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Epitope Cross-Reactivity Frequently Differs between Central and Effector Memory HIV-Specific CD8\(^+\) T Cells\(^1\)


HIV diversity may limit the breadth of vaccine coverage due to epitope sequence differences between strains. Although amino acid substitutions within CD8\(^+\) T cell HIV epitopes can result in complete or partial abrogation of responses, this has primarily been demonstrated in effector CD8\(^+\) T cells. In an HIV-infected Kenyan cohort, we demonstrate that the cross-reactivity of HIV epitope variants differs dramatically between overnight IFN-\(\gamma\) and longer-term proliferation assays. For most epitopes, particular variants (not the index peptide) were preferred in proliferation in the absence of corresponding overnight IFN-\(\gamma\) responses and in the absence of the variant in the HIV quasispecies. Most proliferating CD8\(^+\) T cells were polyfunctional via cytokine analyses. A trend to positive correlation was observed between proliferation (but not IFN-\(\gamma\)) and CD4 counts. We present findings relevant to the assessment of HIV vaccine candidates and toward a better understanding of how viral diversity is tolerated by central and effector memory CD8\(^+\) T cells. *The Journal of Immunology, 2007, 178: 3750–3756.

Efforts to produce an effective HIV vaccine have been hampered by the extreme genetic diversity and evolutionary capabilities of HIV (1). In addition to escape from effective immune responses, tremendous regional and global genetic diversity (\(>30\%\) amino acid divergence between subtypes) poses a significant problem for HIV vaccine design: that vaccine-elicited immune responses may be unable to provide coverage for the diversity of strains a vaccinee may encounter in nature (2). Although some evidence for cross-reactivity in cellular immune responses exists (3–6), how this will translate into cross-reactive protection from infection or disease progression remains controversial. Containment of challenge virus in a nonhuman primate was lost due to a single amino acid mutation in a dominant CD8\(^+\) T cell epitope (7). In HIV infection, CD8\(^+\) T cells place substantial selection pressure on the virus to evade this response, which can result in disease progression (8, 9). To overcome the ability of HIV to evade immune responses, an improved understanding of the relationship between viral diversity and protective immunity is needed.

CD8\(^+\) T cell epitopes can become unrecognizable to the immune system through mutation within an infected individual and throughout the epidemic if transmitted without reversion to the consensus sequence (10). Amino acid mutation can lead to aberrant epitope processing, suboptimal binding to HLA class I alleles, or inefficient TCR recognition. Substitutions at any residue in an epitope, and particularly the middle residues, can change the threedimensional epitope shape and affect TCR recognition (11). When compared with the index epitope, in vitro recognition of variants ranges from equivalent responsiveness to complete abrogation, with a range of intermediates, depending on the patient, epitope, and variant under evaluation (12–16). One recent study suggests accommodation of variation is a characteristic of responses restricted by protective HLA alleles (17). However, in the majority of studies, epitope variation has been compared in overnight ELISPOT assays or cytotoxic target cell killing assays, and therefore little is known about how other functional aspects of CD8\(^+\) T cell responses are affected by epitope variation.

The functional heterogeneity of virus-specific memory CD8\(^+\) T cells has long been recognized. Most data suggest the existence of distinct effector memory (Tem)\(^3\) and central memory (Tcm) CD8\(^+\) T cell populations, defined on the basis of timing of effector functions and homing potential of these cells (18). Although Tem are typically cytotoxic, home to the periphery, rapidly secrete IFN-\(\gamma\), and have low proliferative potential, Tcm home to the lymph node, secrete IL-2, retain proliferative capacity, and eventually differentiate to display effector functions. Recent studies have begun to assess multiple parameters of HIV-specific CD8\(^+\) T cell responses (19, 20). This is in part because although CD8\(^+\) T cells can contain HIV replication in some cases (21–23), ongoing viral replication and disease have also been found in the face of seemingly robust CD8\(^+\) T cell responses (24, 25). Several studies have found a lack of correlation between IFN-\(\gamma\) responses and viral load, even using whole HIV proteome screening (26–28). Moreover, specific defects have been found in proliferation and perforin expression in HIV-specific CD8\(^+\) T cells, while CD8\(^+\) T cells with a central

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\(^3\)Abbreviations used in this paper: Tem, effector memory CD8\(^+\) T cell; Tcm, central memory CD8\(^+\) T cell; SEB, *Staphylococcus aureus* enterotoxin B; SFU, spot-forming unit.

