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Different Vaccine Vectors Delivering the Same Antigen Elicit CD8+ T Cell Responses with Distinct Clonotype and Epitope Specificity

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Prime-boost immunization with gene-based vectors has been developed to generate more effective vaccines for AIDS, malaria, and tuberculosis. Although these vectors elicit potent T cell responses, the mechanisms by which they stimulate immunity are not well understood. In this study, we show that immunization by a single gene product, HIV-1 envelope, with alternative vector combinations elicits CD8+ cells with different fine specificities and kinetics of mobilization. Vaccine-induced CD8+ T cells recognized overlapping third V region loop peptides. Unexpectedly, two anchor variants bound H-2Dd better than the native sequences, and clones with distinct specificities were elicited by alternative vectors. X-ray crystallography revealed major differences in solvent exposure of MHC-bound peptide epitopes, suggesting that processed HIV-1 envelope gave rise to MHC-I/peptide conformations recognized by distinct CD8+ T cell populations. These findings suggest that different gene-based vectors generate peptides with alternative conformations within MHC-I that elicit distinct T cell responses after vaccination. The Journal of Immunology, 2009, 183: 0000–0000.

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3 Abbreviations used in this paper: Env, HIV-1 envelope; Ad, adenovirus; βl, β1, microglobulin; BCG, Mycobacterium bovis bacillus Calmette-Guérin; CM, central memory; EM, effector memory; V3, third V region.
for HIV-1 transmission, in contrast to the CXCR4-tropic HIV-I_{HIN} (9, 16). We have used Env as the substrate for recombinant vector-based vaccines and have studied prime-boost combinations with DNA or recombinant Mycobacterium bovis bacillus Calmette-Guérin (rBCG) priming, followed by recombinant adenovirus (rAd) boosting.

In this study, we first identified functional peptides related to the immunodominant V3 loop peptide of HIV-I_{BaL}, that bind well to the H-2D\d restriction element. These peptides were used to make a set of H-2D\d-peptide tetramers that enabled the detection and characterization of disparate subpopulations of HIV-specific CD8\T cells induced by DNA or rBCG priming before rAd boosting compared with rAd Env vector immunization alone. Structural analysis and TCR sequencing were used to examine the molecular basis for differential recognition of specific H-2D\d-peptide complexes by distinct populations of CD8\T cells.

Materials and Methods

Cell culture and peptide induction of surface MHC-I expression

A TAP-defective cell line, LKD8, expressing H-2D\d (21), was propagated in DMEM supplemented with 10% FCS, 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 1% nonessential amino acids, and 50 \mM 2-ME. Cell cultures were incubated with indicated peptides overnight either with or without the addition of human β2-microglobulin (β2m). Cells for flow cytometric analysis in all studies were incubated with the viability dye ViVid (Molecular Probes) (22). Subsequently, cells were stained with anti-CD8 (BD Pharmingen), anti-CD49d (2B11; Beckman Coulter), anti-CD107b (53A3; BD Pharmingen), and anti-CD107a (10e6; Beckman Coulter). Stained cells were analyzed using a modified BD LSR II flow cytometer with FlowJo software (Tree Star), and the results are shown as Δ mean fluorescence intensity.

Production and preparation of rBCG, rAd, and plasmid DNA-expressing modified HIV-1 Env

We used a previously characterized vector encoding gpl140ΔCFI31V12 and prepared a rBCG vaccine expressing this modified Env gene.

Immunization

BALB/c mice purchased from The Jackson Laboratory were maintained in the Vaccine Research Center Animal Care Unit, National Institute of Allergy and Infectious Diseases, National Institutes of Health, under pathogen-free conditions. The animal studies were approved by the Vaccine Research Center Animal Care and Use Committee and conducted in accordance with all federal and National Institutes of Health policies and regulations. Mice were immunized with 10\5 viral particles of rAd and primed with DNA or rBCG intramuscularly (supplemental Table S-I).

