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Mutations in a Dominant Nef Epitope of Simian Immunodeficiency Virus Diminish TCR:Epitope Peptide Affinity but not Epitope Peptide:MHC Class I Binding

Evan M. Cale,* Heidi S. Bazick,* Tony A. Rianprakaisang,* S. Munir Alam,†,‡ and Norman L. Letvin*

Viruses like HIV and SIV escape from containment by CD8+ T lymphocytes through generating mutations that interfere with epitope peptide:MHC class I binding. However, mutations in some viral epitopes are selected for that have no impact on this binding. We explored the mechanism underlying the evolution of such epitopes by studying CD8+ T lymphocyte recognition of a dominant Nef epitope of SIVmac251 in infected Mamu-A*02+ rhesus monkeys. Clonal analysis of the p199RY-specific CD8+ T lymphocyte repertoire in these monkeys indicated that identical T cell clones were capable of recognizing wild-type (WT) and mutant epitope sequences. However, we found that the functional avidity of these CD8+ T lymphocytes for the mutant peptide: Mamu-A*02 complex was diminished. Using surface plasmon resonance to measure the binding affinity of the p199RY-specific binding. We explored the mechanism underlying the evolution of such epitopes by studying CD8+ T lymphocyte recognition of epitope peptide:MHC class I binding. However, mutations in some viral epitopes are selected for that have no impact on this tions that impart a selective advantage on viruses facing this

CD8+ T lymphocytes play a critical role in controlling the replication of HIV-1 and SIV in infected individuals. CD8+ T lymphocytes are capable of limiting HIV-1 replication in vitro (1, 2). This CD8+ T lymphocyte function is most striking in PBMCs of HIV-1 controller subjects (3). Moreover, the in vivo expansion of an oligoclonal population of virus-specific CD8+ T lymphocytes is associated with early viral clearance in HIV-1–infected humans (4, 5) and in SIV-infected rhesus monkeys (6, 7). Finally, in vivo Ab-mediated depletion of cells expressing CD8 in SIV-infected rhesus macaques is associated with a loss of control of viral replication and rapid disease progression (8). This series of observations makes a compelling case for the importance of these cells in HIV-1 containment.

The intense pressure exerted on HIV-1 and SIV by epitope-specific CD8+ T lymphocytes results in the selection of mutations that impart a selective advantage on viruses facing this cellular immune response. Virus escape from CD8+ T lymphocytes was first demonstrated in the early 1990s in HIV-1–infected individuals (9–13), and growing evidence of this phenomenon led to the conclusion that the CD8+ T lymphocyte-mediated selection of mutations is a hallmark of HIV-1 infection (14). Selection for mutations in MHC class I-restricted epitopes has been demonstrated during acute (15–18) and chronic (11, 19–21) stages of HIV-1 and SIV infection. In an AIDS vaccine study in rhesus monkeys, virus escape from virus-specific CD8+ T lymphocytes resulted in the failure of a vaccine-induced cellular immune response to control virus replication (22). These observations highlight the tremendous obstacles that viral escape from CD8+ T lymphocyte recognition imposes on designing effective HIV-1 vaccines based on cellular immunity.

A number of mechanisms have explained how mutations in MHC class I-restricted epitopes allow viruses to evade CD8+ T lymphocyte responses. The most common mechanism is decreased binding of mutated epitope peptides to MHC class I molecules (11–13, 20, 22–28), resulting in the failure of virus-infected cells to present epitope peptides on their surface. Other mutations, usually those that immediately flank the epitope sequence, interfere with normal intracellular peptide processing by altering proteasomal processing efficiency (25, 29), by interfering with the actions of aminopeptidases responsible for trimming the N-terminal end of the epitope peptides (30), or by inhibiting normal association of the epitope peptides with TAP. Finally, some mutations were shown to alter TCR recognition of the epitope peptide:MHC class I (pMHC) complex on the surface of infected cells (13, 31–37), resulting in suboptimal CD8+ T lymphocyte responses to mutated epitopes and even antagonistic cellular responses to the wild-type (WT) epitopes.

Many investigators who reported a reduced functional capacity of CD8+ T lymphocytes, when stimulated with altered epitope peptides in vitro, simply presumed that epitope-escape mutations

*Division of Viral Pathogenesis, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215; †Department of Medicine, Duke Human Vaccine Institute, Duke University School of Medicine, Durham, NC 27710; and ‡Department of Pathology, Duke University School of Medicine, Durham, NC 27710

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Address correspondence and reprint requests to Dr. Norman L. Letvin, Division of Viral Pathogenesis, Beth Israel Deaconess Medical Center, 330 Brookline Avenue, ECLS 1043, Boston, MA 02215. E-mail address: nletvin@bidmc.harvard.edu

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Abbreviations used in this article: DRM, detergent-resistant microdomain; ITM, isoleucine-to-methionine substitution at position 7; ITT, isoleucine-to-threonine substitu- tion at position 7; βm, β2-microglobulin; GOG, octyl-β-D-glucopyranoside; pMHC, epitope peptide:MHC class I; S3L, serine-to-leucine substitution at position 9; SPR, surface plasmon resonance; T2S, threonine-to-serine substitution at position 2; WT, wild-type; YRF, tyrosine-to-phenylalanine substitution at position 9.

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alter the CD8+ T lymphocyte TCR affinity for mutant pMHC complexes (17, 38–41). Although this hypothesis provides a mechanistic explanation for how these epitope mutations may impart a selective advantage for viruses, it remains to be demonstrated that the altered functional profiles of virus-specific CD8+ T lymphocytes are, in fact, due to altered affinities of epitope-specific TCRs for mutant epitope pMHC complexes. A number of investigators have attempted to directly address the strength of the interaction of different epitope pMHC complexes for cognate TCRs using surface plasmon resonance (SPR) technologies, but these studies have been limited to measuring the interactions of only one or a few cloned TCRs (35, 42). Recent advances for studying TCR binding to pMHC complexes using SPR have been facilitated by the introduction of the Biosensor L1 chip, which can directly immobilize lipid bilayers on the sensor surface (43). Purified detergent-resistant microdomain (DRM) preparations from T lymphocytes, which are enriched in TCRs and their associated signal-transduction molecules (44–47), can be applied to the L1 chip, effectively immobilizing TCRs from purified CD8+ T lymphocytes for SPR analysis of binding to epitope pMHC ligands. A major advantage of this technique is that one does not need prior knowledge of the clonal composition of the TCR repertoire to carry out this type of analysis. Therefore, this technique can be used to determine the average binding affinity of a particular epitope pMHC complex for the entire bulk repertoire of epitope-specific TCRs from CD8+ T lymphocytes sampled ex vivo.

