefficients were quite variable from a low of $r = 0.31$ ($p = 0.134$) for the A2 peptides to a high of $r = 0.83$ ($p < 0.0001$) for A6. Peptide A1 stimulated extremely strong responses in only one individual (supplemental Fig. 1).[5] Peptide D2 was recognized by T cells from 11

Table II. *CD4 or CD8 T cells proliferative response to recombinant HA Ags as shown as frequency of CFSE^{low} and the percentage of cells that are CD45RO$^+$*

			Protein					
	Medium		H1		H3		H5	
Subject	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8
003C								
Frequency	1.36	2.94	32.2	7.04	90.9	92.9	20.6	1.97
%CD45RO	46.6	26.8	92	66.9	90.5	70.8	90.5	46.9
025C								
Frequency	3.14	19.2	86.7	0.85	53.4	2.89	26.7	20.8
%CD45RO	77.7	98.8	95.6	98.9	97.2	98.8	95.1	99.6
039F								
Frequency	1.09	0.76	44.2	1.2	9.59	0.21	11.7	0.45
%CD45RO	52	38.9	93.2	77.1	91.6	63.6	92.8	76.2
237A								
Frequency	0.88	0.28	2.25	0.54	7	0.74	31	10.6
%CD45RO	18.7	50	77.5	48.8	89.4	51.7	93.3	87.1
246A								
Frequency	0.44	0.06	14.7	2.87	32.7	15.2	23.5	20.4
%CD45RO	52	100	93.2	68.2	92.6	78.4	92.8	90
255A								
Frequency	1.34	0.37	7.7	0.43	13.4	0.65	25.6	2.04
%CD45RO	89.7	54.2	95.7	53.8	95.7	55.2	89.2	68.7

Frequency of CD45RO$^+$ CD4$^+$ or CD8$^+$ T cells in CFSE^{low} lymphocytes population when stimulated with recombinant influenza antigens. PBMC were incubated with either H1, H3, or H5 at 1 μg/ml for 7 days and analyzed by flow cytometry.

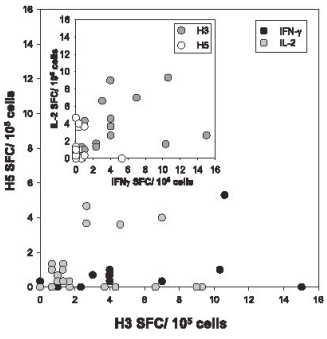

FIGURE 4. ELISPOT analysis of IL-2/IFN-γ SFC. Responses to H5 were characterized by modest levels of IL-2 and low levels of IFN-γ producing T cells consistent with an immature phenotype. *Inset* shows relatedness between IL-2 and IFN-γ production in those subjects that responded well to H3 but not H5. Cells were stimulated with 1 μg/ml protein.

subjects with a slight bias toward H5 responsiveness (Table I and supplemental Fig. 1).[5] This was in contrast to the responses observed with peptide F3 in which 11 subjects responded, but with an H1 bias (Fig. 6*D*). Dose-dependent cross-reactivity was observed with four subjects in response to the H1 peptide of both H1 and H5 HAs (Fig. 6*G*). Such was not always true, however, as titrated responses to the H1-F3 peptide were observed but not to H5-F3 (Fig. 6*J*). In general, responses seemed biased toward the H1 peptides (Fig. 6), consistent with environmental and vaccine exposure to H1 strains in the population. One of the most closely related H1 and H5 sequences, peptide E5, produced relatively moderate responses with cells from 7 of 24 subjects and strong responses to either peptide from two (Fig. 6, *F* and *L*). The peptides with a high degree of relatedness are similar to previous results (39, 51).