Flow cytometric analysis of tetramer staining and intracellular cytokine production

PBMC and spleen cells (10\6) were simultaneously and sequentially incubated with PE- and/or allophycocyanin-conjugated H-2D\d tetramers containing human β2m for 15 min at room temperature; cells were then stained for CD3 (BD Pharmingen), CD8 (BD Pharmingen), CD16/32 (Beckman Coulter), CD4 (BD Pharmingen), CD62L (eBioscience), CD127 (eBioscience), KLRG-1 (BD Pharmingen), CD8 (BD Pharmingen), CD16/32 (Beckman Coulter), CD44 (BD Pharmingen), CD62L (eBioscience), CD127 (eBioscience), KLRG-1 (BD Pharmingen), CD8 (BD Pharmingen), and CD16/32 (Beckman Coulter). The following synthetic peptides were used for tetramer production: Env-modified PA9 (IGPGRAFYA), Env-modified P10 (IGPGRAFYTT), native PT9 (IGPGRAFYTT), native PT9 (IGPGRAFYTT), P1810 (RIGPGRAFYVT), and motif control (AGPARAAAL) (National Institute of Allergy and Infectious Diseases Tetramer Core Facility). In some experiments, immune spleen cells were incubated with the peptide (2 \mM), anti-CD28 (2 \mM) for 24 h to induce T cell proliferation. Data analysis and statistics

All comparisons between recombinant and control groups and between immunization groups were conducted using ANOVA tests assuming variances with the JMP program (SAS Institute). Data are expressed as the mean ± SD.

Results

Identification of variant V3 peptide epitopes elicited by HIV-1_{BaL} Env immunization

Because previous studies of the immune response in BALB/c mice to HIV-I_{HIN} Env revealed that a decamer peptide spanning the V3 loop, RGPGRAFVTI (P18I10), was immunodominant for the H-2D\d-restricted CD8\T cell response (20), we asked whether the HIV-I_{BaL} Env (29) was cross-reactive, and whether the two responses were of comparable magnitude (Fig. 1A and Table S-I). Mice primed with HIV-I_{BaL} expressed in a DNA plasmid vector and boosted with rAd showed a recall response to the pool of overlapping 15-mer peptides representing the entire BaL Env protein and to the specific BaL-derived peptides PT9 and PT10, but did not respond to the P18I10 peptide. Mice immunized with the corresponding HIV-I_{HIN} vaccine responded to the cognate peptide pool as well as to P18I10, but failed to respond to either PT9 or PT10. However, the recall response (as measured by the percentage of CD8\T cells producing intracellular IFN-γ) to either native PT9 or PT10 in HIV-I_{BaL}-immunized mice was consistently weaker than the response to the P18I10 peptide following HIV-I_{HIN} immunization (5.6 ± 2.8% for PT9 and 4.6 ± 2.5% for PT10 as compared with 15.3 ± 1.5% for P1810). Thus, responses to each of the vaccines were specific for the delivered peptide epitopes.

Inspection of the amino acid sequence of the HIV-I_{BaL} V3 loop suggested that processed peptides derived from this region might not bind to the H-2D\d-presenting element to the same degree as the immunodominant peptide derived from the HIV-I_{HIN} isolate.

TCR clonotype analysis

Small, live CD161+/CD19+/CD32+ CD8\T tetramer-positive spleen cells (10,000 per condition) were sorted to greater than 98% purity using a modified FACS DIVA (BD Biosciences) (25). Unbiased analysis of TCR gene expression was conducted, as described previously, using a strand-switch anchored RT-PCR with TCRα and TCRβ C region primers (26). All sequences were analyzed with reference to the international ImMunoGeneTics information system website V-align (http://imgt.cines.fr).