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memory phenotype (IL-2, proliferation) have been found to correlate with long-term nonprogression (29, 30).

In viral infections where quasispecies are typical, it was unknown whether pools of Tcm and Tem viral-specific CD8+ T cells share specificity for the same variant epitopes. We explored the impact of viral diversity on these subsets, defined herein using assays that measure cardinal features each (long-term proliferation, impact of viral diversity on these subsets, defined herein using Table II. Epitope characteristics

Patients were antiretroviral therapy-naive at the time of sampling and of East African decent. Clinical and demographic characteristics have been summarized (Table I). HLA class I allele haplotypes were determined by sequence-based typing.

**Materials and Methods**

**Patient characteristics**

All patients in this study are part of a well-characterized cohort of female sex workers based in Nairobi, Kenya (31), and all subjects gave informed consent to participate in this study in accordance with the Institutional Review Boards of the Universities of Manitoba and Nairobi. All patients were antiretroviral therapy-naive at the time of sampling and of East African decent. Clinical and demographic characteristics have been summarized (Table I). HLA class I allele haplotypes were determined by sequence-based typing.

**Peptides**

Epitope-specific responses were mapped using an HIV Env overlapping peptide library. Naturally occurring variations (four to six per epitope) of optimal epitopes were selected on the basis of frequency in selected Kenyan sequences (Ref. 32 and F. A. Plummer, unpublished data), and on variant frequencies in available HIV-1 group M sequences (http://hiv-web.lanl.gov/content/hiv-db/mainpage.html) (Table II). Peptides were synthesized via F-moc chemistry (Sigma-Genosys) and diluted in RPMI 1640 and DMSO (<1% final concentration). HIV-negative subjects (n = 6) were tested by ELISPOT and proliferation to rule out responses not specific to peptides; all subjects were negative in these assays (data not shown).

**Sample processing**

Fresh PBMC were isolated for use in ELISPOT and flow cytometric proliferation assays. Plasma viral loads were determined by Versant DNA 3.0 viral load assay (Bayer Roche). Proliferation supernatants were collected at day 7 for analysis of cytokine and chemokine levels.

| Table I. Patient characteristics |
|-------------------------|------------------|-----------------|-----------------|-----------------|-----------------|
| Age (Years) | Years Infected | CD4 Count (cells/μl) | Log Plasma Viral Load (copies/ml) | HLA-A Frequencies* | HLA-B Frequencies |
| Mean | 41.6 | 12.6 | 422.5 | 4.14 | 16 alleles (2301,28; 3001,28; 0301,17; 2902,17; 6601,17; 3002,11; 2301,11; 6802,11) | 17 alleles (4201,33; 07x,27; 5703,22; 801,17; 1401,17; 15x,17; 5801,11; 5301,11) |
| Median | 41 | 12 | 386 | 4.23 | 13 alleles (1701,47; 0401,24; 0701,17; 1801,17; 08x,17; 0201,12; 0002,12; 0702,12) |
| Range | 30–58 | 1–18 (n = 5 in early infection) | 61–1003 | 2.40–5.52 | |

*p Number of alleles represented in 18 of 19 patients studied, followed by alleles found in more than one individual, with the frequency (percentage) that the allele was observed. “x” indicates a number of alleles in this family were observed.

