

Hepatitis C Virus Immune Escape via Exploitation of a Hole in the T Cell Repertoire¹

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Hepatitis C virus (HCV) infection frequently persists despite eliciting substantial virus-specific immune responses. Thus, HCV infection provides a setting in which to investigate mechanisms of immune escape that allow for viral persistence. Viral amino acid substitutions resulting in decreased MHC binding or impaired Ag processing of T cell epitopes reduce Ag density on the cell surface, permitting evasion of T cell responses in chronic viral infection. Substitutions in viral epitopes that alter TCR contact residues frequently result in escape, but via unclear mechanisms because such substitutions do not reduce surface presentation of peptide-MHC complexes and would be expected to prime T cells with new specificities. We demonstrate that a known *in vivo* HCV mutation involving a TCR contact residue significantly diminishes T cell recognition and, in contrast to the original sequence, fails to effectively prime naive T cells. This mutant epitope thus escapes *de novo* immune recognition because there are few highly specific cognate TCR among the primary human T cell repertoire. This example is the first on viral immune escape via exploitation of a “hole” in the T cell repertoire, and may represent an important general mechanism of viral persistence. *The Journal of Immunology*, 2008, 181: 6435–6446.

Chronic viral infections, such as those caused by HIV, hepatitis C virus (HCV),³ and hepatitis B virus (HBV) are among the leading causes of death in the world (1). HCV infection is a particularly important model for elucidating mechanisms of viral persistence because most HCV infections persist but some spontaneously resolve in the first year. This effect permits comparison of host responses to viral evasion tactics between those who do and those who do not control infection.

Acute HCV infection usually elicits a CD8 T cell response yet evades clearance in ~75% of infected individuals (2–4). If infection persists, the T cell responses generated during acute infection typically decline, and chronic infection is characterized by a low frequency of virus-reactive CD8 T cells in peripheral blood (2, 3,

5). The mechanisms contributing to decline of the immune response during chronic infection have not been fully elucidated, but viral evolution over the course of infection can contribute to persistence, enabling escape by mutation of key epitopes targeted by T lymphocytes. In the chimpanzee model of HCV infection as well as in humans, a strong association between viral persistence and the development of escape mutations has been demonstrated (6–8).

Evasion of the immune response via substitution within or near T cell epitopes occurs during HCV, HBV, SIV, and HIV infections via decreased MHC binding and impaired Ag processing for presentation, both of which decrease epitope density on the cell surface (7–16). Substitutions in TCR contact residues could also affect T cell recognition, but these substitutions would be expected to lead to only transient immune escape because the new epitope is still present on the cell surface and should be recognized by a distinct repertoire of T cells. *De novo* generation of CD8 T cell responses specific for some but not all escape variants has been demonstrated for HIV (17). Model systems have suggested mechanisms by which substitutions affecting TCR contact residues might prevent efficient *de novo* recognition by T cells bearing high affinity TCR in the context of a competent immune system. One possibility is that the substituted epitope is less well recognized because the initial Ag has already elicited a subset of T cells cross-reactive with but of lower affinity for the new Ag that dominate the response to the substituted epitope. Such a mechanism, which we term “repertoire fixation,” is analogous to the phenomenon of original antigenic sin that has been applied to T cell responses to reinfection with a microbial variant of the pathogen to which an individual was previously exposed (18, 19). An alternative mechanism involves generation of an epitope for which the T cell repertoire contains a paucity of high affinity TCRs, a so-called relative “hole” in the TCR repertoire. This response may occur if, for example, the peptide epitope or the peptide-MHC complex mimics

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³ Abbreviations used in this paper: HCV, hepatitis C virus; HBV, hepatitis B virus; DC, dendritic cell; CD62L, CD62 ligand; MFI, mean fluorescence intensity.

self. This mechanism was proposed to explain differential responsiveness to foreign Ag such as the synthetic polymer poly(Glu50Tyr50) (GT) by different strains of MHC congenic mice (20). Escape through impaired T cell recognition via this mechanism has not been demonstrated for infection by any pathogenic organism.

We report in this study evidence for escape by a naturally occurring mutation in a T cell epitope that only affects TCR contact without altering peptide processing or MHC affinity. We show that the naive T cell repertoire in most individuals lacks clonotypes capable of high-avidity cognate recognition of the escape epitope sequence. Our results provide evidence in humans that HCV can evade the immune response by exploiting a relative hole in the T cell repertoire.

Materials and Methods

Participants

The Risk Evaluation Assessment of Community Health (REACH) prospective study of young injection drug users in Baltimore, MD, examined the incidence and risk factors for HCV infection, as previously described (21). Participants eligible for the study were anti-HCV Ab negative, between 15 and 30 years of age, and acknowledged use of injection drugs. Participants were invited to co-enroll in a substudy of acute hepatitis C, and those who consented had blood drawn for isolation of serum, plasma, and PBMC in a protocol designed for monthly follow up. At each visit, participants were provided counseling to reduce the risks of drug use. The REACH protocol and the HCV substudy protocols were approved by the Institutional Review Boards of the Johns Hopkins Schools of Medicine and of Hygiene and Public Health. In addition, peripheral blood leukocytes were obtained from HLA-A2⁺ healthy volunteers from the general population either by leukapheresis or venipuncture according to protocols approved by the University of Washington Institutional Review Board. All subjects gave written informed consent. Donors were initially screened for HLA-A2 expression by flow cytometry of PBMC using an A2-specific mAb (BB7.2). The expression of HLA-A2 was subsequently confirmed by molecular typing in the Clinical Immunogenetics Laboratory at the Puget Sound Blood Center (Seattle, WA). Markers for infectious agent exposure (HBsAg, anti-HBc, anti-HCV, anti-HTLV-1/II, anti-HIV-1/2, HCV-PCR, HBV-PCR, HIV-PCR) were assessed by the Puget Sound Blood Center (Seattle, WA). Only HCV-, HBV-, HIV-negative donors were included for the naive population.

Hemigenomic HCV sequencing and analysis

From 140 to 280 μ l of serum or plasma, the 5.2-kb region from the 5' untranslated region to the NS3/NS4A junction was cloned as previously described (22). For each specimen, 33 clones were assigned to clonotypes by using a previously described gel-shift assay, and two clones representing the modal clonotype were sequenced, with a third clone used as needed to resolve discrepancies (23). Sequences were assembled into contigs using Aligner (CodonCode). Sequence data were obtained at the point of initial viremia and ~6 mo later, as previously described (2).

IFN- γ ELISPOT assay

HCV-specific CD8⁺ T cell responses were quantified by ELISPOT assay as previously described (24) with the following modifications. The 96-well polyvinylidene plates (Millipore) were coated with 2.5 μ g/ml recombinant human anti-IFN- γ Ab (Endogen) in PBS at 4°C overnight. Previously frozen PBMC were added at 200,000 cells/well or 30,000 T cells from lines in 140 μ l of R10 medium (RPMI 1640 (Sigma-Aldrich), 10% FCS (Sigma-Aldrich), and 10 mM HEPES buffer (Sigma-Aldrich) with 2 mM glutamine, and antibiotics 50 U/ml penicillin-streptomycin). The PBMC obtained 6 mo after initial viremia were tested for IFN- γ production in response to serial dilutions of synthetic peptides representing the viral sequence present at initial viremia (KLVALGINAV, termed L_{p5}) or at 6 mo following initial viremia (KLVAMGINAV, termed M_{p5}). Comparison of the L_{p5} and M_{p5} epitopes was performed using 10-fold dilutions of those peptides from 10 to 0.001 μ M. The plates were incubated for 20 h (PBMC) or 5 h (T cell lines) at 37°C, 5% CO₂. Plates were then washed, labeled with 0.25 μ g/ml biotin-labeled anti-IFN- γ -Ab (Endogen), and developed by incubation with streptavidin-alkaline phosphatase (Bio-Rad) followed by incubation with BCIP/NBT (Bio-Rad) in Tris buffer (pH 9.5). The reaction was stopped by washing with tap water, and the plates were dried before counting on an ELISPOT reader (Cellular Technology). The assay was performed at least in duplicate and background was not more than 15

spot-forming colonies per million PBMC. Responses were considered positive if the number of spots per well minus the background was at least 25 spot-forming colony per million PBMC (24). A control of pooled CMV, EBV, and influenza Ags termed CEF Control Peptide Pool, obtained from the National Institutes of Health AIDS Research and Reference Reagent Program, and PHA were used as positive controls (25). Responses to the CEF Control Peptide Pool were quantifiable and varied by less than 10% over time. Responses to PHA were uniformly positive.