We recently characterized the coevolution of the immunodominant SIV Nef epitope p199RY (YTSGPGIRY) and the p199RY-specific CD8+ T lymphocyte population in a cohort of SIVmac251-infected rhesus monkeys that express the p199RY-restricting MHC class I allele Mamu-A*02 (48). Using 454 sequencing technology, we demonstrated that virus-escape mutations were selected before day 21 postinfection and that mutant viruses completely replaced those harboring the WT epitope sequence in chronic infection. Tetramer-binding studies indicated that the p199RY-specific CD8+ T lymphocytes in these monkeys cross-recognized the WT epitope sequence and two mutations, yet these polyclonal specific cells were not capable of containing viral evolution.

In the current study, we further characterized the evolution of the p199RY-specific CD8+ T lymphocyte response in SIVmac251-infected Mamu-A*02+ rhesus monkeys by defining the clonal composition of these cells. Furthermore, we evaluated possible mechanisms to explain how mutant viruses can persist in the presence of the p199RY-specific CD8+ T lymphocyte response by measuring the binding affinity of mutant epitope peptides for Mamu-A*02 and measuring the proliferative, cytotoxic, and cytokine-producing responses of p199RY-specific CD8+ T lymphocytes upon in vitro stimulation with the WT and mutant epitope peptides. Finally, we applied a novel SPR technique for measuring the equilibrium binding of WT and mutant peptide:Mamu-A*02 complexes to Biacore L1 chip-immobilized TCR-enriched DRMs prepared from PBMCs sampled from SIVmac251-infected Mamu-A*02+ rhesus monkeys. These studies demonstrated how mutant viruses can persist in the presence of CD8+ T lymphocyte populations that are capable of recognizing mutant epitope sequences. Moreover, these results raise important implications concerning how novel vaccines aimed at eliciting CD8+ T lymphocyte responses with broad epitope-sequence recognition should be evaluated for both breadth of recognition of potential epitope mutations and for functional efficacy in preventing the emergence of mutated viruses that evade the CD8+ T lymphocyte response.

### Materials and Methods

**Animals and SIVmac251 challenge**

All animals used in this study were Indian-origin rhesus monkeys (Macaca mulatta). They were housed in accordance with the guidelines of the Institutional Animal Care and Use Committee for Harvard Medical School and the Guide for the Care and Use of Laboratory Animals (49). Monkeys were challenged with a 1/3000 dilution of uncloned SIVmac251 by the i.v. route (50).

**Peptides and oligonucleotides**

Synthetic peptides were obtained at >95% purity from Quality Control Biosciences or New England Peptide. These peptides included the WT SIVmac251 Nef p199RY (YTSGPGIRY), p199RY epitope-sequence variants T2S (YTSGPGMRY), Y9F (YTSGPGIRF), Y9A (YTSGPGIRF), Y9S (YTSGPGIR), the WT SIVmac251 epitope Nef p56 (YTYYEAVRY), and an irrelevant peptide p11B (ALSEGCTPYDIN). High-performance liquid chromatography-purified oligonucleotides were obtained from Bio-source International or Invitrogen.

**Monomers, tetramers, and Abs**

WT, T2S, and Y9F p199RY peptide:Mamu-A*02 monomers and tetramers were constructed as previously described (48) and conjugated to PE-labeled or allophycocyanin-labeled streptavidin (ProZyme). The following Abs were used in the study: anti–CD8α-FITC (clone SK1), anti–CD3–allophycocyanin (SP34.2), anti–CD3–PE-Cy7 (SP34.2), anti–CD4–PerCP/Cy5.5 (L200), anti–CD8α–allophycocyanin-Cy7 (SK1), anti–CD3–Pacific Blue (SP34.2), anti–IFN-γ–PE-Cy7 (B27) (all from BD Biosciences), and anti–CD69–ECD (TP1.55.3; Beckman Coulter).

**Immunofluorescence purification of Mamu-A*02 protein**

An immunofluorescence coupled to the anti-MHC class I Ab clone W6/32 was prepared. Briefly, Protein A-Sepharose (all reagents used for immunofluorescence purification of Mamu-A*02 were from Sigma, unless otherwise noted) was hydrated in borate buffer and applied to a 1.5-cm-diameter Kontes Flex column (Fisher Scientific) to form a 10-ml bed. Two to three mg/ml of bed volume of W6/32 Ab (custom prepared by Strategic Biosolutions) resuspended in borate buffer was added to the column, allowed to incubate for 1 h, and allowed to drain. The column was washed with borate buffer until the OD280 of the eluate was <0.02, washed with 200 mM triethanolamine (pH 8.2), incubated for 45 min in 20 mM di-methyl pimelimidate/200 mM triethanolamine (pH 8.2), and drained. The column was washed twice with 20 mM ethanolamine (pH 8.2), incubating for 5 min between washes, and then washed with borate buffer, followed by 0.02% sodium azide/PBS. Mamu-A*02 transfectedants of the MHC class I-deficient human cell line 721.221 were lysed in a buffer containing 2 mM PMSF, 1% Nonidet P-40, 20 mM Tris-base, and 150 mM sodium chloride. Lysates were centrifuged for 20 min at 25,000 × g, and the supernatant was filtered through a 0.8-μm filter. This preparation was sequentially passed through 10-ml Kontes Flex columns containing Sepharose CL4B and Protein A-Sepharose, both of which were hydrated in wash buffer containing 10 mM Tris-base (pH 8.0) and 1% Nonidet P-40. The columns were stripped with elution buffer containing 50 mM diethylamine in 150 mM sodium chloride (pH 11.5), 1% octyl-β-D-glucopyranoside (OGP), and 0.02% sodium azide (Fisher Scientific) and neutralized with wash buffer. The lystate was again passed through each column and then passed twice through the W6/32 immunofluorescence column, and this column was washed with wash buffer. The final column wash with 0.4% OGP, the Mamu-A*02 protein was eluted with elution buffer while maintaining the eluate at pH 7.0–7.5. The eluted Mamu-A*02 was concentrated using Centriprep YM-30 Filter Units (Millipore) and stored at −20°C in 50% glycerol.

**Mamu-A*02:peptide–binding assay**

WT p199RY peptide was subjected to chloramine T-catalyzed iodination (all reagents used for the binding assay were from Sigma, unless otherwise noted). Briefly, 12.5 μl of 8 μg/ml peptide diluted in 0.05% Tween 20 was mixed with 5 μl of 0.2 M NaClO (1% [v/v] MP Biomedicals) and then mixed with 5 μl of 100 μg/ml chloramine T, followed by 5 μl 100 μg/ml sodium metabisulfite, with a 1-min incubation between the addition of each reagent. Twenty-five microliters of 0.05% Tween 20 was added, and the labeled peptide was filtered through PBS-hydrated Sephadex G10 using columns provided in the Axygen Multi-Spin Separation Kit (Fisher Scientific).
The TCR repertoire and clonality of Nef p199RY-specific CD8+ T lymphocytes was determined in a test assay using SYBR Green to determine GGTATCAACGCAGAGT, and the following conditions: 95°C for 15 s, according to the manufacturer’s directions. The resulting cDNA libraries underwent PCR preamplification for 10–28 cycles using the Advantage 2 PCR Enzyme System (Clontech), Preamp Primer (5′-AAGCAGT-GGTATCAACGCAGAGT-3′), and the conditions following: 95°C for 15 s, 65°C for 30 s, and 68°C for 6 min. The number of cycles of preamplification was determined in a test assay using SYBR Green to determine the maximum number of cycles that amplify cDNA in the log-linear amplification range (51).