| Table II. Epitope characteristics |
|-------------------------|------------------|-----------------|-----------------|-----------------|-----------------|
| Epitope (Protein, Position in HXB2 Env) | Previously Shown HLA Restriction(s) | Frequency of Recognition as a 15-mer in Epitope Mapping Study (n = 61) | Frequency of Los Alamos Sequences (% of 630) | Frequency in Selected Kenyan Sequences (% of >50) | ELISPOT Responses/ Patients Tested | Proliferation Frequency Responses/ Patients Tested |
| IF9 (gp41, 843-51) | B*07 | 40% | IPPRIRQQGF | 23.7 | 40.0 | 10/11 | 6/11 |
| EL9 (gp41, 584-92) | B*14, A*32 | 10% | ERYLKDQQL | 49.2 | 40.0 | 2/2 | 2/2 |
| RL9 (gp41, 557-65) | Cw*03, B*51 | 15% | RAIEAQQHL | 50.0 | 51.3 | 5/5 | 1/5 |
| RS9 (gp120, 298-306) | B*07 | 15% | RPPNNTKES | 36.0 | 42.5 | 2/5 | 1/5 |
| TW9 (gp120, 37-45) | A*11, A*03, B*03 | 25% | TVYYGVFVW | 86.3 | 95.2 | 6/6 | 6/8 |

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ELISPOT assays

ELISPOT plates were coated with anti-IFN-γ mAb (Mabtech) and blocked with RPMI 1640 10% FBS (R-10). PBMC resuspended in R-10 were stimulated overnight with 10 μg/ml peptide, 1 μg/ml Staphylococcus aureus enterotoxin B (SEB), or medium alone (background). Plates were developed on day 2 of the assay as previously described (33). Dried plates were counted on an automated ELISPOT reader (Autoimmun Diagnostica). Responses were shown to be CD8 mediated by intracellular cytokine staining in a portion of patients (data not shown). A positive ELISPOT assay was defined as spot-forming units (SFU) greater than twice background, at least 50 SFU/million PBMCs, and a positive SEB response, and presented as background-subtracted SFU/million PBMCs.

Proliferation assays

PBMC were resuspended in PBS and loaded with CFSE as per the manufacturer’s protocol (Molecular Probes). CFSE-loaded PBMCs were stimulated for 7 days with 10 μg/ml peptide, 1 μg/ml SEB, or medium alone (background). On day 7, PBMCs were washed, fixed, and stained with CCR7-PE (BD Biosciences), CD45RA-energy-coupled dye (Beckman Coulter), and CD8-allophycocyanin (BD Biosciences). Samples were acquired on a FACS Calibur instrument and data were analyzed using CellQuest Pro (BD Biosciences). Some proliferation assays were stained for intracellular IFN-γ production. Golgiplug (BD Biosciences) was added 12 h before the completion of these assays; cells were permeabilized and stained with CD8-PerCP complex (BD Biosciences) and IFN-γ-allophycocyanin (BD Biosciences). At least 20,000 events were collected per sample. A positive proliferation assay was defined as CD8⁺ CFSE⁺⁺ cells (upper left quadrant) more than twice background levels and >1% of CD8⁺ cells and presented as background-subtracted percentages of proliferated CD8⁺ cells. The mean and median background unstimulated proliferation levels were 1.36 and 0.46% of CD8⁺ T cells, respectively, while the mean and median positive peptide responses were 11.07 and 5.74% of CD8⁺ T cells, respectively.

Cytokine bead arrays

Day 7 proliferation supernatants were tested for cytokine and chemokine levels (IFN-γ, IL-2, IL-7, IL-10, TNF-α, RANTES, and MIP-1β) using Cytokine Bead Array Flex Sets (BD Biosciences), according to the manufacturer’s instructions. These assays were acquired on a BD Biosciences LSR II flow cytometer and analyzed using FCAP 2.0 software.

Viral quasispecies cloning and sequencing

Where samples were available, proviral DNA was isolated from whole blood using DNA kits (Qiagen). Over 1 kb of HIV-1 Env was amplified in a nested PCR, using outer primers 5′-gcttatttctagatcccctcctg-3′ and 3′-aggtaacctgaggtctgactggaaagcctac-5′ and 3′-agttattctagatcccctcctg-3′ and inner primers 5′-gaagaggacagacgaggaagctca-3′ and 3′-ggcttaggcatctcctatggcaggaagaag-5′ in the first round, and inner primers 5′-ggcttaggcatctcctatggcaggaagaag-3′ and 3′-ggcttaggcatctcctatggcaggaagaag-5′ in the second round. Final PCR products were cloned using the TOPO-TA cloning system (Invitrogen Life Technologies) and up to 32 clones were sequenced per patient in both directions and run on an ABI Prism 3100 Genetic Analyser (Applied Biosystems). Sequences were analyzed using Sequencher version 4.0.5 (Gene Codes).