Intracellular cytokine staining and degranulation assay

The assay was essentially performed as previously described (26, 27). Briefly, 2×10^5 T cells were stimulated for 5 h in 96-well plates using 4×10^5 peptide-pulsed T2 cells. CD107a-PE (BD Biosciences) and 10 μ g/ml brefeldin A (Sigma-Aldrich) was added at the beginning of the stimulation period. After 5 h, cells were stained for CD107a and costained for CD8. Cells were then fixed, permeabilized, and stained with Abs against IFN- γ , TNF- α , or IL-2 (BD Biosciences), using Fix/Perm and Perm/Wash solution (BD Biosciences). Cells were then washed and analyzed on a FACSCalibur flow cytometer using CellQuest software (BD Biosciences).

MHC-peptide binding assays

EBV-transformed cell lines were used as the primary sources of HLA molecules. Cells were maintained in vitro and HLA molecules purified by affinity chromatography as previously described (28). Quantitative assays to measure the binding of peptides to purified HLA A*0201 molecules are based on the inhibition of binding of a radiolabeled standard peptide (28). Briefly, 1–10 nM radiolabeled peptide was coincubated at room temperature with 1 μ M to 1 nM purified HLA A*0201 in the presence of 1 μ M human β_2 -microglobulin (Scripps Laboratories) and a mixture of protease inhibitors. After a 2-day incubation, binding of the radiolabeled peptide to the corresponding HLA*A0201 molecule was determined by capturing HLA*A0201-peptide complexes on Greiner Lumitrac 600 microplates (Greiner Bio-one) coated with the W6/32 Ab, and measuring bound cpm using the TopCount microscintillation counter (Packard Instrument). A 5-fold difference in binding is considered significant in this assay.

mRNA electroporation

The mRNA constructs containing the epitope region derived from autologous virus of subject 28 at initial viremia (KLVALGINAV, L_{p5}) or the KLVAMGINAV sequence obtained 6 mo after infection (M_{p5}) were designed. Clones with either the KLVALGINAV or KLVAMGINAV sequence served as a template. The precise composition of these PCR products was confirmed by sequencing. Optimization of viability and transfection efficiency was performed by electroporation of EBV-transformed B cells with GFP mRNA using the Amaxa electroporation system. Cultured B cells ($5\text{--}10 \times 10^6$) were washed with PBS and resuspended at $5\text{--}10 \times 10^6/100 \mu$ l in human B cell nucleofector solution. The cells were then electroporated (pulse program P016), with 5 μ g of either L_{p5} or M_{p5} mRNA derived from subject 28. The B cells were incubated for 16 h at 37°C/5% CO₂ in 20% FBS before use in intracellular cytokine secretion assays.

Bulk stimulation of PBMC

To establish CD8 T cell lines, cryopreserved or fresh PBMC ($4\text{--}10 \times 10^6$) were stimulated with 10 μ g/ml of synthetic HCV peptide and 0.5 μ g/ml of the costimulatory Abs anti-CD28 and anti-CD49d (BD Biosciences) in R20 (20% human serum) medium. Recombinant IL-2 (25 IU/ml) was added on day 2 and every other day thereafter. T cells were counted and restimulated with an equal number of irradiated allogeneic PBMC and 10 μ g/ml synthetic HCV peptide after 10 and 20 days in culture.

Induction of T cell lines from healthy, HCV-seronegative individuals and dendritic cell (DC) priming of CD8⁺ T cells from HCV exposed subjects

T cell lines were generated as previously described with some minor modifications (29). Briefly DC were derived from adherent monocytes, cultured for 5 days in Cellgenix DC medium, supplemented with 1% human serum, 800 IU/ml GM-CSF (Chiron) and 1000 IU/ml IL-4 (R&D Systems). On day 3, 1.5 ml of fresh medium, supplemented with 1600 IU/ml GM-CSF and 1000 IU/ml IL-4, was added to each well. On day 5 of the DC culture, cells were harvested, resuspended for maturation in fresh DC medium and supplemented with a cytokine mix of 800 IU/ml GM-CSF 1000 IU/ml IL-4, 10 ng/ml LPS from *Escherichia coli* O55:B5 (Sigma-Aldrich) and 50 IU/ml IFN- γ (Intermune). Parallel to the maturation phase, DC were also incubated with 10 μ g/ml peptide. DC were incubated in 6-well plates and used for T cell stimulation the following day.

On day 0 of the T cell culture, naive CD8 T cells were obtained by first depleting CD45RO⁺ cells using anti-CD45RO microbeads (Miltenyi Biotec) and LD columns, followed by a positive selection step using anti-CD8 microbeads and LS columns. This approach resulted in a >95% pure CD45RO⁻/CD8⁺ population. For subject 28, CD8⁺ T cells were isolated using anti-CD8 microbeads (Miltenyi Biotec) and LS columns. Mature, peptide-pulsed DC were washed and incubated with the CD8 cells (1×10^6 /ml) at a ratio from 1:2 to 1:10 in complete T cell medium (RPMI 1640 medium supplemented with 12.5 mM HEPES, 4 mM L-glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin (Invitrogen), 50 μ M 2-ME (Sigma-Aldrich), and 10% human serum) supplemented with 100 μ M 1-methyltryptophane (Sigma-Aldrich). Each group consisted of a minimal concentration of 1×10^6 T cells, distributed equally either on 96-well V-bottom plates or 24-well plates ($2-5 \times 10^5$ T cells/well, respectively).

On day 4 of culture, IL-7 and IL-15 (R&D Systems) was added with fresh medium at a final concentration of 5 ng/ml. Cell lines were fed every 2–3 days with fresh medium and cytokines until harvest or restimulation. For restimulation, autologous PBMC were pulsed with peptide (10 μ g/ml) for 2 h and irradiated with 30 Gy. Each T cell line was harvested, washed, resuspended in fresh medium without cytokines, and mixed with 1×10^7 irradiated PBMC in a 6-well plate (final volume 2 ml). 24 h later 2 ml of fresh medium was added containing cytokines IL-2 (50 IU/ml; Chiron), IL-7 (5 ng/ml), and IL-15 (5 ng/ml). At 7–9 days after the last restimulation the T cell lines were evaluated by tetramer staining or restimulated as described.

Tetramer staining, titration, and dissociation

Tetramer staining was routinely performed using previously optimized concentrations of MHC tetramer PE (Beckman Coulter). Both tetramers for the L_{p5} and M_{p5} variant were synthesized using the same batch of MHC monomers and controlled for equivalent labeling with PE. Approximately 1×10^5 T cells per sample were stained for 30 min at room temperature, followed by incubation with CD8 Ab (BD Biosciences). For tetramer titration, T cells were stained with graded concentrations of tetramer (25 min, 37°C), followed by staining with anti-CD3 and anti-CD8 Ab on ice. To analyze the tetramer dissociation rate, cells stained with tetramer and then were incubated for various times at 37°C with an HLA-A2 blocking Ab (clone BB7.2, 275 μ g/ml; BD Biosciences) added to prevent tetramer re-binding. At each time point an aliquot was immediately fixed using cold PBS/1% paraformaldehyde. Serial dilution of tetramer was performed using half log dilutions.

Antagonism studies

A T cell line was generated by stimulating naive T cells with the L_{p5} peptide and restimulated using the L_{p5}, M_{p5}, and the control peptide as indicated. As control peptide the sequence FVFDRLPV, which is derived from the Human Proteasome component C2 97 and has an HLA A*0201 binding capacity of 1.0 nM (30). For antagonism studies using CFSE, T cells were labeled with the optimal concentration of CFSE (Invitrogen) and stimulated and restimulated as indicated. FACS analysis for CFSE dilution was performed 5 days later.

Statistical analysis

The percentage of L_{p5} or M_{p5} tetramer-positive T cells was analyzed for each Ag using generalized estimating equations with an exchangeable correlation structure (31). Comparisons of naive and memory T cells were made using unpaired *t* test and Fisher's exact test. Differences were considered significant for values of *p* < 0.05.