Protein expression, structure determination, and crystallographic refinement

The soluble extracellular segment (aa 1–275) of H-2D\d was expressed in Escherichia coli as inclusion bodies, solubilized, and refolded in vitro with similarly expressed murine β2m and either the PA9 or P10 peptide, essentially as described previously for the H-2D\d and HIV-I_{BaL} complex (27). Crystals were frozen in liquid nitrogen after dipping in paratone oil and examined by synchrotron radiation at beamline X29A at the National Synchrotron Light Source at Brookhaven National Laboratory. Data were collected from single crystals in a nitrogen stream at 100 K, and were indexed, scaled, and merged using HKL2000. The PA9-containing complex crystallized in space group P2_1_2_1 with one complex (H chain, 2m) and either the PA9 or PI10 peptide, in the asymmetric unit, and a Matthews coefficient of 2.50. The P10 complex in space group P2_1_2_1 also had one complex per asymmetric unit, and a Matthews coefficient of 3.14. Data collection and refinement statistics are reported in Table S-III. The structures were readily solved by molecular replacement with MOLREP of the CCP4 suite, using the H-2D\d/β2m complex from IQ03 from which both peptide and Ly49A had been removed. Refinement was conducted in CNS 1.2, manual fitting of each of the peptides was accomplished with Coot (28), and molecular graphics figures were prepared with PyMOL (http://pymol.sourceforge.net/). The PA9 and P10 complexes were determined to 2.4 and 2.1 Å, respectively, with corresponding R_{crys}/R_{free} of 22.8/27.5 and 21.9/25.1. Coordinates of the refined models and structure factors have been deposited in the protein data bank (D\d-PA9, 3E6F and D\d-PI10, 3E6H). Side chain accessibility was calculated with AREAIMOL of the CCP4 suite.

Data analysis and statistics

All comparisons between recombinant and control groups and between immunization groups were conducted using ANOVA tests assuming variances with the JMP program (SAS Institute). Data are expressed as the mean ± SD.

The online version of this article contains supplemental material.
Because PA9 binds H-2D\(^d\) better than the putative endogenously generated PT9, and because PI10 binds H-2D\(^d\) better than the putative endogenously generated PT10, we expected that tetramers prepared with these variant peptides would have greater stability and would be more effective reagents with which to monitor specific T cells. However, it remained possible that subtle differences in either the proportion of molecules bound by the higher affinity peptides or the conformations of the epitopic residues of these peptides when bound to H-2D\(^d\) might influence either the specificity of the T cells elicited or the ability of such T cells to be detected with specific tetramers.

We analyzed the fine specificity of the HIV-1\(_{\text{BaL}}\) response to PA9, PT9, PT10, and PI10 using intracellular cytokine staining for IFN-\(\gamma\), IL-2, and TNF-\(\alpha\) (Fig. S1). In all vaccine vectors, the amino acid sequence encoded in the functional epitope was IG PGRAFYTT, which includes both PT9 and PT10. The native PT9 and PT10 peptides, which have apparently lower affinities for H-2D\(^d\), elicited no triple cytokine-positive CD8\(^+\) T cells in HIV-1\(_{\text{BaL}}\) Env-immunized mice. However, the higher affinity, anchor-variant peptides, PA9 and PI10, elicited a significant proportion of triple-positive cells (46 and 47\%, respectively). All the V3 peptides specifically stimulated immune CD8\(^+\) T cells and not CD4\(^+\) T cells (data not shown), whereas a pool of HIV-1\(_{\text{BaL}}\) Env gp120 peptides stimulated both CD4\(^+\) and CD8\(^+\) T cells.

**FIGURE 1.** CD8\(^+\) T cells from HIV Env-immunized mice show specificity for individual virus isolates, exhibit distinct potencies, and recognize variant antigenic peptides that associate differently with H-2D\(^d\). A. Mice were primed with DNA encoding either HIV-1\(_{\text{BaL}}\)- or HIV-1\(_{\text{IIIB}}\)-modified Env and boosted with rAd expressing either HIV-1\(_{\text{BaL}}\) or HIV-1\(_{\text{IIIB}}\) Env, as described in Materials and Methods and Table S-II. Splenocytes were harvested 14 days after boosting, stimulated in vitro for 6 h with the V3 epitope peptides (2.5 \(\mu\)M) P18I10 (RGPGRAFYVT), PT10 (IGPGRAFYTT), or PT9 (IGPGRAFYVT); pools of overlapping 15-mer peptides spanning HIV-1\(_{\text{BaL}}\)-Env (Bal peptide pool) or HIV-1\(_{\text{IIIB}}\)-Env (IIB peptide pool); or an irrelevant Ebola Env peptide as a control (29); and then stained for intracellular IFN-\(\gamma\) production, as described previously (22). Functional profiles of CD8\(^+\) T cell responses to native and variant peptides in HIV-1\(_{\text{BaL}}\)-Env-vaccinated mice are shown in Fig. S1. B. Peptide-induced surface expression of H-2D\(^d\) with V3 loop-related peptides. TAP-negative LKD8 cells were incubated with the indicated concentrations of each peptide and stained with mAb 34-5-8, as described in Materials and Methods. Control peptide is WKEATTTLLCASDAK. Results are shown as the mean fluorescence intensity over background (\(\Delta\) mean fluorescence intensity). Red lines indicate peptides used for tetramer construction.