Quantitative PCR of the TCR VB gene region
cDNA from each preamplified cDNA sample was equally distributed into 46 individual wells of a 96-well PCR plate. Each reaction mixture contained 1 μl of the cDNA (equally-distributed from the preamplification cycle), 0.2 μM family-specific primers (listed in Ref. 52), the VB9 probe (52), CBQ reverse primer (52), ROX normalizer dye, and SureStart Taq DNA polymerase (Stratagene). Real-time PCR was carried out on preamplified cDNA for 50 cycles using the MX4000 Multiplex Quantitative PCR System (Stratagene) under the following conditions: 95°C for 10 min and 50 cycles of 95°C for 10 s, 58°C for 30 s, reading of fluorescence, and 72°C for 30 s.

Sequencing of the TCRβ CDR3 region
The TCRβ families in each cDNA sample was determined by sequencing the CDR3 region of TCR clones. Preamplified cDNA generated for use in the VB quantitative PCR assay was used as a template for a second round of PCR using an individual VB forward primer and a CBQ reverse primer. The cDNA was amplified for 30 cycles in a Perkin Elmer 9600 GeneAmp PCR system under the following conditions: 90°C for 1 s, 55°C for 30 s, and 68°C for 60 s, with a final 10-min extension at 68°C. These PCR reactions were cleaned of excess primers using the QiAquick PCR Purification Kit (Qiagen) and cloned using the pGEM-T Easy Vector System (Promega), according to the manufacturer’s directions. Plasmids were transformed into JM109 competent bacteria (Promega) and plated on ImMedia Amp Blue agar (Invitrogen). White colonies from each plate were selected and grown in ImMedia Amp media (Invitrogen) overnight at 37°C with shaking. Cloned plasmids were purified using the QuickLyse Miniprep Kit (Qiagen) and sequenced using the pGEM-T vector following the inserted PCR products.

CFSE dilution cell-proliferation assay
PBMCs sampled from SIVmac251-infected Mamu-A*02* rhesus monkeys were separated from EDTA-preserved whole blood by Ficoll density-gradient centrifugation and were quantified using the Guava EasyCyte Plus instrument (Millipore). Cells were resuspended in PBS + 5% FBS containing 5 μM CFSE (Invitrogen) for 5 min, washed three times in room-temperature PBS + 5% FBS, and resuspended in RPMI 1640 supplemented with 10% heat-inactivated FBS and penicillin/streptomycin (Life Technologies). A total of 2 × 10^5 lymphocytes/well was added to a 96-well round-bottom culture plate and cultured for 6 d in the presence of 20 U/ml IL-2 (Hoffman-LaRoche) and serial log dilutions of WT, T2S, or Y9F p199RY epitope peptides, 1 μg/ml phytoceramide/lipid A (Sigma, as a positive control), or supernatants of Y9F p199RY peptide for 10 d to generate effector cells. Then the cultures were washed to remove unbound peptide/MHC/2m complexes were removed by washing, and radiation was measured with an Evolution 200 plate reader (Bio-Tek Instruments) at an interval pulse-off time of 30 s (Misonix Sonicator 3000). The cell

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membrane preparation was extruded 11 times through a 100-nm poly-carbonate filter and immediately used for SPR-binding assays. In earlier studies with T cell hybridomas, we showed that TCRs are enriched in DRM fractions and, following capture on Biacore sensor chip, bind specifically to soluble peptide–MHC monomeric complexes (data not shown).

**SPR measurements**

SPR-binding analyses using T cell DRMs were carried out as previously described for protein–ligand interactions (43, 53–59) and for reconstituted G protein-coupled receptors in liposomes (55). DRMs were captured on a Biacore L1 sensor chip, which uses an alkyl linker for anchoring lipids. Before capturing lipids, the surface of the L1 chip was cleaned with a 60-s injection of 40 mM OGP at 100 μl/min, and the chip and fluidics were washed with excess buffer to remove any traces of detergent, as described previously (59). TCR-expressing DRMs were captured by slowly injecting the T cell DRM preparation at 5 μl/min over the L1 sensor chip. Monomeric pMHC complexes constructed with the WT, T2S, and Y9F p199RY epitope peptides were injected at 50 μM/min for 3 min at concentrations ranging from 25 to 100 μg/ml (WT pMHC) or 25 to 200 μg/ml (T2S pMHC, Y9F pMHC). The specific binding signal was obtained by subtracting nonspecific signal from p56 pMHC monomers injected at the same concentrations. The global curve fitting to the Langmuir equation was used for calculation of kinetic parameters: $k_{on}$, $k_{off}$, $K_d$, as described earlier (35, 53). All SPR measurements were carried out on a Biacore 3000 instrument, and data analyses were done using BIAevaluation 4.1 software (GE Healthcare).

**Results**

*The Nef p199RY epitope of SIVmac251 evolves in a sequential manner following infection of Mamu-A*02* rhesus monkeys*

We previously described the evolution of the Nef p199RY epitope in a cohort of five infected Mamu-A*02* rhesus monkeys (48). We averaged the frequencies of the most commonly detected mutations across the entire cohort to determine the general pattern of sequence evolution in the p199RY epitope following infection. The most common p199RY epitope sequences detected by 454 pyrosequencing and by traditional Sanger sequencing bore mutations at position 2 (a threonine-to-serine substitution, T2S), position 3 (a serine-to-leucine substitution, S3L), position 7 (isoleucine-to-methionine, I7M, and isoleucine-to-threonine, I7T, substitutions), and position 9 (a tyrosine-to-phenylalanine substitution, Y9F) (Fig. 1A). These epitope mutations emerged in a consistent, sequential pattern during SIVmac251 infection (Fig. 1B). The transmitted WT sequence, which predominated during the earliest time-points following infection, gradually decreased in prevalence to near undetectable levels in chronic infection, whereas an early-emerging mutation (S3L) gradually increased in prevalence and persisted in chronic infection. In addition to S3L, a late-emerging mutation (Y9F) became the predominant sequence of the virus during chronic infection. Transient mutations that were selected after day 21, including the I7T, I7M, and T2S mutations, also decreased in prevalence during chronic infection. Therefore, the epitope variants of the virus could be categorized as those that were initially highly prevalent and gradually waned in frequency (WT), those that emerged early (S3L), and those that emerged late (Y9F) during infection to become fixed in the virus quasispecies. There were also mutations that were transiently selected in the viral quasispecies (I7M, I7T, and T2S).