Results

The HLA A*0201-restricted T cell epitope spanning positions 1406 to 1415 in the HCV NS3 protein (KLVALGINAV, NS3₁₄₀₆) was commonly recognized in our cohort of HCV-infected patients identified prospectively, with 10 of 19 (53%) HLA A*0201-positive subjects with acute HCV infection bearing T cells specific for this epitope as measured by IFN- γ ELISPOT assay (data not shown). Viral sequencing for one of our subjects who had T cells specific for NS3₁₄₀₆ (subject 28) indicated that over time this epitope had been substituted at leucine at position five with methionine to become KLVAMGINAV. This substitution has been observed in HLA A*0201-positive subjects with chronic HCV infection (32, 33). To assess the potential impact of the methionine substitution on T cell recognition, bulk PBMC obtained from Sub-

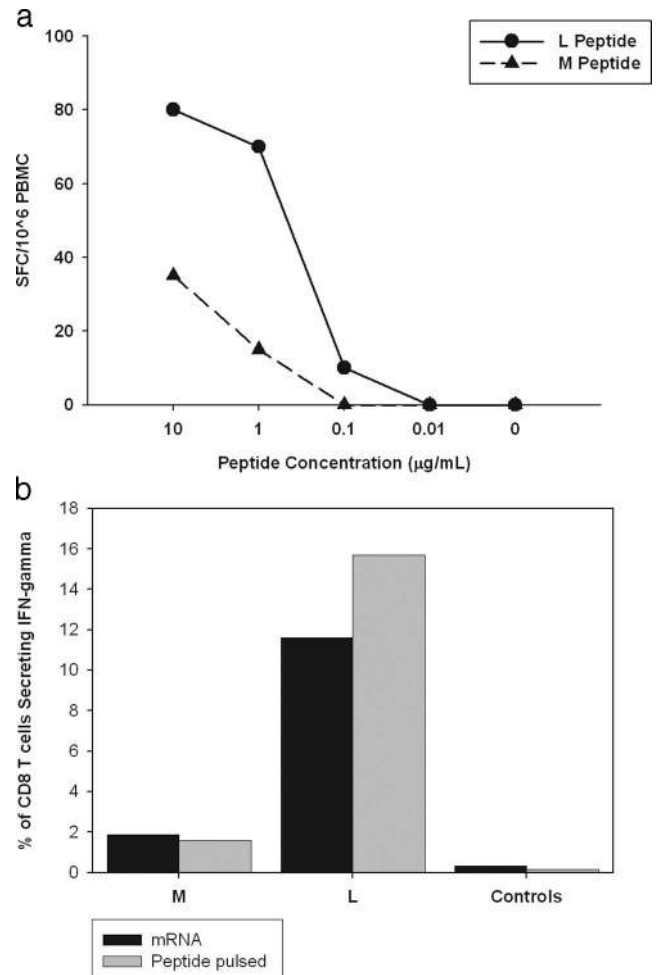


FIGURE 1. Decreased recognition of the methionine variant (M_{p5}) of the HLA A*0201-restricted T cell epitope NS3₁₄₀₆ is not due to loss of Ag processing and presentation or masking of low level responses. *a*, IFN- γ ELISpot was performed using PBMC obtained from subject 28 1 year following HCV infection when M_{p5} was the dominant form of virus. Responses for PBMC incubated with the L_{p5} peptide (●), which was present upon initial viremia, and M_{p5} peptide (▲), which was dominant 6 mo following infection, demonstrate decreased recognition of the M_{p5} variant. *b*, Constructs containing either the L_{p5} or M_{p5} sequence were introduced via electroporation into EBV-transformed cells from subject 28. Immunogenicity of the construct-containing EBV cells (■) labeled mRNA was assessed via intracellular cytokine staining for IFN- γ using NS3₁₄₀₆ specific-T cell lines generated from subject 28. As a positive control, the same T cell line was tested for recognition of L_{p5} and M_{p5} peptides pulsed onto the surface of the autologous EBV-transformed cells (▣) labeled peptide-pulsed. There was no recognition of mock-transfected EBV-transformed cell (■) controls or EBV-transformed cells not pulsed with peptide (□) controls. Recognition of the constructs as well as control constructs known not to represent processing mutations were similar to recognition when the peptides were pulsed onto the cell surface at a concentration of 0.001 M, indicating that the M_{p5} substitution did not prevent processing.

ject 28 at ~1 year after initial viremia, after the methionine-substituted variant had become the dominant viral sequence, were tested for IFN- γ production by ELISPOT in response to serial dilutions of the KLVALGINAV (L_{p5}) peptide or the KLVAMGINAV (M_{p5}) peptide (Fig. 1*a*). A 1 to 2 log left shift in the dose-response curve was observed, suggesting the substitution of leucine with methionine represented an escape mutation. Similar data were observed using PBMC obtained from subject 28 at

~6 mo after initial viremia, another point when the methionine-substituted variant was the dominant viral sequence (7).

To determine whether the decreased recognition reflected lower affinity binding by M_{p5} to HLA A*0201, quantitative assays of binding of the L_{p5} and M_{p5} variant peptides to purified HLA A*0201 were performed. These direct binding assays are based on the inhibition of binding of a radiolabeled standard peptide and, as previously described, a 5-fold or greater difference in binding capacity is significant (28). The assay demonstrated that the L_{p5} and M_{p5} peptides had comparable A*0201 binding capacity with inhibitory concentrations of 50% of 5.0 and 2.3 nM, respectively. Therefore, the markedly decreased recognition of the M_{p5} could not be explained by decreased HLA A*0201 binding. Indeed, the substitution at position five is predicted to interact with the TCR more than the MHC based on both algorithms and the crystal structure analysis of other HLA-A2-peptide complexes (34).

Impaired Ag processing cannot explain the observed decreased recognition of peptide pulsed onto the cell surface because pulsing bypasses the requirement for intracellular processing. However, it is possible that the M_{p5} substitution completely prevents Ag processing and presentation, thereby mitigating the development of a strong M_{p5} -specific response in vivo following the L_{p5} to M_{p5} epitope conversion in the patient. To determine whether the M_{p5} substitution prevents presentation, mRNA encoding either the L_{p5} or M_{p5} variant of NS3₁₄₀₆ was transfected into autologous EBV-transformed B cells to serve as APCs in an intracellular cytokine secretion assay (Fig. 1*b*). B cells transfected with the L_{p5} or M_{p5} variant mRNA both stimulated IFN- γ secretion from an NS3₁₄₀₆-specific T cell line recognizing both the L_{p5} and M_{p5} variants of NS3₁₄₀₆ similarly to B cells pulsed directly with peptide, indicating that the reduced response to the methionine substitution in vivo did not reflect the inability of the M_{p5} containing NS3₁₄₀₆ epitope to reach the cell surface and be presented to the T cell.

Because decreased MHC binding or failure to undergo processing and presentation failed to explain the reduced recognition of the methionine-substituted peptide, we investigated other mechanisms that could result in impaired T cell recognition of M_{p5} . To determine whether a minor population of T cells with greater specificity for M_{p5} were being masked by the more abundant L_{p5} -specific T cells when tested directly ex vivo, we performed in vitro restimulation of PBMC obtained from subject 28 at ~6 mo of viremia, which was after he developed M_{p5} as the dominant form of the virus. The PBMC were incubated with the M_{p5} or L_{p5} peptide for two 10-day cycles and subsequently tested for recognition of the M_{p5} or L_{p5} peptide in an ELISPOT assay for IFN- γ (Fig. 2*a*). Independent of the peptide used for restimulation, both T cell lines recognized targets pulsed with the L_{p5} peptide better than the M_{p5} peptide. To determine whether the preference for L_{p5} could be overcome by using a more potent APC, either L_{p5} - or M_{p5} -pulsed mature DC were used to restimulate and expand CD8 T cells isolated from PBMC obtained from subject 28 after the M_{p5} sequence became dominant in vivo (Fig. 2*b*). Stimulation with L_{p5} peptide-pulsed DC and subsequently PBMC resulted in a robust expansion of Ag-specific T cells from 0.6% of CD8⁺ T cells specific for L_{p5} directly ex vivo to 60% of the cells specific after three rounds of stimulation. However M_{p5} -pulsed DC did not induce a response that demonstrated greater specificity for M_{p5} than L_{p5} , despite the T cells having been exposed in vivo to M_{p5} and the magnitude of the resulting response (12%) was clearly reduced compared with the 60% of CD8 T cells specific for the Ag after L_{p5} peptide stimulation (Fig. 2*b*). Thus, regardless of the peptide used for expansion, the mode of stimulation, or the assay used to assess IFN- γ production, T cells derived from subject 28 at a time when the M_{p5} mutation dominated their HCV sequences no less consistently dis-

played better recognition of the L_{p5} peptide than did T cells derived of the M_{p5} peptide. Despite even longer in vivo stimulation with the M variant and very low precursor frequency of T cells specific for NS3₁₄₀₆, similar recognition patterns were observed when PBMC from the 1-year time point were used. For the L_{p5} expanded T cell line, the pattern of reduced cytokine production in response to M_{p5} vs L_{p5} held for T cell degranulation as well with reduced CD107a expression on the T cell surface in response to M_{p5} vs L_{p5} (Fig. 2*c*). In contrast, the M_{p5} expanded line from PBMC at the 1-year time point displayed no significant degranulation in response to cells pulsed with either Ag. Given the low precursor frequency, confirmation of the poor capacity of M_{p5} to induce T cell degranulation was obtained using T cell lines derived from another host in which there was no exposure to M_{p5} in vivo. The T cell lines could also be expanded well using L_{p5} (Fig. 2*d*). M_{p5} induced poor degranulation vs L_{p5} of T cells derived from this subject as well.