Results

We observed several proliferative and IFN-γ responses to Env epitopes and selected variants (Table II) and found no correlation between the magnitude of responses between assays (r² = 0.036, p = 0.72, Spearman rank correlation) (Fig. 1A). Many variants were not recognized in either assay (63 of 163 variants tested, Fig. 1B). Of the variants where at least one response was observed, a large proportion of responses were IFN-γ in the absence of proliferation (38 of 100, 38%), while proliferation in the absence of IFN-γ was rarer (12 of 100, 12%). For variant epitopes that elicited both IFN-γ and proliferation responses (50 of 100), we further classified these as strong or weak responses, using cutoffs of 10% proliferation and 500 SFU/million PBMCs (Fig. 1C). Although approximately half of variant responses correspond well (strong/strong or weak/weak), the remainder show some preferential recognition by one of the assays. Taken together, the lack of correlation and moderate degrees of correspondence suggest that variant cross-reactivity...
and SFU values per million PBMCs are shown in the epitopes in IFN-γ ELISPOT and CFSE-based proliferation assays. Percentages of CD8^+^/CFSE^low^ cells are shown in the upper left quadrant, and SFU values per million PBMCs are shown in the lower right corner of each FACS plot. CD8-allophycocyanin fluorescence intensities are shown on the y-axis, CFSE fluorescence intensity on the x-axis. All FACS plots are gated on CD8^+^ cells. Moving down each figure compares reactivity of particular epitope variants and moving horizontally compares the responsiveness of a particular patient to a panel of variants. Data pertaining to the following index peptides and variants (Table I) are shown: RPNNNTRKS (gp120 298-306) (A), RAIEAQQHL (gp41 557-65) (B), IPRRIRQGF (gp41 843-51) (C), and TVYYGPWW (gp120 37-45) (D).

FIGURE 2. Cross-reactivity of selected naturally occurring variants of epitopes in IFN-γ ELISPOT and CFSE-based proliferation assays. Percentages of CD8^+^/CFSE^low^ cells are shown in the upper left quadrant, and SFU values per million PBMCs are shown in the lower right corner of each FACS plot. CD8-allophycocyanin fluorescence intensities are shown on the y-axis, CFSE fluorescence intensity on the x-axis. All FACS plots are gated on CD8^+^ cells. Moving down each figure compares reactivity of particular epitope variants and moving horizontally compares the responsiveness of a particular patient to a panel of variants. Data pertaining to the following index peptides and variants (Table I) are shown: RPNNNTRKS (gp120 298-306) (A), RAIEAQQHL (gp41 557-65) (B), IPRRIRQGF (gp41 843-51) (C), and TVYYGPWW (gp120 37-45) (D).

may differ between overnight IFN-γ and proliferation assays, opposing CD8^+^ T cell phenotypes.

Several examples of this preference can be observed in the epitopes and variants studied. Four variants of RAIEAQQHL (gp41 557-65) differ at position five, where alanine (index peptide) is substituted for other small, hydrophobic residues (valine, glycine, and threonine). Despite this similarity, these substitutions have divergent effects on proliferative and IFN-γ responses (Fig. 2A, Table II). Although the index epitope is recognized by all five patients in ELISPOT, only one had a corresponding proliferative response. However, the inverse was observed for RAIEAQQHL, where two of five were ELISPOT positive and proliferation was observed in four of five. Where both assays were positive for both variants (patient 1616), RAIEAQQHL proliferation was 4-fold lower and ELISPOT 1.5-fold higher when compared with RAIEAQQHL. For patient 1625, RAIEAQQHL-specific ELISPOT responses were 10-fold higher than to RAIEAQQHL, but proliferation was only observed to the latter variant. The second most prevalent variant RAIEAQQHM (20% of HIV-1 group M) showed a similar pattern of reactivity as the index peptide, commonly recognized in ELISPOT (100%) but not proliferation. RAIEVQQHL is relatively rare, present in only 0.3% of HIV-1 M sequences, while RAIEAQQHL is present in 50%.