The WT p199RY epitope peptide and p199RY epitope peptides bearing mutations selected in SIVmac251-infected Mamu-A*02* rhesus monkeys bind to Mamu-A*02 with equal affinity

Many previously characterized HIV and SIV mutations that evolve in immunodominant CD8 T lymphocyte epitopes become fixed in the virus quasispecies because the epitope peptides have a decreased affinity for the MHC class I molecule that presents the peptides on the surface of infected cells (17, 20, 22, 24). To determine whether this was also true for the mutant epitope peptides representing selected viral variants detected in the cohort of SIVmac251-infected Mamu-A*02* rhesus monkeys, these peptides were synthesized and evaluated for their ability to bind to Mamu-A*02.

We used an assay in which purified soluble Mamu-A*02 protein was incubated with 125I-labeled WT p199RY peptide and serial log dilutions of unlabeled competitor peptide in the presence of excess β2-m for 48 h in a 96-well plate. This reaction mixture was then incubated with plate-bound anti-MHC class I Ab and washed to remove unbound peptides, and radioactivity was measured to determine the amount of radiolabeled peptide remaining associated with Mamu-A*02. Data from these peptide:Mamu-A*02 affinity assays indicated that all of the mutant epitope peptides selected in the SIVmac251-infected monkeys competed comparably with the radiolabeled WT index peptide for binding to Mamu-A*02 (Fig. 2, blue lines). Furthermore, this competitive
binding was comparable to that of unlabeled WT epitope peptide (Fig. 2, red line). Epitope peptides that incorporated two other mutations that were not selected for in this cohort of monkeys, but were previously shown to affect epitope peptide binding to Mamu-A*02 [Y9A and Y9S (60)], had reduced binding to the Mamu-A*02 protein in the assay (Fig. 2, black lines). Finally, an irrelevant peptide control, p11B (ALSEGCPYDIN), bound very weakly to Mamu-A*02, and this binding was not dependent on the dose of the peptide used in the competition assay (Fig. 2, green line). Therefore, these studies indicated that mutations selected in the p199RY epitope in SIVmac251-infected Mamu-A*02 monkeys did not result in reduced binding affinity of the epitope peptides for Mamu-A*02, suggesting that the epitope mutations allowed the evolving virus to evade CD8+ T cell recognition by a mechanism other than reduced Ag presentation on the surface of SIV-infected cells.

CD8+ T lymphocytes specific for either WT or mutant Nef p199RY epitope peptides share a restricted TCR Vβ repertoire

We previously used tetramer staining to determine whether CD8+ T lymphocytes sampled from SIVmac251-infected monkeys could recognize two prototype mutant p199RY epitope peptide sequences: one that was transiently selected during SIVmac251 infection (T2S) and one that became fixed in the viral quasispecies during chronic infection (Y9F). We found that p199RY-specific CD8+ T lymphocytes had the capability to cross-recognize the WT epitope and both mutant epitopes. Moreover, CD8+ T lymphocytes able to recognize the mutant p199RY epitopes were present on day 21 postinfection, before the mutants were readily demonstrable in the viral quasispecies (48). We sought to define the clonal composition of these WT and mutant p199RY-specific CD8+ T lymphocytes.

To investigate the clonal diversity of the Nef p199RY-specific CD8+ T lymphocytes, we initially evaluated the TCR Vβ repertoire of PBMCs specific for the WT, T2S, and Y9F epitope sequences. Because the frequency of p199RY-specific CD8+ T lymphocytes was often too low to perform this analysis on cells sampled directly ex vivo (Supplemental Table I), PBMCs sampled from monkeys at three time-points postinfection were divided into three aliquots and separately stimulated with the WT, the T2S, or the Y9F epitope peptide to expand these epitope-specific CD8+ T lymphocyte populations. After a 12-d culture, cells were sorted using tetramers constructed with the same peptide used in the in vitro stimulation. mRNA was harvested from these cells and subjected to real-time quantitative PCR using a panel of 46 Vβ family- and subfamily-specific forward primers and a common reverse primer to quantify the contribution of each Vβ family and subfamily to the entire p199RY-specific CD8+ T lymphocyte repertoire.

The TCR Vβ repertoires expressed by the WT- and mutant peptide-exposed, sorted CD8+ T cell populations were extremely narrow, consisting of only one to three Vβ families (Fig. 3). As shown in the study of the representative monkey BH25, CD8+ T cells exposed to the WT, T2S, and Y9F epitope peptides primarily expressed the TCR Vβ13A family, regardless of when the cells were sampled from the monkey (Fig. 3A–C). Data on mutant epitope peptide-exposed, sorted CD8+ T lymphocytes could not be generated for cells sampled on day 21 after repeated attempts, perhaps because the CD8+ T lymphocytes exposed to the intense cytokine stimulation and large antigenic burden during acute infection were apoptotic (Fig. 3A) (61, 62). The appearance of a T2S-specific CD8+ T lymphocyte population sampled on day 196 that uses Vβ24 may represent the late de novo generation of a cell population specific for the T2S mutant epitope peptide on day 84 postinfection (Fig. 3C).

The WT-, T2S-, and Y9F-specific CD8+ T lymphocyte Vβ repertoires in the other four monkeys were similar to those observed in monkey BH25 (Fig. 3D). In all evaluated monkeys, the WT-specific CD8+ T lymphocytes sampled on days 17–28 postinfection only used the TCR Vβ13A family. In monkeys AJ82, BH25, and CA53, the Vβ repertoires for the WT, T2S, and Y9F peptide-exposed, sorted CD8+ T lymphocytes sampled on day 84 postinfection were similarly restricted to the Vβ13A family. However, in monkeys BE86 and BR32, the use of additional Vβ families (Vβ19 and Vβ17, respectively) was detected that may represent de novo-generated CD8+ T lymphocyte clones induced by exposure of the CD8+ T lymphocyte pool to viruses that had accumulated p199RY mutations. Finally, during chronic infection, epitope peptide-exposed, sorted CD8+ T lymphocytes sampled on days 184–196 postinfection continued to be dominated by cells expressing TCRs using the Vβ13A family, although each monkey had minor populations of CD8+ T lymphocytes expressing other Vβ genes (Vβ19, Vβ22B.2, Vβ16, and Vβ24). Together, these data indicated that WT and mutant p199RY-specific CD8+ T lymphocytes shared a narrow Vβ repertoire that was present prior to viral evolution and that persisted following the selection of viruses with multiple mutations in the p199RY epitope.