To determine whether the weaker recognition of M_{p5} observed were due to impaired engagement of TCR by the M_{p5} /A*0201 complex, the avidity of TCR in T cell lines from subject 28 for the L_{p5} or M_{p5} /A*0201 complex was analyzed. Recent analyses indicate that the dissociation rate best correlates with "functional avidity" as defined by the cytokine response of a T cell (35). Therefore, we analyzed tetramer off-rate by performing dissociation experiments in the presence of an HLA-blocking Ab to prevent rebinding of the tetramer. For cells expanded with L_{p5} , the rate of dissociation of L_{p5} tetramer from the TCR was 90-fold slower than that of the M_{p5} tetramer. The dissociation rate of the L_{p5} tetramer from cells expanded with M_{p5} remained slower with a 7-fold reduction in off-rate vs the M_{p5} tetramer (Fig. 3*a*). Regardless of the peptide used for restimulation to expand the reactive cells, the TCR of the resulting cells always bound L_{p5} tetramer better than M_{p5} tetramer. Serial dilution of tetramers also demonstrated uniformly better binding of L_{p5} tetramer (Fig. 3*b*). Thus, T cells derived from a chronically infected individual that were primed against the L_{p5} variant in vivo demonstrated a higher avidity for L_{p5} , even after the M_{p5} variant became dominant in vivo and after repeated in vitro stimulations with M_{p5} .

We next considered two distinct mechanisms that could account for these unexpected observations. M_{p5} could potentially be less well recognized because L_{p5} had already elicited a subset of T cells cross-reactive with but of lower affinity for the new Ag that dominate the response to the substituted epitope. Such a mechanism termed repertoire fixation is analogous to the phenomenon of original antigenic sin that has been applied to T cell responses to reinfection with a microbial variant of the pathogen to which an individual was previously exposed (18, 19). Alternatively, the failure to mount a better response to M_{p5} could represent poor intrinsic immunogenicity of this variant. This intrinsically reduced capacity to stimulate could result from a hole in the repertoire, defined as an absence or paucity within the primary repertoire of T cells bearing high-affinity TCR with specificity for that variant.

To investigate these potential mechanisms, we evaluated recognition of L_{p5} and M_{p5} by the primary naive T cell repertoires in HLA A*0201-positive individuals never exposed to HCV. In such HCV-naive individuals, a similar response to both L_{p5} and M_{p5} epitopes would be expected if both peptides had similar immunogenicity because repertoire fixation resulting in a preference for the L_{p5} epitope could not have occurred. In contrast, a hole in the T cell repertoire for M_{p5} would be manifest by diminished M_{p5} recognition by the HCV-naive T cell repertoire. We have recently defined optimal in vitro conditions for induction of Ag-specific T cell responses from the naive repertoire (29). CD45RO⁻CD8⁺ T cells from HCV-negative individuals were used as the responding

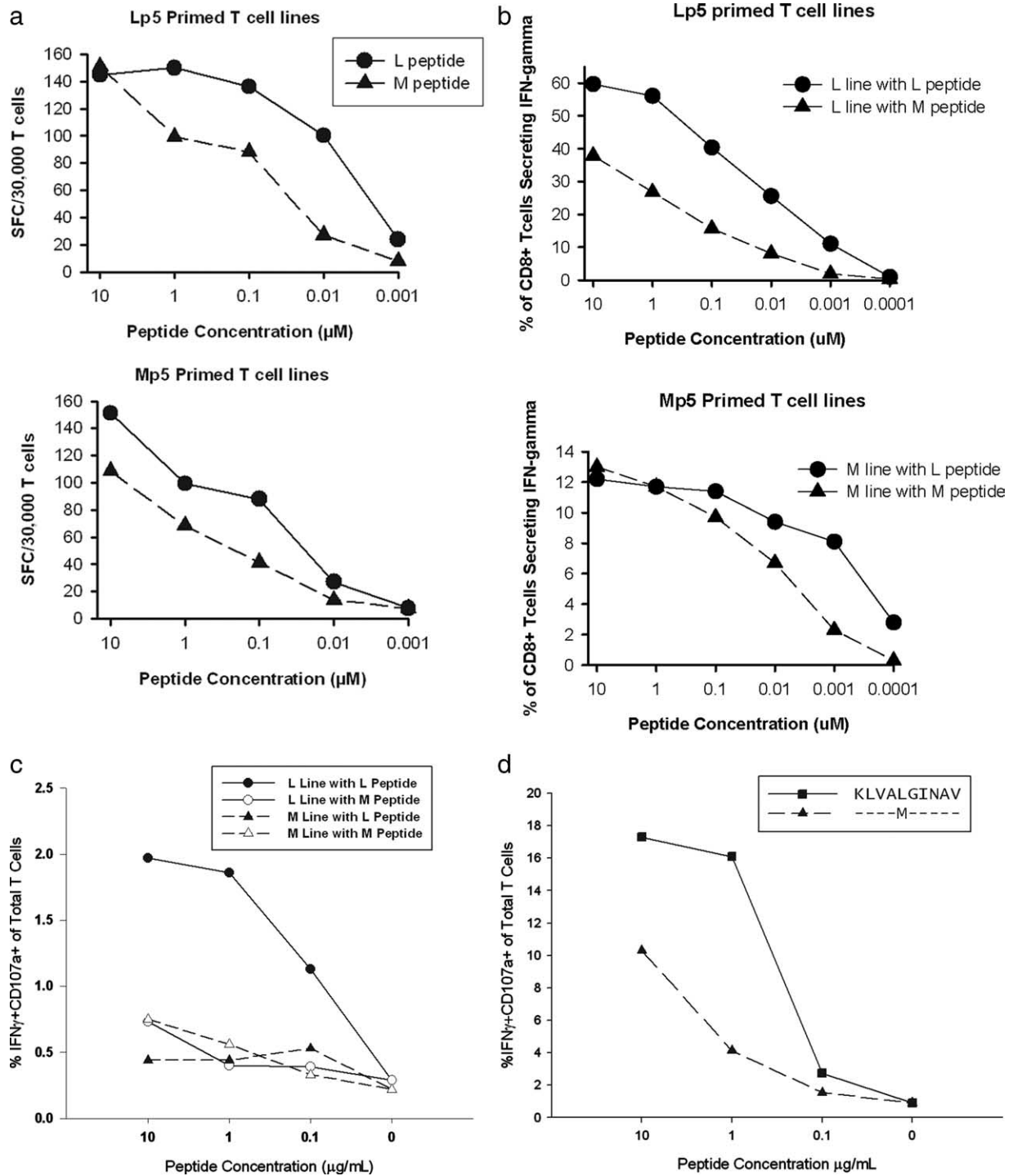


FIGURE 2. Prolonged CD8 T cell restimulation with M_{p5} peptide failed to select a T cell line that recognized M_{p5} better than L_{p5} . *a*, PBMC were obtained from subject 28 ~6 mo after initial viremia when the M_{p5} form of the virus was dominant. Those PBMC were stimulated in vitro with either the L_{p5} (*top*) or M_{p5} (*bottom*) peptide and supplemental IL-2 for two 10-day cycles and were subsequently tested for recognition of the M_{p5} or L_{p5} peptide. Independent of the peptide used for stimulation, both T cell lines recognized targets pulsed with the L_{p5} peptide better than with the M_{p5} peptide in an ELISPOT assay for IFN- γ . *b*, CD8 T cells isolated from subject 28 after he developed M_{p5} as the dominant form of the virus were restimulated with either L_{p5} -pulsed (*top*) or M_{p5} -pulsed (*bottom*) DC followed by two 7-day cycles of stimulation with autologous PBMC pulsed with the same peptides as were pulsed on the DC. The response of both the L_{p5} and M_{p5} peptide-stimulated CD8 T cells was subsequently assessed via IFN- γ intracellular cytokine staining using L_{p5} (●) or M_{p5} (▲) as Ags. L_{p5} remained better recognized. *c*, PBMC from subject 28 taken from the 1-year time point were cultured in the presence of either L_{p5} or M_{p5} peptide. The precursor frequency of T cell specific for L_{p5} or M_{p5} was extremely low at this time point. However, after two cycles of stimulation with the peptide or the variant peptide, cells were tested for recognition of both peptides as demonstrated by IFN- γ production and CD107a degranulation, in serial peptide dilutions in an intracellular cytokine assay. *d*, PBMC from an additional subject were used to generate an L_{p5} -specific T cell line as described in *c*. The T cell line recognized L_{p5} peptide better than M_{p5} peptide, as measured by IFN- γ production and degranulation.