In comparing T cells from 24 subjects stimulated with peptides derived from various H5 mutants (Table I and Fig. 7), structural relatedness could not explain all responses. These were sometimes

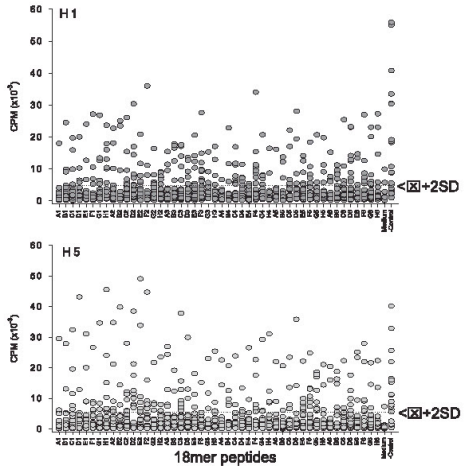

FIGURE 5. Proliferative responses by 24 subjects to 48 peptides each from H1 and H5. Negative controls consisted of medium alone and positive controls consisted of the respective recombinant protein Ag, either H1 or H5. Dotted line indicates positive threshold (mean plus 2 SDs of negative controls).

strikingly different (Fig. 7, *A–C*), ranging from widely dispersed responses (E3 vs F8 in Fig. 7*A*) to those that were markedly linear (A5 vs B10 in Fig. 7*B*). The former peptides (E3 and F8) differ by only a single residue (R15G), whereas peptides A5 and B10 differ by two residues K4R and N15S, respectively (Table I). Even a very conservative single amino acid substitution in the core of the peptide (K11R) produce significantly different results with cells from subjects 053C and 255A (H4 vs A10; Fig. 7, *F* and *I*). Thus, single amino acid changes in H5 can convert a nonresponder to a responder and visa versa. This has implications for pandemic spread of particular mutant strains.

Discussion

In a group of healthy blood donors, we have shown that the H5 HA of avian influenza virus is recognized in vitro and responses seem to correlate with responses to either the H1 or H3 strain Ags. The basis for the latter responses is thought to be vaccination and environmental exposure over recent decades; responses to H5, in contrast, are puzzling given that little epidemiologic evidence of exposure to H5 avian virus exists in the United States population. The nature of H5 responsiveness is of relevance not only to vaccination efforts (are they likely to be effective?) but also to concerns regarding the likelihood of a major avian influenza pandemic. Our focus has been on CD4, helper T cells because of their central role in promoting both B cell (neutralizing Ab) and CD8 T cell (elimination of infection) responses. Responses to H5 and the most closely phylogenetically related H1 are distinguished solely by a somewhat lower frequency of IFN-γ-producing cells, but IL-2 levels produced in response to H5 recombinant Ag appear comparable, indicative of at least the potential for an effective immune response. Using synthetic peptide from H1 and H5, we show that T cells from most individuals within our study cohort were able to respond to at least one epitope in proliferative assays and responsiveness to any given H5 epitope seemed to be mostly correlated with the related H1 peptide, suggesting the possibility of cross-recognition of phylogenetically conserved epitope sequences. Structural relatedness, on further analysis, however, could not explain the qualitative and quantitative differences we observed on an epitope by epitope basis.

In studying human immune responses in vitro, no evidence has been uncovered that human T cells can respond without previous exposure, either through vaccination or environmental contact. Thus, all data regarding human T cell responses in vitro represent secondary immune responsiveness, which is consistent with secondary challenge experiments in animals. Therefore, whereas responsiveness to H5 recombinant Ag was not anticipated, the fact that it seemed correlated with responses in most cases to previously encountered H1 and H3 was reasonable. Higher level evaluation showed that the response to recombinant H5 was mediated in vitro by helper (CD4) T cells as shown by CFSE staining and flow cytometric phenotyping; furthermore, responding CD4 T cells were of the memory subset ($CD45RO^+$) and by ELISPOT could secrete reasonable levels of IL-2 and IFN-γ, even though, as noted above, the precursor frequency of the latter was lower relative to those found with T cells stimulated with either H1 or H3 recombinant Ags. The response was class II (HLA-DR) restricted as shown by the fact that proliferation could be blocked by isotype-specific anti-HLA-DR Ab, L243, as expected. Despite the fact that all donors studied shared the HLA-DR1 and/or DR4 genes, no relationship could be observed between response patterns and HLA phenotype. Taken together, all of these data suggest that the response of human helper T cells to H5, as measured in vitro, does not