![FIGURE 2](http://www.jimmunol.org/) Most mutations in the p199RY epitope did not reduce the binding affinity of the epitope peptides to Mamu-A*02. 125I-labeled WT p199RY peptide was incubated with Mamu-A*02 protein, human β2m, and serial dilutions of the indicated unlabeled p199RY epitope peptide variants. Mamu-A*02 peptide complexes were captured in a 96-well plate, and the amount of radioactive peptide associated with Mamu-A*02 was determined using a scintillation counter. The percent inhibition of binding of the radiolabeled index peptide by each of the unlabeled p199RY peptide variants was calculated and plotted according to the concentration of the competing, unlabeled peptide. Red, unlabeled WT peptide; blue, unlabeled mutant epitope peptide detected in SIVmac251-infected Mamu-A*02+ monkeys by 454 pyrosequencing and Sanger sequencing; green, unlabeled peptide that does not bind to Mamu-A*02 (p11B: ALSEGCPYDIN); black, unlabeled p199RY sequence-derived peptides (Y9A: YTSPGIR and Y9S: YTSPGIR); and white, p199RY peptide-exposed, sorted CD8+ T lymphocytes from day 84 postinfection

WT, T2S, and Y9F p199RY-specific CD8+ T lymphocytes share identical CDR3 sequences

We then sought to determine the clonal composition of these cell populations more precisely by evaluating the TCRβ CDR3 sequence heterogeneity of these Vβ13A-expressing CD8+ T lymphocytes. cDNA used for the Vβ repertoire analyses was further amplified using a Vβ13A-specific forward primer and a Cβ reverse primer, and the TCRβ CDR3 region was cloned and sequenced.

Cloning and sequencing of the CDR3 regions of WT-, T2S-, and Y9F-exposed, sorted CD8+ T lymphocyte populations revealed that the TCRβ-chains in each of these variant epitope-specific cell populations shared identical CDR3 sequences (Fig. 4). In monkey AJ82, eight of the detected CDR3 clonotypes in cells sampled on

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day 21 postinfection were shared between WT- and Y9F-exposed, sorted CD8+ T lymphocytes. Moreover, the more common CDR3 clonotypes detected in cell populations sampled on day 21 persisted in the WT and mutant epitope peptide-exposed, sorted CD8+ T lymphocyte populations sampled at later time-points. There was also sharing of identical CDR3 sequences in cells exposed to different epitope peptides at later time-points postinfection. For example, clonotype CASSLGGHPMNTEAFF was shared among all three epitope peptide-exposed, sorted cell populations. Interestingly, the clonotypic profile of WT, T2S, and Y9F peptide-exposed CD8+ T lymphocytes changed dramatically by day 184 postinfection in this monkey. None of the CDR3 clonotypes detected in cells sampled on day 84 was present in cells sampled on day 196, and the CDR3 sequences were restricted to two newly detected clonotypes (CASSQRDDQPYF and CASSRDWELTAQLFF), one of which was detected in T2S-exposed, sorted cells sampled on day 21 of infection. The populations of WT and mutant epitope peptide-exposed, sorted cells shared these two CDR3 clonotypes.

This phenomenon of CDR3 sequence sharing between WT and mutant epitope-specific CD8+ T lymphocytes was also seen in monkeys BE86 (Fig. 4), BH25, BR32, and CA53 (Supplemental Fig. 1).
clonotypes (CASSYRVLALDPQYF and CASSMRTGTDPQYF) were detected in the WT-, T2S-, and Y9F-exposed, sorted cell populations, and others were shared between two of the three peptide-exposed, sorted CD8+ T lymphocyte populations. Other clonotypes not detected in cells sampled on day 21 were present in cells sampled on day 84. Although it is possible that these may represent de novo-generated p199RY-specific CD8+ T lymphocytes, it is also possible that they were not detected on day 21 because of limited sampling of CDR3 clones. By day 196, all but 2 of the 11 defined CDR3 sequences were shared between the WT and Y9F peptide-exposed, sorted cells (Fig. 4). Two of these clonotypes (CASSLGGHPMNTEAFF and CASSLRGLGEPQTQYF) were also present in the T2S peptide-exposed, sorted cell population. It is likely that we would have detected more shared clonotypes in the T2S-specific cells sampled on day 196 postinfection had more CDR3 clones been available for sequencing.

CDR3 sequencing was also performed on peptide-exposed, tetramer-sorted CD8+ T lymphocytes that made use of other TCRβ gene families. cDNA used for Vβ repertoire analysis was amplified by PCR using a Vβ13A-specific forward primer and a CB reverse primer. The resulting product was cloned, and the TCR β CDR3 region was sequenced (Supplemental Fig. 2). In monkey BE86, Vβ17-expressing cells sampled from monkey BR32 on day 84 shared the CDR3 sequence CASSEEIQETQYF, regardless of whether the cells were cultured with the WT, T2S, or Y9F epitope peptide.

Thus, the CDR3-sequencing analysis conducted on WT, T2S, and Y9F peptide-exposed, sorted CD8+ T lymphocyte populations showed that identical clonal populations of cells were present in all three epitope-specific CD8+ T lymphocyte populations. Interestingly, clonotypes that were present in these epitope-specific cell populations early in the course of infection, prior to the emergence of viruses containing p199RY escape mutations, persisted as the infecting viruses underwent sequential mutations. These data confirmed the cross-reactivity of p199RY epitope-specific CD8+ T lymphocytes with emerging viruses bearing epitope mutations (48).

Fewer p199RY epitope-specific CD8+ T lymphocytes proliferate in response to stimulation with the T2S and Y9F epitope peptides than with the WT epitope peptide. The Vβ repertoire and CDR3 sequencing data suggested that p199RY-specific CD8+ T lymphocytes should have the capacity to recognize the WT, as well as the mutant, epitope sequences. We hypothesized that the epitope mutations that developed in SIVmac251-infected Mamu-A*02+rhesus monkeys may confer a selective advantage to these mutant viruses in the presence of these cross-reactive epitope-specific cells because the TCRs of these cells may not interact as efficiently with the mutant epitope sequences as they do with the WT epitope sequence. Therefore,
we initially evaluated the proliferative capacity of p199RY-specific CD8+ T lymphocytes sampled from four monkeys in response to stimulation with the WT, T2S, and Y9F epitope peptides. Cells were collected from four SIVmac251-infected Mamu-A*02+ rhesus monkeys, loaded with CFSE, and split into three separate aliquots. Each group of cells was stimulated in vitro with serial log dilutions of the WT, T2S, or Y9F epitope peptide. Cells were then stained with tetramers constructed with the matching epitope peptide used for stimulation and evaluated for proliferation by flow cytometric detection of CFSE dilution in CD3+CD8+ tetramer+ cells.