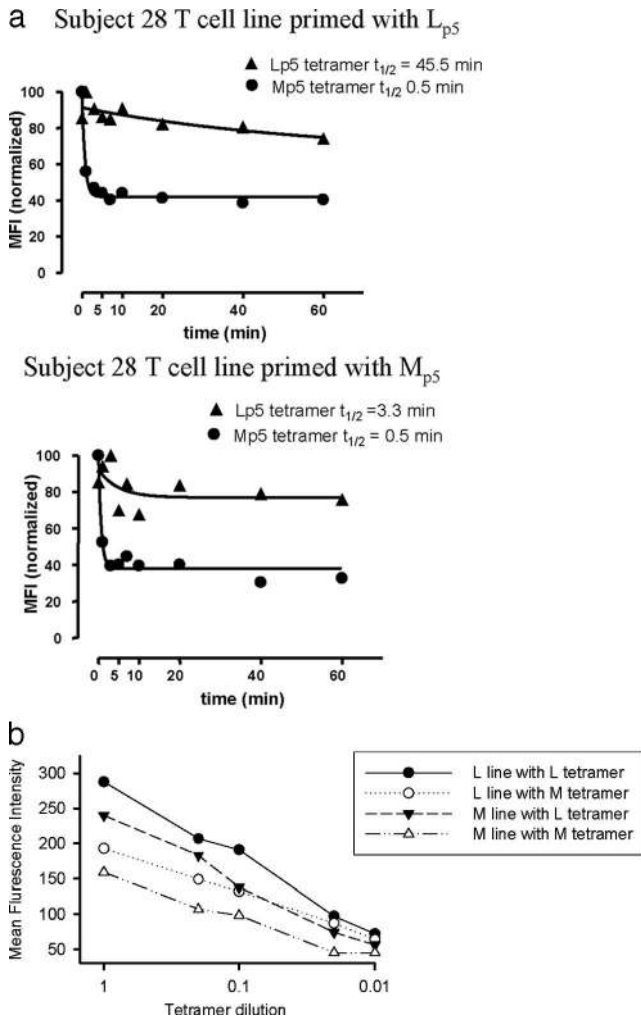


FIGURE 3. TCR from Subject 28 have decreased avidity for the M_{p5} /HLA-A*0201 complex. *a*, Binding of TCR from subject 28 to L_{p5} and M_{p5} on HLA A*0201 was assessed by dissociation rates using the T cell lines described in Fig. 2. Tetramer off-rate experiments were performed by staining L_{p5} and M_{p5} peptide-primed T cell lines with L_{p5} or M_{p5} tetramer, washing, then incubating the cells with excess HLA-A2 Ab that prevented rebinding of any tetramer that had fallen off. An aliquot of each sample was removed following 0, 1, 2, 5, 7, 10, 20, 40, and 60 min of incubation and the MFI of L_{p5} and M_{p5} tetramer bound to the TCR for each line was recorded. Data were used to determine the time it took for half the tetramer to dissociate ($t_{1/2}$) and demonstrated higher avidity of both L_{p5} - and M_{p5} -primed T cell lines for the L_{p5} tetramer than the M_{p5} tetramer. *b*, Serial dilution of tetramers were performed using half-log dilutions of tetramer and again demonstrated higher avidity of both L_{p5} - and M_{p5} -primed T cell lines for the L_{p5} tetramer than the M_{p5} tetramer.

population to the L_{p5} or M_{p5} peptides. In seven independent experiments, the outcome of priming of T cells from five different HCV unexposed donors was assessed. Assays were set-up in multiple parallel wells, and T cells were incubated with DC pulsed either with the L_{p5} or the M_{p5} peptide followed by one or two rounds of stimulation with peptide-pulsed PBMC. Peptide concentrations ranging from 100 ng/ml to 10 μ g/ml were used. The percentage of tetramer-positive cells after two cycles of stimulation resulting from each of 38 attempts to prime responses from naive T cells is indicated in Fig. 4. Because we sampled the different naive hosts a different number of times, we used a statistical method called generalized estimating equations. This statistical method determines the statistical significance based on the number

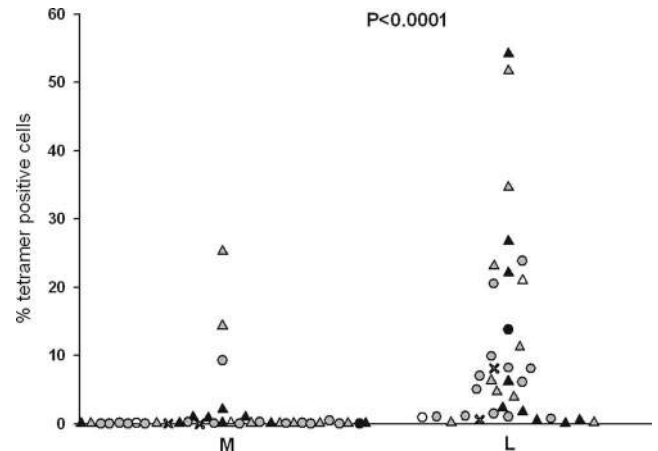


FIGURE 4. M_{p5} ineffectively primes naive T cells. $CD45RO^-/CD8^+$ (naive) T cells from HCV-negative individuals were primed under optimal conditions for generation of an Ag-specific response with L_{p5} or M_{p5} and the generation of Ag-specific responses assessed by tetramer staining. Priming was performed with five different donors in multiple experiments and in parallel groups for L_{p5} and M_{p5} . An L_{p5} -specific response was obtained in 36 of 38 cell lines and the majority of responses were vigorous. In contrast, a total of 14 of 38 lines generated using M_{p5} had a specific response for that variant as detected by tetramer staining, but nearly all were very low level responses and at the limit of detection in the assay. The L_{p5} - and M_{p5} -specific responses were compared via generalized estimating equation analysis and the L_{p5} -specific responses were significantly more frequent and of higher magnitude than M_{p5} -specific responses. $p < 0.0001$. A symbol is assigned to each individual patient.

of subjects sampled and the number of times each subject is sampled. The results demonstrate a dramatic difference between the ability of the HCV-naive T cell population to respond to L_{p5} vs M_{p5} . An L_{p5} -specific response was obtained in 36 of 38 T cell lines, with the majority of responses vigorous. In contrast, stimulation with the M_{p5} variant did not result in any detectable response in three of five donors. A response was detected by MHC tetramer staining in 14 of the 38 attempts to generate T cell lines using M_{p5} peptide for stimulation, but these responses were nearly all of very low magnitude, residing at the limit of detection of the assay. Additionally, the M_{p5} and L_{p5} peptide batches used to stimulate in the priming experiments were both reevaluated for maintenance of integrity via mass spectrometry after completion of the majority of experiments, which demonstrated that M_{p5} had not degraded and, importantly, that the failure to prime a response to M_{p5} could not be explained by M_{p5} oxidation or another chemical modification. This diminished ability of M_{p5} to prime naive T cells is unusual among peptides with maintained HLA binding capacity as demonstrated by our previous work with this assay with Melan-A, Wilms tumor Ags of multiple HLA restrictions, and an HIV gag epitope (29, 36 and M. Wolff, unpublished data). T cells capable of recognizing the heteroclitic HLA-A0201-restricted Melan-A epitope (L_{26-35}) are relatively frequent, which has allowed us to assess the robustness and reproducibility of the culture system (37). Effective use of this priming system has also been described for the Wilms tumor Ag 1, a transcription factor, which is being explored as an immunological target for leukemic cells (38). Proliferation of Ag-specific T cells specific for several WT-1 epitopes with different HLA restrictions has been detected (36). We have also evaluated the induction of T cell responses against the HLA-A0201-restricted epitope HIVgagP17/76–84 in healthy donors and were able to induce T cell responses against this epitope (M. Wolff, unpublished data). Thus, this method has been