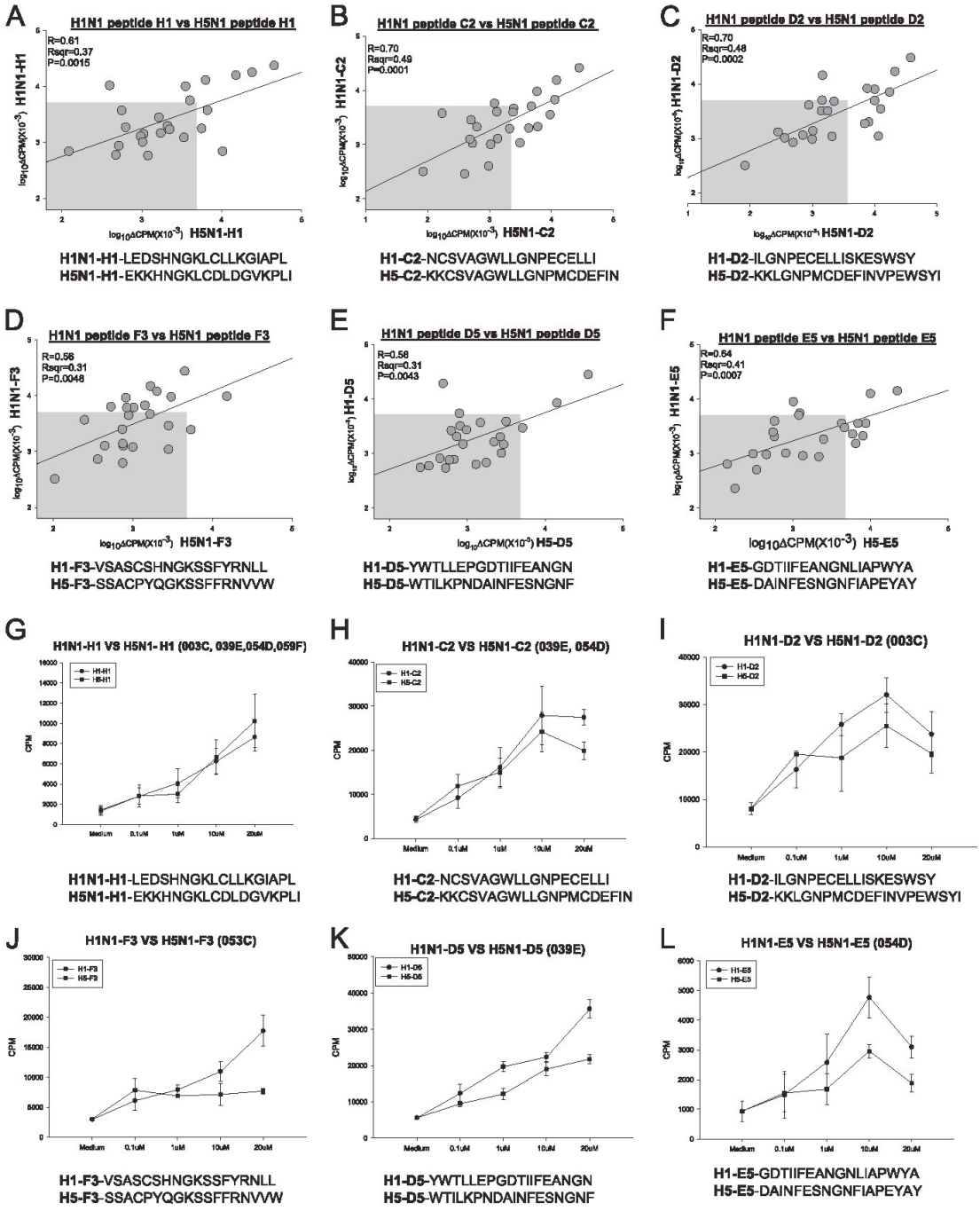

FIGURE 6. T cell proliferative response to H1 and H5 18-mer peptides are biased toward H1. Pairwise comparison of peptide specific responses by 24 subjects; peptide sequences are indicated in the graph and in Table I (*A–F*). Titration curves of H1 peptide response in comparison to corresponding H5 peptide (*G–L*). Subject identifiers are indicated in the heading of each graph. *G* and *H*, Responses by indicated subjects are presented as the mean ± SE of the pooled individual responses. Gray box indicates statistical cutoff for nonresponders. Statistical values were calculated using SigmaPlot 2000.