In cells from all four SIVmac251-infected monkeys, the WT epitope peptide stimulated the greatest percentage of WT tetramer+ cells to divide (Fig. 5). The T2S epitope peptide stimulated a smaller percentage of T2S tetramer+ cells to divide, and the Y9F epitope peptide stimulated an even smaller percentage of Y9F tetramer+ to divide. Differences in proliferative capacity were most notable when cells were stimulated with low peptide concentrations, whereas proliferation in response to stimulation with WT, T2S, and Y9F peptides was comparable in cells from three of the four monkeys when exposed to high peptide concentrations (Fig. 5). Therefore, a smaller percentage of mutant p199RY peptide-stimulated epitope-specific CD8+ T lymphocytes proliferated compared with CD8+ T lymphocytes stimulated with the WT p199RY epitope peptide.

Y9F peptide-exposed, sorted CD8+ T lymphocytes kill matching peptide-sensitized target cells less efficiently than do WT and T2S peptide-exposed, sorted CD8+ T lymphocytes

We also evaluated the capacity of CD8+ T lymphocytes to lyse epitope peptide-pulsed target cells after exposure of these cells to WT, T2S, and Y9F p199RY epitope peptides. PBMCs collected from four Mamu-A*02+ rhesus monkeys chronically infected with SIVmac251 were split into three aliquots, each of which was cultured in the presence of the WT, T2S, or Y9F p199RY epitope peptide. These cells were then cocultured with a [35Cr]-loaded Mamu-A*02–expressing cell line pulsed with serial log dilutions of the matching p199RY epitope peptides used for in vitro stimulation. p199RY-stimulated CD8+ T lymphocytes from all four monkeys were capable of lysing target cells pulsed with the WT, T2S, and Y9F epitope peptides (Fig. 6). In all four monkeys, the WT and T2S peptide-exposed, sorted CD8+ T lymphocytes lysed peptide-sensitized target cells with comparable efficiency. However, the Y9F peptide-exposed, sorted CD8+ T lymphocytes lysed Y9F epitope-sensitized target cells less efficiently than did the WT and T2S peptide-exposed, sorted CD8+ T lymphocyte populations. This difference in target cell killing was most notable when high peptide concentrations were used to sensitize target cells for lysis by effector cells from monkeys 00C076 and CP10, whereas differences in target cell killing were most notable when low peptide concentrations were used to sensitize the target cells for lysis by effector cells from monkey AY32. The Y9F peptide-exposed, sorted CD8+ T lymphocytes from monkey CF55 were less efficient in lysing target cells pulsed with all four concentrations of peptide. Thus, the Y9F epitope-peptide-exposed, sorted CD8+ T lymphocytes were consistently less efficient in lysing peptide-pulsed target cells than were the WT and T2S peptide-exposed, sorted CD8+ T lymphocytes.

Fewer p199RY-specific CD8+ T lymphocytes produce IFN-γ in response to stimulation with the T2S and Y9F epitope peptides than in response to stimulation with the WT epitope peptide

We also evaluated cytokine production by CD8+ T lymphocytes upon stimulation with the variant p199RY epitope peptides. PBMCs from three Mamu-A*02+ rhesus monkeys chronically infected with SIVmac251 were split into three aliquots, and each aliquot of cells was stimulated with serial log dilutions of the WT, T2S, or Y9F p199RY epitope peptides in the presence of brefeldin A and monensin to prevent intracellular protein transport and secretion. Cells were then stained with tetramers constructed with the matching p199RY epitope peptide used for stimulation, fixed and permeabilized, and then stained with an Ab specific for the cytokine IFN-γ. The percentage of tetramer+ cells that produced IFN-γ was determined by flow cytometry.

We also evaluated the capacity of CD8+ T lymphocytes to lyse epitope peptide-pulsed target cells after exposure of these cells to WT, T2S, and Y9F p199RY epitope peptides. PBMCs collected from four rhesus monkeys chronically infected with SIVmac251 were split into three aliquots, each of which was cultured in the presence of the WT, T2S, or Y9F p199RY epitope peptide. These cells were then cocultured with a [35Cr]-loaded Mamu-A*02–expressing cell line pulsed with serial log dilutions of the matching p199RY epitope peptides used for in vitro stimulation. p199RY-stimulated CD8+ T lymphocytes from all four monkeys were capable of lysing target cells pulsed with the WT, T2S, and Y9F epitope peptides (Fig. 6). In all four monkeys, the WT and T2S peptide-exposed, sorted CD8+ T lymphocytes lysed peptide-sensitized target cells with comparable efficiency. However, the Y9F peptide-exposed, sorted CD8+ T lymphocytes lysed Y9F epitope-sensitized target cells less efficiently than did the WT and T2S peptide-exposed, sorted CD8+ T lymphocyte populations. This difference in target cell killing was most notable when high peptide concentrations were used to sensitize target cells for lysis by effector cells from monkeys 00C076 and CP10, whereas differences in target cell killing were most notable when low peptide concentrations were used to sensitize the target cells for lysis by effector cells from monkey AY32. The Y9F peptide-exposed, sorted CD8+ T lymphocytes from monkey CF55 were less efficient in lysing target cells pulsed with all four concentrations of peptide. Thus, the Y9F epitope-peptide-exposed, sorted CD8+ T lymphocytes were consistently less efficient in lysing peptide-pulsed target cells than were the WT and T2S peptide-exposed, sorted CD8+ T lymphocytes.

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The binding of WT epitope peptide:Mamu-A*02 complexes to p199RY-specific TCRs is associated with slower dissociation kinetics and lower equilibrium dissociation binding $K_d$ compared with T2S and Y9F epitope peptide:Mamu-A*02 complexes

Although we observed that the majority of WT p199RY-specific CD8+ T lymphocytes cross-recognized T2S and Y9F epitope peptides, our functional studies indicated that the CD8+ T lymphocytes responded differently to stimulation with each of the variant epitope peptides. We hypothesized that the lower proliferative, cytotoxic, and cytokine-secreting activities of p199RY-specific CD8+ T lymphocytes in response to T2S and Y9F epitope peptide exposure compared with WT epitope peptide exposure were a consequence of a low TCR affinity for the mutant epitope peptide:MHC complexes. We used a novel SPR strategy to determine the affinities of the WT, T2S, and Y9F epitope ligands for the TCRs expressed by the entire p199RY epitope-specific CD8+ T lymphocyte population of each of the evaluated monkeys. We isolated CD3+CD8+ lymphocytes from each of the four rhesus monkeys chronically infected with SIVmac251, using flow cytometric sorting to deplete cells that bound to a tetramer constructed with p56, a Mamu-A*02–restricted subdominant Nef epitope that has no cross-reactivity with p199RY- or CD8+ T lymphocytes cross-recognized T2S and Y9F epitope peptide:Mamu-A*02 monomer; the Y9F monomer had a 2–4-fold lower affinity (Fig. 8A). Steady-state analysis and curve fitting to the Langmuir equation were used to derive $K_d$ (Fig. 8B, Supplemental Table II), association rate $k_{on}$ (Fig. 8C, Supplemental Table II), and dissociation rate $k_{off}$ (Fig. 8D, Supplemental Table II) of each monomer for binding to the TCR-enriched DRMs. The strongest-binding WT peptide:Mamu-A*02 monomer had a 3–8-fold lower $K_d$ than did the Y9F peptide: Mamu-A*02 monomer, and the Y9F monomer had a 2–4-fold