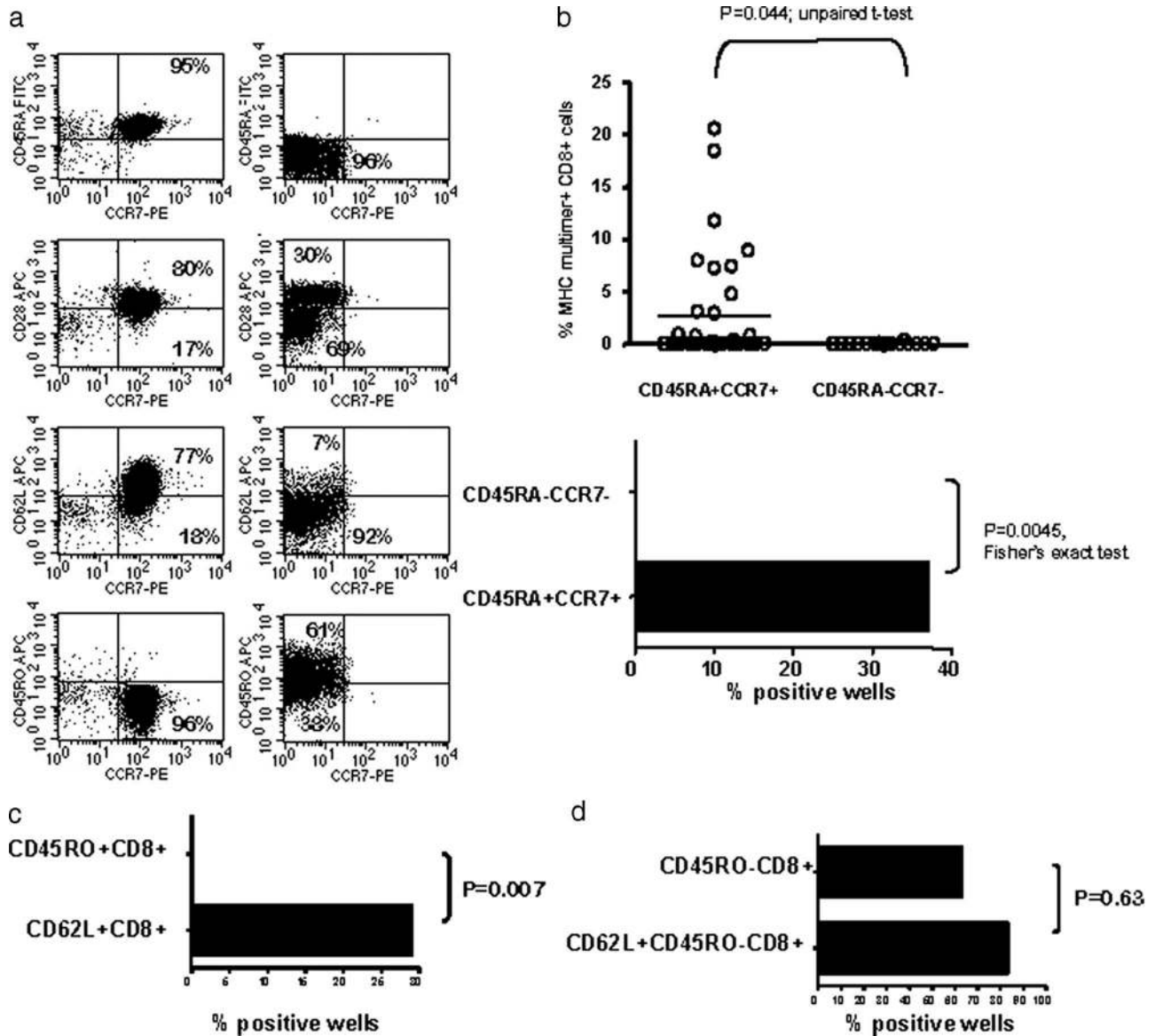


FIGURE 5. Responses to L_{p5} in HCV-naive individuals derive from the naive T cell population. *a*, Immunophenotyping of $CD45RA^+CCR7^+$ T cells sorted cells showed a high purity of $CD45RA^+CCR7^+CD28^+CD62L^+$ naive T cells (*left column*), whereas a $CD45RA^-CCR7^-$ control population was a $CD45RO^+$ memory subpopulation (*right column*). *b*, $CD45RA^+CCR7^+$ and $CD45RA^-CCR7^-$ sorted T cells were expanded and restimulated for two cycles. Only the naive $CD45RA^+CCR7^+$ population yielded a response to the L_{p5} HCV epitope, as measured by magnitude (*top*) or percent positive (*bottom*). *c*, $CD8^+$ cells were isolated by negative selection and then positively selected for CD62L or CD45RO expression. No L_{p5} -specific response was obtained from the $CD45RO^+$ memory population, whereas a statistically significant number of wells were positive in the $CD62L^+$ naive group. *d*, Cells isolated by CD45RO depletion followed by $CD8^+$ selection were compared with $CD8^+$ cells obtained from PBMC by negative selection, depleted of $CD45RO^+$ cells, then positively selected for CD62L expression. Following expansion and restimulation for two cycles, a similar number of tetramer-positive wells was detected from both populations containing naive T cells.

highly successful in priming naive T cells specific for other Ags and the lack of priming with M_{p5} is unusual.

Because the $CD45RO^+CD8^+$ population contains not only naive T cells but also $CD45RA^+CCR7^-T_{EMRA}$, a population of late effector memory cells, we performed additional purifications to confirm that the observed responses truly arose from the naive T cell population. Additional experiments using CD45RO, CD45RA, CD62 ligand (CD62L), and CCR7 as selective markers were performed. Immunophenotyping of $CD45RA^+CCR7^+$ sorted cells showed a high purity of $CD45RA^+CCR7^+CD28^+CD62L^+$ naive T cells (Fig. 5*a*, *left column*), whereas a $CD45RA^-CCR7^-$ control population could be characterized as a $CD45RO^+$ memory subpopulation (Fig. 5*a*, *right column*). When these two populations of cells were expanded and restimulated for

two cycles with L_{p5} , only the naive $CD45RA^+CCR7^+$ population yielded a response to the L_{p5} HCV epitope, which was statistically significant regardless whether the magnitude (Fig. 5*b*, *top*) or percentage of positive responses (Fig. 5*b*, *bottom*) was taken into account. Additionally, $CD8^+$ cells were first isolated by negative selection and then positively selected for CD62L or CD45RO expression. The $CD62L^+$ fraction contains both naive T cells ($CD62L^+CD45RO^-$) and central memory cells ($CD62L^+CD45RO^+$), whereas the $CD45RO^+$ fraction contains effector and central memory cells. As demonstrated in Fig. 5*c*, it was not possible to generate an L_{p5} -specific response from the $CD45RO^+$ population, whereas a statistically significant number of wells were positive in the $CD62L^+$ group, determined by Fisher's exact test ($p = 0.007$). Finally, cells isolated by CD45RO depletion

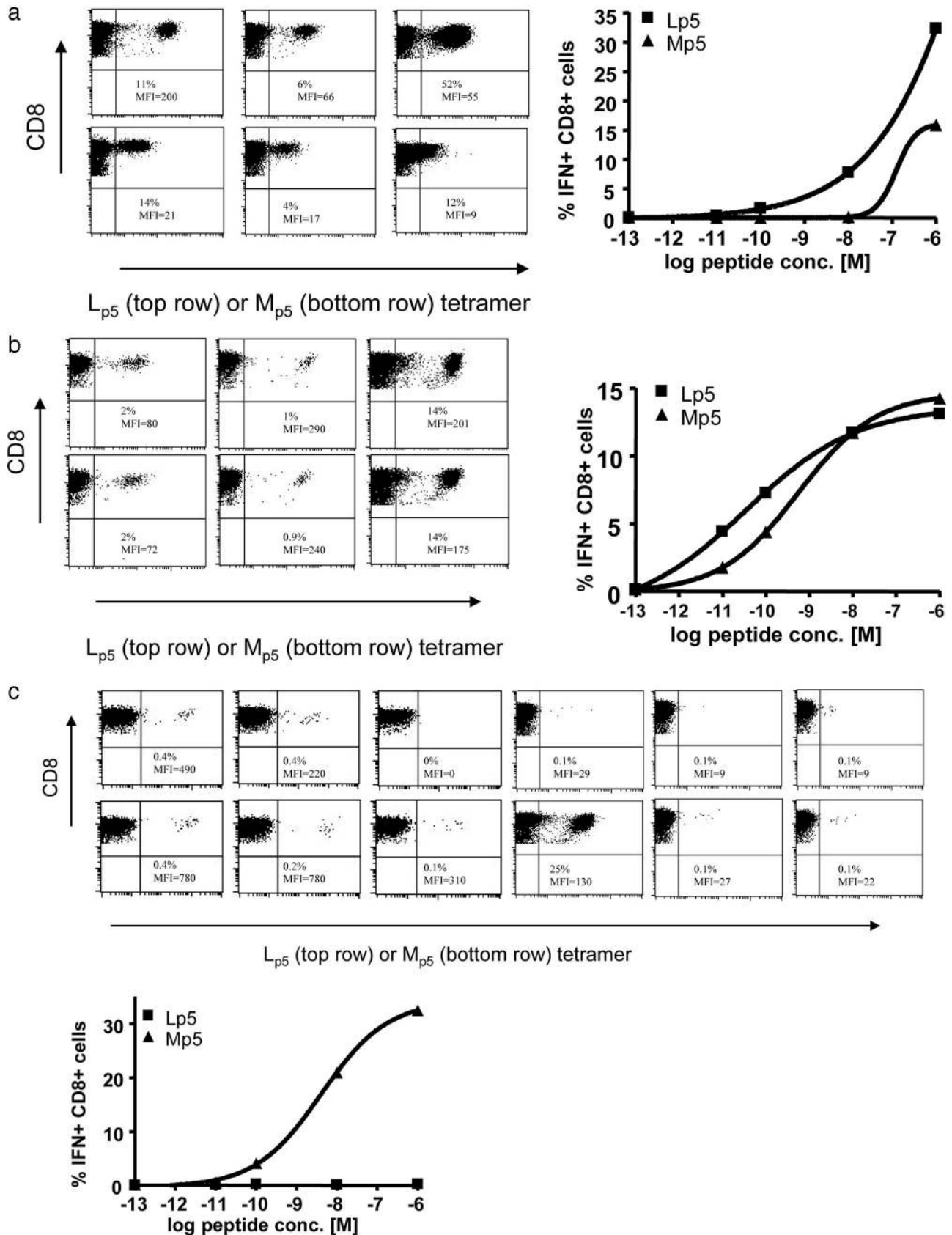


FIGURE 6. M_{p5} primes naive T cells that are primarily cross-reactive. Each T cell line shows the percentage of T cells specific for NS3₁₄₀₆ per MFI (*right lower quadrant*). The percentage of tetramer-bound cells is shown using L_{p5} (*top row*) or M_{p5} (*bottom row*). Representative intracellular cytokine staining data are shown (*right*) for one line of the FACS plots in each panel. *a*, Priming naive cells with L_{p5} peptide resulted in L_{p5} -specific responses that were cross-reactive for the M_{p5} epitope as shown for three representative cell lines. The percentage of T cells specific for L_{p5} was usually higher and the MFI was always higher for L_{p5} than M_{p5} tetramer. Although cross-reactive, better IFN- γ production was observed in response to L_{p5} vs M_{p5} . *b*, For the T cells lines generated with M_{p5} peptide, parallel staining with either M_{p5} / or L_{p5} /A*0201 MHC tetramers demonstrated that eight of the cell lines