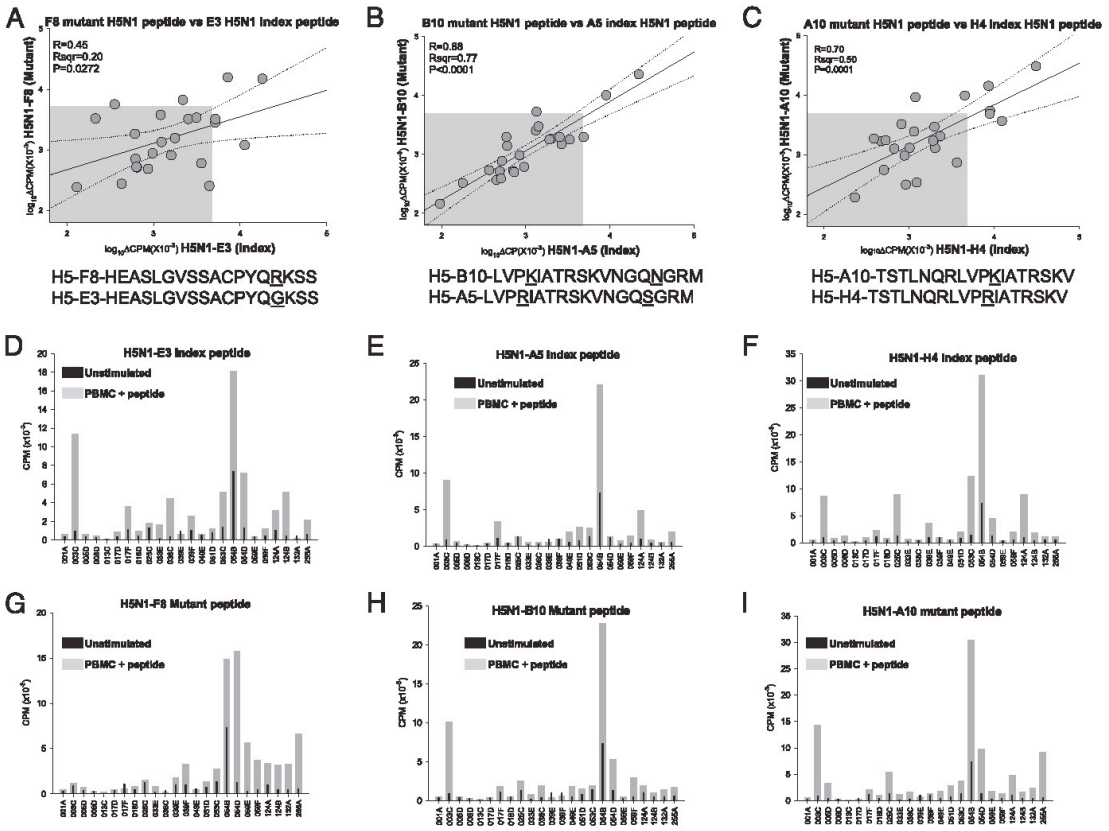

FIGURE 7. Comparison of the effects of mutations in H5 (underlined) and the index (A/Vietnam) sequence of H5; single amino acid mutations results in dramatic changes in T cell proliferation (*A–C*). Individual T cell proliferative responses to each synthetic peptide (▨) are shown for 24 subjects compared with medium controls (■) (*D–I*). Responses to A/Vietnam index peptides are shown in *D–F*; responses to mutant peptides are shown in *G–I*. Note differences in responsiveness by subjects 053C and 255A to index peptide H4 (*F*) and mutant peptide A10 (*I*).

differ significantly from responses observed with other stimulatory Ags from influenza virus or other protein Ags such as tetanus toxoid. This was unexpected because the U.S. population has not been environmentally challenged with H5 avian influenza virus convincingly.