For RPNNNTRKS (gp120 298-306), RPYNNTTRQS generated substantial proliferative responses in all four individuals where proliferation was observed, while the ELISPOT data only corresponded in one instance (Fig. 2B). An interesting intermediate is observed in the immunogenicity of RPYNNTTRKS, where a slight increase in proliferation was observed in most patients, but not in ELISPOT. This suggests that while the Y substitution leads to some proliferation, when combined with the Q substitution, proliferation can increase by >10-fold (patients 1054 and 1500). Furthermore, as with RAIEAQQHL, RPYNNTTRQS is relatively rare in nature (Table I), and found almost exclusively in clade D sequences, which are relatively uncommon in Kenya.

The index epitope IPRRIRQGF (gp41 843-51) and IPRIIRQGA were the most commonly recognized of the tested variants (Fig. 2C, Table II). Although the frequencies of responses were similar between assays, proliferative responses to IPRRIRQGA were relatively stronger in most patients. For example, while patient 1054’s ELISPOT responses were comparable between IPRRIRQGF and IPRRIRQGA, proliferation to IPRRIRQGA was 8-fold higher. Patient 1430’s ELISPOT to IPRRIRQGF was 3-fold higher and proliferation was 1.5-fold lower as compared with IPRRIRQGA. Whereas ELISPOT responses to IPRRIRQGF and IPRRIRQGA correlate strongly (r^2 = 0.85, p = 0.0018), this correlation is lost in proliferation (r^2 = 0.72, p = 0.2) (data not shown). This indicates that although a position 9 mutation allows presentation, as is evident in ELISPOT, proliferation responses can nevertheless be affected, and also that seemingly cross-reactive variants may not be when another immunological parameter is measured. Some individual differences were observed for this epitope, recognition of IPRRIRQGF in both assays in patient 1500 only, and of IPRRIRQGA in patient 1430 only. As with previous epitopes, the proliferation variant IPRRIRQGA is relatively rare in nature (5.4% of HIV-1 M).

Underlined, red residues indicate amino acids varying from the index peptide epitope. Representative of data from five of five individuals (A), four of four individuals (B), three of five individuals (C), and six of six individuals (D) are displayed. Levels of RANTES, MIP-1β, and IFN-γ (picograms per milliliter) as measured in supernatants of day 7 proliferation assays are displayed for RPNNNTRKS and variants (B).
TVYYGVPVW (gp120 37–45) is unique in terms of displaying high levels of cross-reactivity and conservation (Fig. 2D). Virtually all variants were recognized by all individuals in both IFN-γ and proliferative assays. Some variability in the magnitude of ELISPOT or proliferation was observed between variants, but this was minimal as compared with other epitopes. The TVYYGVPVW version of this epitope is dominant in nature (86.3% of HIV-1 M, Table II), and in Kenyan sequences. Furthermore, when compared with the other epitopes tested, TVYYGVPVW variants comprise a large proportion of instances where IFN-γ and proliferation correspond well with one another (Fig. 1).

We assessed HIV-specific Tcm CD8+ T cell responses to determine expression of immune molecules in addition to proliferation. Several IFN-γ, RANTES, TNF-α, and MIP-1β responses were measured in day 7 proliferation supernatants. Variants with the strongest proliferation responses also usually expressed the highest chemokine and cytokine levels at day 7 (data not shown). For RPNNNTRKS (Fig. 2B), chemokine and cytokine levels at day 7 are selectively increased in proliferation-positive assays (n = 3 patients). Intracellular cytokine staining for IFN-γ in proliferating cultures demonstrates that in all double-positive assays, IFN-γ production occurred in the proliferating subset of CD8+ T cells (data not shown). Together, these data suggest that proliferating Tcm are differentiating in vitro to express multiple CD8+ T cell effector functions.