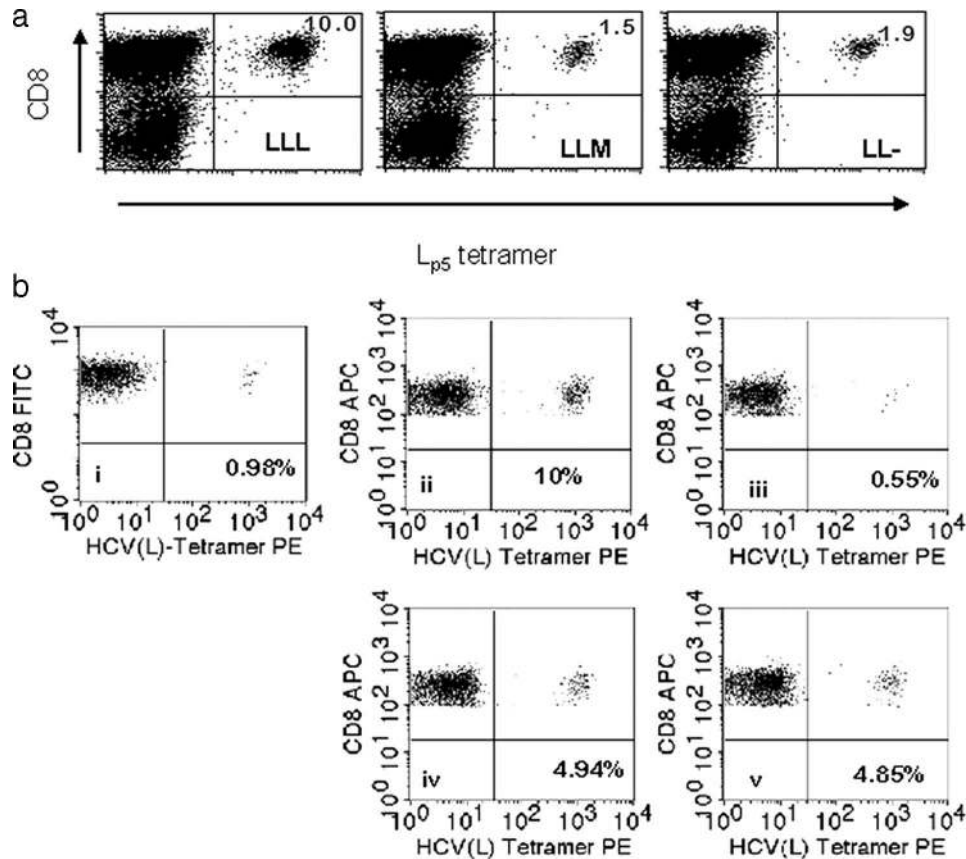


FIGURE 7. M_{p5} acts as a partial agonist and not as an antagonist. *a*, M_{p5} fails to boost an L_{p5} -specific response. Using naive T cells that had been primed with L_{p5} -pulsed DC and expanded with L_{p5} -pulsed PBMC, a third round of stimulation was performed using either L_{p5} (LLL) or M_{p5} (LLM) and compared with a third round of culture with medium alone (LL-). Both L_{p5} - and M_{p5} -bearing tetramers were used to assess the percentage of cells specific for each Ag with no significant differences in recognition pattern. The percentage of CD8⁺/ L_{p5} tetramer-positive T cells did not differ significantly between LLM and LL-, demonstrating that M_{p5} expanded-specific T cells no better than medium alone. *b*, The M_{p5} peptide did not boost the L_{p5} -specific response, but does not have an inhibitory effect relative to a control peptide of comparable HLA-A*0201 binding either. A T cell line was generated by stimulating naive T cells with the L_{p5} peptide and the percentage of L_{p5} variant-specific T cell is shown in the FACS plot (i) as 0.98%. This line was divided and restimulated with the equal number of autologous PBMC pulsed with one of four peptide combinations; L_{p5} only (ii), M_{p5} only (iii), an equal mixture of L_{p5} and control peptide (iv), or an equal mixture of L_{p5} and M_{p5} (v) are also shown. Evaluation by tetramer staining 1 wk after stimulation showed that M_{p5} peptide did not boost or inhibit the L_{p5} -specific response relative to control peptide (L_{p5} -specific T cells 4.94% vs 4.85%).

followed by CD8⁺ selection were compared with CD8 cells obtained from PBMC by negative selection, depleted of CD45RO⁺ cells, and then positively selected for CD62L expression, yielding essentially completely naive cells. When these two populations were expanded and restimulated for two cycles, a similar number of tetramer-positive wells were detected ($p = 0.63$, determined using Fisher's exact test) from both populations of naive T cells (Fig. 5*d*). In summary, stimulation of flow sorted naive CD45RA⁺CCR7⁺ T cells yielded similar results to those obtained with CD45RO⁻CD8⁺ T cells, whereas no responses were detected from the CD45RO⁺ memory population of these HCV-negative donors, confirming that the responses were elicited from priming of naive T cells.

We then assessed whether any T cells responding to M_{p5} reflect the existence of a potential M_{p5} -specific repertoire rather than just cross-reactivity to M_{p5} . Priming naive cells with L_{p5} peptide re-

sulted in L_{p5} -specific responses that generally included a population of cells cross-reactive for the M_{p5} epitope as shown for three representative lines (Fig. 6*a*, left panel). Although cross-reactive, recognition of L_{p5} was better than of M_{p5} as reflected by tetramer binding and functional analysis by intracellular IFN- γ staining (Fig. 6*a*, right panel). For the few T cell lines successfully generated with the M_{p5} peptide, parallel staining with either M_{p5} / or L_{p5} /A*0201 MHC tetramers demonstrated that 8 of the 14 lines were cross-reactive to the L_{p5} epitope (three of which are shown in Fig. 6*b*), although only six lines demonstrated a higher mean fluorescence intensity (MFI) after staining with the M_{p5} -multimer than the L_{p5} -multimer and one line showed a much larger percentage of M_{p5} -specific cells (25% vs 0.1%), indicating that these cells were more specific for M_{p5} . Assessment of the functional response (IFN- γ production) of the one T cell line displaying a high percentage (25%) of M_{p5} tetramer-positive cells demonstrated that

were cross-reactive to the L_{p5} epitope, displaying a similar or higher MFI, when stained with the L_{p5} multimer, as shown for three representative lines. The percentage of T cells specific for L_{p5} and M_{p5} and the MFI were usually equivalent. Responses vigorous enough to assess for cytokine production were very rare, but better or equivalent IFN- γ production was observed in response to M_{p5} as compared with L_{p5} for the lines that recognized both. *c*, Six of the 14 lines generated by M_{p5} priming demonstrated a higher MFI after staining with the M_{p5} multimer than the L_{p5} multimer and one line a much larger percentage of M_{p5} -specific cells (25% vs 0.1%). This result suggests these lines were more specific for M_{p5} and where assessed, also produced more IFN- γ in response to M_{p5} .