Therefore, we examined whether peptides from this region of the BaL V3 loop would bind to H-2D\(^d\) in an epitope stabilization assay. The H-2D\(^d\) peptide-binding motif, determined first by analysis of peptides that copurify with H-2D\(^d\) (30, 31) and further characterized in x-ray structures of H-2D\(^d\) complexed with the IIB-depleted peptide P18I10 (27), consists of G at position 2, P at position 3, R at position 5, and a C-terminal hydrophobic residue at position 9, 10, or 11. Because H-2D\(^d\) is known to bind well to both nonamer and decamer peptides, and because the C-terminal anchor residue strongly influences peptide binding, we evaluated a set of synthetic 9-mer and 10-mer peptide variants for their ability to bind to H-2D\(^d\) (Fig. 1B). Using LKD8, a TAP-deficient H-2D\(^d\)-positive cell line, as an indicator (21), we observed a hierarchy of binding, as follows: PA9 > PL9 > P9 > P18I10 > PT9 > PI10 > PT10. Thus, several nonamer and decamer variants of the PT9 and PT10 sequences found in the BaL Env immunogen bind H-2D\(^d\) with higher apparent affinity than either the native 9-mer or 10-mer.
of the Dd-PI10 tetramer (Fig. 2, B and C), and their ability to produce functional responses after immunization with HIV-1 
vector vaccines (Fig. S1), we further analyzed immune responses with the two tetramers that were not cross-reactive, Dd-PA9 and Dd-PI10. Responses were measured following different immunization schemes with rAd, DNA, and rBCG vectors, either alone or in DNA/rBCG prime-rAd boost combinations (Fig. 3). rAd elicited higher frequency responses than either DNA (p < 0.001) or rBCG (p < 0.0001) as detected by the percentage of Dd-PA9 tetramer-positive cells (Fig. 3A). A similar result was observed, at lower magnitude, with the Dd-PI10 (Fig. 3B) and Dd-PT10 tetramers (data not shown).

To study the effects of prime-boost immunization, we next monitored the CD8+ T cell responses after DNA/rAd and rBCG/rAd (Fig. 3C). Boosting of DNA- or rBCG-primed mice with rAd (Table S-II) resulted in differential timing of the peak response, as detected with either the Dd-PA9 or Dd-PI10 tetramers. At 4 days after the boost, the Dd-PI10-responsive CD8+ T cell subset in the rBCG/rAd group was preferentially elicited (mean value, 5.8 ± 1.5%; blue lines in left panel in Fig. 3C), and lower levels were achieved with DNA/rAd or rAd alone without priming (red and green lines in left panel in Fig. 3C, respectively; both p < 0.0001). This Dd-PI10 tetramer-binding CD8+ T cell population decreased by day 8 postboost and remained stable until day 90. A switch in the dominance of CD8+ T cell populations from Dd-PI10 to Dd-PA9 specificity in mice immunized with rBCG/rAd was observed 14 days after rAd vector boosting. Although the peak Dd-PI10 response occurred earlier than the peak Dd-PA9 response (day 4 as compared with day 14), the magnitude of the Dd-PA9 response was significantly greater than the maximal Dd-PI10 response (24.6 ± 10.5% as compared with the Dd-PI10 response described above). The rBCG-vector control/rAd group showed results very similar to those for the rAd-alone vector group (data not shown).