The clinical relevance of these responses was explored by correlating available CD4 cell counts (n = 16) and plasma viral loads (n = 14) with optimal proliferation and IFN-γ responses, and the presence or absence of proliferation (Fig. 3). There were no trends observed between viral loads and either optimal proliferation (r² = −0.343, p = 0.28) or IFN-γ responses (r² = 0.098, p = 0.76). CD4 cell counts and optimal proliferation responses demonstrated a trend toward a positive correlation (r² = 0.422, p = 0.09), but no trend between CD4 cell counts and optimal IFN-γ response was observed (r² = 0.103, p = 0.69). We next compared overnight IFN-γ responders with CD4 cell counts above or below 400/μl for the presence or absence of proliferation to at least one variant. Although all nine individuals with CD4 counts >400 had proliferation responses, only three of seven individuals with CD4 counts <400 proliferated (p = 0.019, Fisher’s exact test, Fig. 3B), suggesting that proliferation responses are more often absent in individuals with lower CD4 counts.

RPYNNTRQS is rarely observed in nature (Table II) and is almost entirely confined to clade D sequences, which have been observed at low frequencies in Kenya. To determine whether patients with preferential proliferation responses to RPYNNTRQS were infected with this variant at the assay time point, even at low levels, we cloned and sequenced the HIV quasispecies in this region. By sequencing 30–41 clones, we were able to detect quasispecies strains as infrequent as 2.4–3.3% of the overall autologous quasispecies. These analyses show that for all patients where RPYNNTRQS gave optimal proliferation responses, there was a complete absence of this variant in the autologous quasispecies (Table III). Patient 1500’s primary virus is the index peptide strain of IFN-γ response of 100 SFU/million PBMC was made. Interestingly, the variant to which this patient’s strongest IFN-γ response was made (RPNNNTRKS, 565 SFU/million PBMC), is also absent from the quasispecies at the assay time point. Patient 1410 is also responding to variants not currently present in her autologous quasispecies, although day 7 MIP-1β and IFN-γ responses are observed to RPNNNTRKS, which is similar to the autologous variant RPNNNTRKG. In patient 1054’s homologous quasispecies, RPYNNTRQS is the only variant observed (not tested for in vitro recognition). Together, these data suggest that the presence of RPYNNTRQS at low levels in the autologous quasispecies is unlikely to be responsible for the strong proliferation responses observed to this variant in vitro.

**Discussion**

The extreme genetic diversity of HIV and the failure of immune responses to provide protection against disease progression remain...
at the forefront of obstacles to designing an effective HIV vaccine. By comparing the impact of HIV diversity on T cell responses that have been shown protective against disease progression vs those that may not be protective, we find substantial differences in the consequences of this genetic diversity. Our study demonstrates that the consequences of HIV variation differ between assays measuring key attributes of Tem and Tcm CD8+ T cell subsets and further that different epitope variants may elicit different types of memory responses. Although recent studies have compared CD8+ T cell IFN-γ and cytotoxic responses to variant epitopes (34), these Tem assays usually correlate well. Our study is the first to our knowledge that demonstrates the impact of HIV diversity in the setting of polarized Tem and Tcm CD8+ T cell responses. It is further notable that the lack of correlation is not due to global deficiency in proliferation or IFN-γ responses. Rather, we observed multiple instances of differential proliferation and IFN-γ responses that was variant dependent, suggesting a linkage between variant specificity and resulting response phenotype.

The trend toward correlation between CD4 counts and proliferation, and not overnight IFN-γ, suggests that proliferation responses may play a role in nonprogression during HIV infection. However, because this is a study in chronically infected patients, it could also be that maintenance of high CD4 counts is what explains the presence of HIV-specific CD8+ T cell proliferation. The lack of correlations between proliferation or overnight IFN-γ responses and plasma viral loads may be due to low study numbers or suggestive that these responses are not directly involved in control of HIV replication.

The absence of RPYNNTRQS in the HIV quasispecies of patients with strong, preferential proliferation to this variant may have interesting connotations for vaccine design. It is possible, particularly since we sequenced virus from peripheral blood, that these patients were infected transiently with an HIV strain containing RPYNNTRQS which subsequently escaped through mutation, and that the contemporary proliferation responses are long-term memory responses. However, this may be unlikely given the rarity of this variant in nature (Table II). A more likely hypothesis is that immune responses were mounted to variants in the infecting quasispecies and those CD8+ T cells have TCRs that are cross-reactive with RPYNNTRQS in vitro. The propensity of certain variants for proliferation may indicate that by including these variants in HIV vaccines, one would be able to preferentially induce HIV-specific CD8+ T cells with Tcm-like phenotypes. Although the relative rarity of these variants may detract from their abilities to provide broad population coverage, it is also possible a variant that generates strong in vitro proliferation would be capable of cross-reacting with dominant circulating HIV sequences in a protective manner. Additional experiments to identify subjects infected with HIV strains containing proliferation variants, and analyses of their proliferative and IFN-γ responses, are underway.