In CD8+ T lymphocytes from all three rhesus monkeys that were evaluated, the WT epitope peptide stimulated the greatest percentage of tetramer+ cells to produce IFN-γ (Fig. 7). In contrast, both the T2S and the Y9F epitope peptides stimulated a smaller percentage of p199RY epitope-specific CD8+ T lymphocytes to produce this cytokine. In studies of cells from monkeys 203-04 and 275-06, both the T2S and Y9F epitope peptides stimulated lower percentages of epitope-specific CD8+ T lymphocytes to produce IFN-γ at all four peptide concentrations tested. In studies of cells from monkey IC8, differences in IFN-γ production between the Y9F- and T2S-stimulated cells were also noted at the three highest concentrations of peptide tested. However, in cells sampled from all three monkeys, the mutant p199RY epitope peptides stimulated fewer tetramer+ CD8+ T lymphocytes to produce IFN-γ.
lower $K_d$ than did the T2S monomer (Fig. 8B). The difference in the binding affinities was largely due to the faster off-rates of the T2S and Y9F monomers (Fig. 8D), whereas the association rates for all three monomers were largely the same for DRM-enriched DRMs isolated from CD3⁺CD8⁺ T lymphocytes from all four monkeys (Fig. 8C). Therefore, the weaker functional activity of CD8⁺ T lymphocytes in response to mutant epitope peptide exposure may have been a consequence of the faster dissociation and weaker binding of mutant p199RY epitope:Mamu-A*02 monomers for p199RY-specific TCRs. This may account for the selective advantage for viruses harboring the T2S and Y9F epitope mutations in Mamu-A*02⁺ rhesus monkeys chronically infected with SIVmac251.

**Discussion**

Much of the existing literature that describes SIV and HIV evolution at MHC class I-restricted CD8⁺ T lymphocyte epitopes has focused on those epitope-specific CD8⁺ T lymphocyte responses that are associated with protection from high viral loads and clinical deterioration. These include epitope-specific responses restricted by the relatively protective MHC class I alleles HLA-B27 and HLA-B57 in humans and Mamu-A*01, Mamu-B*08, and Mamu-B*17 in rhesus monkeys (63–65). Moreover, mutations in these epitopes were often shown to reduce peptide:MHC class I affinity, such that presentation of these epitopes to CD8⁺ T lymphocytes is abrogated (11, 28). Although these studies have contributed to our understanding of the mechanisms of immune escape by evolving viruses, these epitopes may not demonstrate the sequence plasticity and potential for immune evasion of other viral epitopes. Moreover, this study focused on an immunodominant epitope restricted by Mamu-A*02, which is a high-frequency MHC class I allele that is not associated with clinical protection from a high viral burden. We also demonstrated that the mechanism by which mutated p199RY epitope sequences evade the immune response is not reduced MHC class I binding and cell surface presentation, but rather reduced TCR affinity for the mutated epitope peptides.

In this study, we showed that differences in p199RY-specific CD8⁺ T lymphocyte proliferation, cytotoxicity against peptide-sensitized target cells, and production of IFN-γ following exposure to WT and mutant p199RY epitope peptides were due, at least in part, to the weak affinities of p199RY-specific TCRs for mutant epitope peptide:Mamu-A*02 complexes. This was demonstrated using a novel SPR technique that uses the Biacore L1 chip to immobilize cell membrane preparations onto the solid sensor surface (43). This technique has many advantages over SPR-based approaches previously used to study pMHC interactions with TCRs. The technique that we used in the current study did not limit us to assessing the pMHC affinity of only one or a few cloned TCRs. Rather, we were able to measure the average affinity of pMHC for TCR of a polyclonal population of CD8⁺ T lymphocytes sampled ex vivo. The lipophilic anchor of the L1 chip directly embeds itself into lipid bilayers, obviating the need for artificial adsorption of membrane preparations onto the sensor chip. In addition, there is no need to anchor to the chip other cell surface proteins that are involved in TCR signaling and engagement of pMHC complexes, such as the CD8 molecule, because these proteins are also enriched in the DRM fractions of detergent-solubilized T cell preparations. Finally, the TCRs were able to move laterally in the immobilized lipid membrane, allowing the assessment of their interactions in a physiologic context. Therefore, this new SPR technique allows measurement of the affinity for pMHC of TCRs expressed in their native environment and conformation.

Previous studies used epitope pMHC tetrameric reagents to approximate the dissociation rates of pMHC complexes from epitope-specific CD8⁺ T lymphocytes (66, 67). In those assays, the cells were initially stained with fluorophore-labeled tetramers bearing epitope peptides of interest, washed, and then incubated in the presence of excess unlabeled tetramer. The total fluorescence of the tetramer⁺ CD8⁺ T lymphocyte population was then determined for cells sampled at selected time-points during incubation with the competing, unlabeled tetramer to determine the dissociation rate of the labeled tetramer. Results obtained by the SPR methods described in this work have not been compared with results obtained from dissociation rates calculated from tetramer-competition assays. However, because it uses a tetrameric rather than a monomeric pMHC complex, the tetramer-competition assay may not be sufficiently sensitive to detect subtle differences in pMHC:TCR affinity.
The T2S and Y9F mutations occurred in residue positions shown to be important for anchoring epitope peptides into the Mamu-A*02 peptide-binding cleft (60). Therefore, it was reasonable to hypothesize that these amino acid substitutions might allow mutant viruses to evade the p199RY-specific CD8+ T lymphocyte response by preventing stable presentation of the mutated peptides by MHC class I for TCR recognition. However, our peptide:MHC-binding assays indicated that these two mutations had no effect on the affinity of either peptide for Mamu-A*02. Indeed, the T→S and Y→F substitutions are both conservative, and studies indicated that there is a preference for either threonine or serine residues at position 2 and tyrosine or phenylalanine residues at position 9 of Mamu-A*02-binding peptides (60). One recent study demonstrated that mutations of MHC anchor residues, which typically do not participate in direct interactions with the TCR, can profoundly affect TCR-binding affinity and the resulting CD8+ T lymphocyte functional repertoire (68). Although the mutations assessed in that report were associated with lower MHC affinity but stronger TCR binding than the WT peptide, these studies indicated that anchor residue mutations can impact the structural conformation of the peptide as it sits in the MHC-binding groove, which can alter TCR engagement of the pMHC complex.