Given the correlated responses to H1 and H5 Ags, we expected a structural basis for such comparability and sought to examine the epitope structures localized within each protein as a potential explanation. On the basis of the National Center for Biotechnology Information alignment of the HA1 segments of the A/New Caledonia H1 and the A/Vietnam H5, we synthesized 48 peptides, 18 aa in length and overlapping by 11 residues each. Peptide homology was not exact but was sufficient to provide the potential for highly similar epitopes to be found within the H5 and H1 counterparts. Significantly, there exists only a 56% homology within the HA1 region with the longest run of identity between H5 and H1 composed of 10 aa. Incidentally, peptides B1, C1, and D1 comprised part or all of this conserved region, and responses to these were undifferentiated from epitopes localized elsewhere. Although extensive homology is found within the HA2 segment reflecting greater conservation (81%), preliminary experiments with H1 and for which overlapping peptides were generated for the entire length of the molecule (both HA1 and HA2 segments) revealed few epitopes that stimulated strong proliferative responses. That is, most epitopes seemed to be localized to the distal HA1 domain wherein, perhaps coincidentally, are also located epitope sites for neutralizing Abs. It is remarkable that the HA2 region is not more immunogenic in H5 as well as H1 or H3, especially given the degree of conservation, which implies lower levels of immune selection pressure on HA2-derived epitopes, most if not all of which should be available through understood mechanisms of Ag presentation. Even in HLA-DR1 transgenic mice challenged intranasally with A/New Caledonia (52), twice as many epitopes were found to be immunogenic within the HA1 sequence (30 per 342 aa) as compared with the HA2 segment (10 per 221 aa). Furthermore, these DR1 mice could be preprimed with an intranasal inoculation of A/New Caledonia/1999 and impart cross-reactivity to a 10 μM concentration of H5N1 synthetic peptides in an in vitro ELISPOT assay counting IL-2 SFC in this same region (51). In humans, Roti et al. (39) detected H5N1 HA-specific MHC class II tetramer-positive CD4 T cells when stimulated with H5 peptides for 14 days with the addition of IL-2 at day 7 and then when needed. Although both studies, in combination, found similar CD4 T cell epitopes in accordance with our data in the HA region of both H1N1 and H5N1, both studies preprimed or "enriched" the CD4 T cell population to evaluate the CD4 T cell responses to a naive influenza strain, in this case H5N1. The approach that we sought was to evaluate the CD4 T cell responses in a cohort of

people without shifting the CD4 T cell repertoire toward either H1N1 or H5N1 to get an intrinsic portrayal of how cross-reactive CD4 T cells might be against a new strain of influenza. We speculate that lowered immune selection pressure, perhaps reflected in the lower phylogenetic variation in HA2, as well as fewer numbers of empirically determined T cell epitopes in HA2 compromise what would otherwise provide an ideal focus for synthetic vaccine development (due to extensive conservation within HA2 among viral isolates), perhaps due to sequence overlap with mouse and human proteins that have engendered tolerant T cells. Caveats to such approaches to "epitope counting" include epitope representation within the peptide query set (ours includes every possible 11-mer and would miss longer sequences) and the representation of responding T cells circulating in the peripheral blood.

We are left with a conundrum as to how H5 responsiveness has arisen. One possibility is that environmental priming has occurred contrary to the seroepidemiology. Perhaps more plausible is that exposure to other influenza viruses, other pathogens, or environmental Ags and H5 may share epitope structures and thus stimulate cross-reactive T cell responsiveness. National Center for Biotechnology Information homology searches with H5 epitopes turned up avian and other influenza sequences, as might be expected, but also sequences associated primarily with gut flora. Thus, we postulate that the presence of anti-H5 T cells may derive from environmental cross-priming, and the human in vitro T cell response to H5 may be more robust than previously expected. Regardless of their origins, such results indicate that H5-responsive precursor cells pre-exist in the human population and as such, may prove conducive to vaccination efforts.

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Disclosures

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