Responses to nearly every variant suggests that the majority of variants tested retain the characteristics needed for HLA presentation. We observe multiple HLA restrictions per epitope and no allele-specific patterns of cross-reactivity, suggesting that presentation of variants by each allele is maintained and comparable. This leads to the hypothesis, supported by the above data (Fig. 2), that TCR-variant interactions are involved in determining the response phenotype. RAIEAQQHL data demonstrate that even relatively conserved amino acids can lead to dramatic differences in recognition between Tcm and Tem assays. The intermediate levels of proliferation to RPYNNTRKS as compared with RPYNNTRQS suggest a change in epitope shape that impacts TCR recognition in an assay-dependent manner. This data further confirms that substitutions at multiple residues can influence CD8+ T cell recognition.

Whether Tcm and Tem pools with unique TCRβ clonotypes are responding to different variant epitopes, or whether phenotypically distinct responses arise through functional plasticity of the same TCRs, remains to be seen. Some flexibility in TCR recognition of epitope variants has been observed in HIV-specific CD8+ T cell responses, particularly in HIV-2 infection (35). However, there is also evidence that the initial HIV-specific CD8+ T cell response contains several TCR clonotypes (36). Because infection with a heterogeneous quasispecies has been observed, particularly in female sex workers (37), and quasispecies diversity tends to accumulate in chronic infection, multiple TCRVβ could theoretically be directed toward different variants within the quasispecies in phenotypically distinct fashions. In this scenario, expanded pools of Tcm and Tem cells with different variant specificities may arise independently at various time points in infection and coexist in parallel pools of cells with different roles in the immune response. This is supported by observations of circulating influenza-specific Tcm and Tem subsets with distinct TCRVβ usages (38), and in this study by the differences in assay kinetics between proliferation and overnight IFN-γ assays. Expression of IFN-γ and other effector molecules in proliferating Tcm at day 7, in the absence of overnight IFN-γ, emphasizes that the timing of responses may be important. Although recent studies have examined polyfunctional responses in short-term assays, whether these responses are representative of the true Tcm pool remains unknown.

The extensive cross-reactivity and conservation of TVYYGGVPW may represent a model of how the host immune response can accommodate the sequence diversity of HIV and therefore be suitable for use in HIV vaccines. Not only is this epitope highly conserved, but any variation that were to exist or arise during infection would likely be covered by a multifunctional immune response. Further identification of such epitopes that cover a broader range of HLA alleles may be an important goal for HIV vaccine design.

The findings of the present study support the contemporary notion that HIV vaccine candidates require thorough immunological evaluation. Although overnight IFN-γ may be appropriate for screening purposes, a more comprehensive functional characterization should follow. This is especially prudent as long-term memory responses appear important for protection in successful vaccines. In volunteers followed many years post-smallpox vaccination, vaccinia-specific T cell responses were still observed with a half-life decay of 8–15 years (39). Assessment of Tcm and Tem responses is especially relevant in HIV infection, where not all responses are effective and several functional defects have been shown to be involved in progression to AIDS (40).

Although the functional dichotomy between Tcm and Tem CD8+ T cells has long been known, we demonstrate apparent differences in specificity between these pools in response to variants of commonly recognized epitopes in a chronic human viral infection. The possibility of separate pools of Tcm and Tcm CD8+ T cells with propensities for recognizing different variant epitopes in a viral quasispecies may open future avenues of research that could use these variant epitopes to elicit protective immune responses through vaccination. HIV genetic diversity remains a major obstacle to successful vaccination, and here we present a more comprehensive assessment of how, in a natural setting of HIV variation, substitutions within HIV epitopes affect protective immune responses.

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