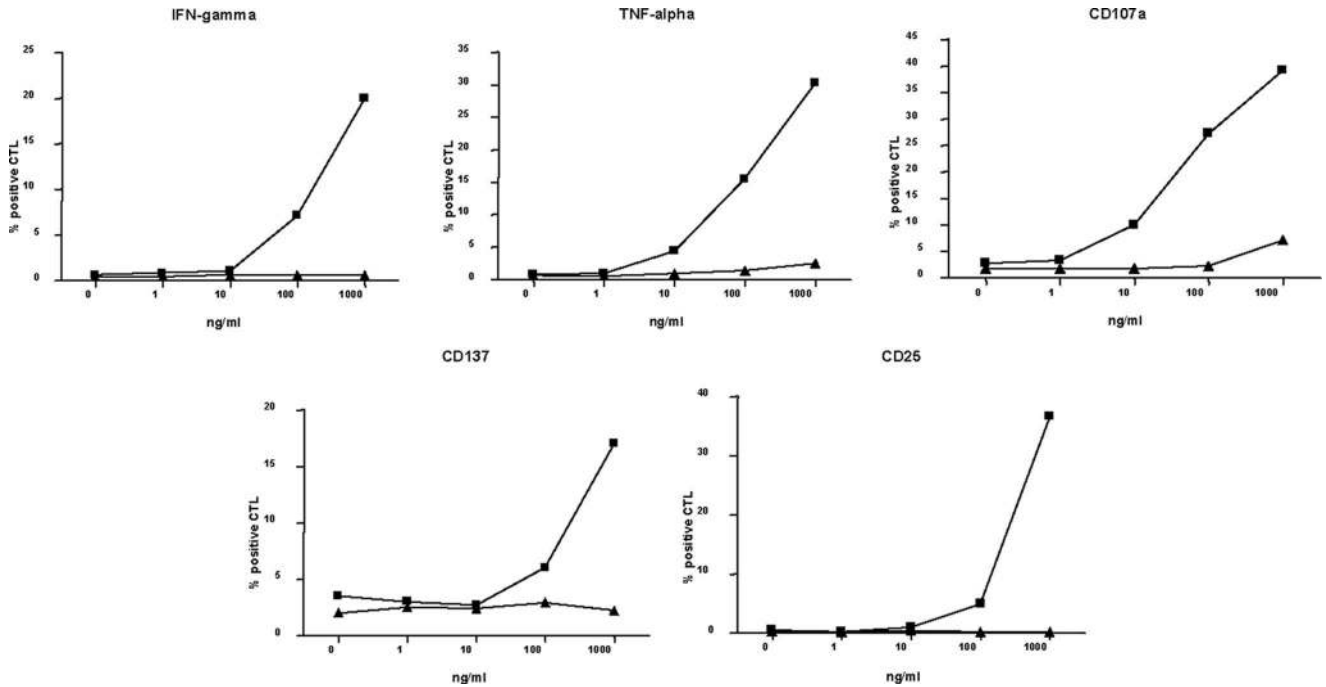


FIGURE 8. Priming naive cells with L_{p5} peptide resulted in L_{p5} -specific responses that were very weakly cross-reactive for the M_{p5} epitope. ■, L peptide; ▲, M peptide. Data are shown for three representative lines in Fig. 6a. Although cross-reactive, better IFN- γ (shown in Fig. 6a) and TNF- α production, degranulation (CD107a), and up-regulation of activation markers (CD137, CD25) was observed in response to the L_{p5} priming as compared with M_{p5} .

these cells exclusively recognized the M_{p5} variant, revealing the existence of very rare truly M_{p5} -specific T cells (Fig. 6c).

That priming of M_{p5} -specific cells was detectable as a rare event demonstrates that the experimental system is sensitive enough to detect such rare responses in the few instances when such cells are present. The highly significant difference ($p < 0.0001$) in the capacity to prime naive T cells from HCV unexposed individuals with the M_{p5} peptide compared with the L_{p5} peptide (Fig. 4) indicates that variant virus containing M_{p5} is highly inefficient at priming naive T cells relative to the virus with the L_{p5} epitope. Thus, in the setting of viral infection, the variant virus containing the M_{p5} mutation can exploit this relative hole in the T cell repertoire in most individuals as an escape mechanism.

After demonstrating that naive T cells were poorly primed by the M_{p5} variant, we next asked whether the M_{p5} variant also failed to boost previously primed responses. There was evidence of poor capacity of M_{p5} to expand preexisting responses to L_{p5} in vivo because viral replacement of leucine with methionine at position 1410 in subject 28 was associated with eventual loss of T cell responses to L_{p5} and M_{p5} . Responses to both peptides declined after the replacement and became undetectable by ~460 days following infection. This decline might at least in part be explained by the possibility that the methionine substitution fails to boost an L_{p5} -specific response as well as fails to prime cells with greater M_{p5} specificity. Indeed, T cells from naive individuals primed and then boosted in vitro with the L_{p5} peptide completely failed to be boosted by the M_{p5} peptide (Fig. 7a). In addition to a failure to expand tetramer-positive T cells, M_{p5} boosting resulted in reduced functional activation in response as assessed by intracellular IFN- γ and TNF- α cytokine staining, CD107a mobilization shift assay for degranulation, and up-regulation of the activation markers CD137 and CD25 (Fig. 8).

Because the M_{p5} variant had poor capacity to activate and boost, we next asked whether TCR engagement of M_{p5} on HLA A*0201 is not only less stimulatory but actually inhibitory, a mechanism that has been described as antagonism for altered peptide ligands

of other viral epitopes (32). Because the L_{p5} HCV variant was present first in vivo, we assessed the effects of M_{p5} on expansion of a previously L_{p5} peptide-primed T cell line. We found that L_{p5} -specific lines were not inhibited at all when the M_{p5} peptide was mixed with L_{p5} in restimulation assays relative to mixture with control peptide (Fig. 7b). The failure of the M_{p5} peptide to inhibit stimulation with L_{p5} was observed in multiple formats of in vitro restimulation using either expansion of tetramer-positive cells or CFSE dilution as readouts (data not shown and Fig. 7). Based on these findings, we conclude that there is no clear evidence for an antagonistic function of the M_{p5} peptide. However, altered peptide ligands can act as partial agonists in some settings and as antagonists in others so the possibility that M_{p5} may have antagonistic function under certain conditions cannot be excluded (39).

Discussion

We identified an escape mutation that arose in vivo in a commonly recognized HCV peptide epitope involving a primary TCR contact residue that could not be explained by impaired HLA binding or loss of processing. A paucity of reactivity against the substituted M_{p5} epitope was consistently observed in HCV naive individuals, all of whom mounted robust responses to the original L_{p5} epitope, suggesting that immune escape via conversion to M_{p5} is exploiting a relative hole in the T cell repertoire. This represents the first example of a viral escape substitution resulting in evasion of the T cell response via exploitation of a hole in the T cell repertoire. Although the hole in the repertoire is not absolute, the paucity of T cell lines with even modest recognition of the M_{p5} peptide that could be generated from five HCV-naive individuals using the M_{p5} peptide suggests the frequency of cells bearing a TCR that can react with this peptide is exceedingly small. Given the diversity and complexity of the TCR repertoire, "absence" of reactivity among the total T cell population is never absolute but rather a spectrum of frequencies and affinities, and will differ between individuals despite sharing the same restricting allele because of

different genetic backgrounds as the source for self-peptides. Numerous examples exist of residual T cells with low affinity and frequency that are specific for self-Ags, even for those Ags shown to be expressed in the thymus. The overall difference in reactivity between the L variant and M variant peptides among the HCV naive subjects is dramatic and highly statistically significant. The host TCR repertoire would be expected to include only a few T cells specific for the M variant if the peptide mimics self or has a conformation that prevents TCR interaction. No self-peptide homologs to M_{p5} were detected in a search of the NR, RefSeq and SwissProt databases, but self-mimicry is often determined by the three-dimensional structure of the peptide-MHC complex and thus cannot generally be predicted based on the primary sequence of the peptide (40, 41). Thus, we could not determine from these studies whether the relative hole in the repertoire is the result of self-mimicry (with resultant tolerance induction) or the failure to generate a primary TCR repertoire specific for M_{p5} because of its conformation.

Despite having assessed and not detected antagonism in a number of in vitro formats, antagonism may be operative in vivo as there is no definitive assay that defines the absence of antagonism. However, the results from our analysis of responses in the HCV naive subjects clearly demonstrate a significant hole in the repertoire independent of any antagonistic effects of the M variant peptide for L-specific responses. Similarly, the results in naive hosts suggest that a hole in the repertoire is exploited for escape rather than that repertoire fixation limits the immune response. Nonetheless, the repertoire fixation and repertoire hole mechanisms are likely not mutually exclusive in vivo and may operate simultaneously. Preexisting repertoire fixation may further decrease the response to an epitope that has relatively few TCR with specificity for it and a paucity of TCR specific for an epitope may enhance repertoire fixation by decreasing competition for expansion with T cells primed to an epitope present earlier.

T cell escape mutations resulting in complete abrogation of HLA binding or disruption of processing will not be present on the surface of the cell and are thus invisible to the immune system. In contrast, substitutions that result in decreased recognition but the continued presence of the viral peptide on the surface of the infected cell, such as those that affect TCR recognition or those that decrease but do not abrogate HLA binding, remain immunologically important because they still represent targets against which host responses can be generated and could be targeted by vaccines or that could be potentially amplified via manipulations that block T cell inhibitory pathways (42, 43). Understanding mechanisms for escape via mutation of TCR contact residues will facilitate development of vaccines containing Ags that stimulate cross recognition of Ags still present on the cell surface while avoiding amino acid substitutions in the vaccine strain that reduce T cell priming and recognition.

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Disclosures

The authors have no financial conflict of interest.

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