We initially hypothesized that the sequential evolution of the p199RY epitope was associated with de novo priming of CD8+ T lymphocytes that were specific for arising mutations. However, our previous study of p199RY-specific CD8+ T lymphocytes from Mamu-A*02+ SIVmac251-infected rhesus monkeys indicated that the p199RY-specific CD8+ T lymphocyte population was capable of cross-recognizing the WT, T2S, and Y9F epitope sequences (48). This was confirmed by the VB repertoire and TCRβ CDR3-sequencing analyses in the current study, which indicated the sharing of VB gene usage and CDR3 clonotypes among WT-, T2S-, and Y9F-specific CD8+ T lymphocyte populations throughout the course of infection. The failure of emerging mutations to prime and drive the expansion of cell populations that were uniquely specific for the mutant epitope sequences may have been a consequence of the lack of CD4+ T cell help, which is required for the priming and maintenance of effective memory T lymphocytes (69–71), in the setting of ongoing SIV replication in CD4+ T cells.

In these studies, the absolute numbers of WT-, T2S-, and Y9F-specific CD8+ T lymphocytes sampled from infected monkeys were too low to permit the isolation of sufficient cellular mRNA from the cells directly ex vivo. Therefore, these cells were expanded in vitro by exposure to each of the variant p199RY epitope peptides before they were sorted by flow cytometry and mRNA was extracted. The use of this procedure raises the concern that the in vitro expansion of cells may bias the Vβ repertoire, as determined in the analyses. To address this concern, the Vβ repertoires of CD8+ T cells specific for an immunodominant Gag epitope were evaluated in cells isolated from monkeys and in cells that were peptide expanded in vitro using the same panel of Vβ family- and subfamily-specific primers (51). The repertoires of the cells sampled ex vivo and the cells expanded in vitro were found to be very similar. However, it should be noted that comparable comparisons of Vβ repertoires obtained from the cells specific for the Nef p199RY epitope described in this study have not been performed.

Because of the cross-reactivity of p199RY-specific CD8+ T lymphocytes for the WT, T2S, and Y9F epitopes, it is reasonable to suppose that these cells were being continually stimulated during the course of infection, even after the WT virus was replaced by emerging mutant viruses. A number of reports described the phenomenon of T cell exhaustion during HIV and SIV infection, where chronic activation is associated with loss of cytotoxic potential, lower proliferative capacity, and reduced production of IFN-γ by virus-specific CD8+ T lymphocytes (72–74). Other studies in mouse models of chronic viral infection showed that various functions of chronically stimulated CD8+ T lymphocytes are lost in a stepwise fashion, with IL-2 and TNF-α production being more sensitive to exhaustion than is IFN-γ production (75–77). This observation is consistent with our inability in the current study to detect by intracellular cytokine staining even modest numbers of cells from chronically infected Mamu-A*02+ monkeys that produced IL-2 and TNF-α.

We noted a discrepancy between the SPR measurements of TCR binding to pMHC and the functional profiles of p199RY-specific CD8+ T lymphocytes upon exposure to the WT, T2S, and Y9F epitope peptides. In cell-proliferation and IFN-γ-production assays, exposure of CD8+ T lymphocytes to the Y9F epitope peptide consistently induced less cell proliferation and cytokine production than did exposure to the T2S epitope peptide. However, the SPR measurements indicated that the T2S-Mamu-A*02 monomers had a faster dissociation rate from and lower equilibrium-binding affinity to p199RY-specific TCRs than did the Y9F-Mamu-A*02 monomers. A number of possible explanations may account for these discordant data. First, the TCR affinity for the WT epitope pMHC complex was much higher than for the T2S and Y9F epitope pMHC complexes, whereas the differences in the affinities between the T2S and Y9F epitope pMHC complexes for TCRs were less pronounced. It is possible that the affinities of the T2S and Y9F epitope complexes for the TCRs were below a threshold required for optimal activation of epitope-specific CD8+ T lymphocytes, whereas the affinity of the TCRs for the WT epitope complex was sufficient to trigger cell activation. Second, other studies documented the absence of a correlation between CD8+ T lymphocyte TCR affinity for epitope pMHC complexes and the functional activity of these cells (78). This observation raises the possibility that factors determining CD8+ T lymphocyte responsiveness, other than TCR:pMHC affinity, may contribute to T cell activation, including the thermodynamics of TCR binding to mutant pMHC complexes, TCR conformational changes or other structural changes that favor the binding of the TCR to one pMHC ligand over another, or even structural changes that may trigger different intracellular-signaling pathways (79–81). Finally, the discrepancy between the T2S and Y9F pMHC-binding affinity to TCRs and functional responses by CD8+ T lymphocytes to these epitope peptides is consistent with the recently proposed model suggesting that optimal T cell activation may require TCR engagement of heterodimeric pMHC complexes consisting of the epitope pMHC and endogenous pMHC complexes (82–84). It is possible that the more rapid dissociation rate of the T2S pMHC may better accommodate the viral/endogenous epitope heterodimer and association with the TCR than does the slower dissociation rate of the Y9F pMHC, whereas the heterodimer pMHC engagement of TCRs is less important for binding of TCRs to pMHC complexes with higher affinity, such as the WT pMHC.

This study has important implications for the design of CD8+ T lymphocyte-based vaccines. A number of strategies have been described for inducing polyclonal and cross-reactive CD8+ T lymphocyte responses that cover the immense breadth of HIV sequence diversity in the infected population worldwide. These strategies include polyepitope vaccines in which a string of epitopes is expressed from a single vaccine vector, centralized gene vaccines in which an ancestral epitope sequence is used as an immunogen, and mosaic vaccines that include sequences derived...
from the in silico recombination of naturally occurring virus strains (85). However, the present studies suggested that epitope-specific CD8+ T lymphocytes that are capable of cross-recognition multiple epitope variants may not always confer protection against the emergence of epitope escape mutations. These results underscore the importance of evaluating the breadth of recognition of CD8+ T lymphocytes elicited by novel vaccines, as well as assessing the functional capacities of these cells and monitoring viral evolution after challenge to determine how effective these vaccine regimens control viral replication and prevent CD8+ T lymphocyte escape in vivo.

The results of these experiments provide an example in which naturally selected CD8+ T lymphocyte escape mutations do not affect MHC class I affinity. Therefore, these mutant epitope peptides may be effective priming immunogens in a vaccine. It is possible that immunization of healthy individuals with epitope sequences containing commonly selected mutations may induce CD8+ T lymphocytes that have a preferential specificity for the mutated epitopes, and these cells may be effective in controlling the emergence of epitope escape mutations following viral challenge.

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