The maturation and differentiation status of Dd-PA9 and Dd-PI10-specific CD8+ T cells was compared between DNA/rAd and rBCG/rAd regimens at the peak of the immune response 14 days after rAd boosting (Fig. 4). The majority of the Dd-PA9-specific CD8+ T cells in the spleen showed an effector cell CD127lowCD62LlowCD44high phenotype in both the DNA/rAd and rBCG/rAd immunization protocols (57.6 and 73.5% of gated cells, respectively; Fig. 4A). The remaining Dd-PA9-specific CD8+ T cells were CD127highCD62LlowCD44high effector memory (EM; 33.7 and 16.4% in DNA/rAd and rBCG/rAd, respectively) and CD127highCD62LhighCD44high central memory (CM; 0.76 and 3.98% in DNA/rAd and rBCG/rAd, respectively). In contrast, of the Dd-PI10-specific CD8+ T cells analyzed at the same time, the majority were EM (66.7 and 31.6% in DNA/rAd and rBCG/rAd, respectively) and CM (11.6 and 28.9% in DNA/rAd and rBCG/rAd, respectively). Thus, at the peak of the immune response, Dd-PA9-specific CD8+ T cells were substantially skewed toward more differentiated effector phenotypes relative to the contemporaneous Dd-PI10-specific CD8+ T cell populations (Figs. 4C and 5).
S2), indicating clear differences between the two distinct CD8⁺ T cell subsets according to MHC-I/peptide specificity.

### Analysis of TCR gene expression in Env V3-specific CD8⁺ T cell populations

To characterize the TCR gene usage of these tetramer-positive cells at 14 days postboost, we analyzed TCRA and TCRB gene expression at the clonotypic level. In rBCG/rAd-immunized mice, the tetramer-positive populations were clonotypically distinct (Fig. 5). Remarkably, Dd-PA9-specific CD8⁺ T cells sorted from two different mice immunized with rBCG/rAd (Table S-II), animals were boosted with rAd (DNA/rAd, red dotted lines). Animals were also immunized with rBCG/rAd (blue dotted lines) or rAd without priming (green dotted lines), as shown in Table S-II, and analyzed for the generation of CD8⁺ T cells specific for Dd-PT10 (left panel) and Dd-PA9 (right panel). Dotted lines show data for each animal, and solid lines show mean values of five animals in each group. Dd-PT10 responses were lower, but showed a similar pattern to those specific for Dd-PA9 (data not shown).

**MHC-peptide structures suggest a basis for recognition by different T cell populations**

To gain further insight into the nature of the MHC-I/peptide epitopes that constituted these different H-2D^d/peptide tetramers, we determined the high resolution x-ray crystal structures of H-2D^d complexed with PA9 and with PI10, and compared these with the previously published structure of H-2D^d bound to the related HIV-1 envelope peptide P18I10 (Figs. 6 and S3). Details of the structure determination and crystallographic refinement are provided in Materials and Methods and in Table S-II. The structures of PA9 and PI10, each complexed with H-2D^d and murine β₂m, were determined to a resolution of 2.4 and 2.1 Å, respectively. P18I10 bound to H-2D^d has been structurally characterized as the trimeric H chain/β₂m/peptide complex (27) and also with the same peptide in complex with the murine NK cell receptor Ly49A (32). We compared the two newly determined Dd-PA9 and Dd-PI10 structures with Dd-P18I10. The comparisons are focused on the α1α2 domain and bound peptide to illustrate the conformational differences of the three different bound peptides. For all three structures, the N-terminal five residues of the peptides superpose precisely (root mean square deviation of 0.053 to 0.127 Å for the three pairwise superpositions), but there is considerable
FIGURE 4. Phenotypes of D^d-PA9-specific and D^d-PI10-specific CD8^+ T cells elicited by immunization with DNA/rAd and rBCG/rAd. The differentiation phenotypes of tetramer-binding CD8^+ T cells specific for D^d-PA9 (A) and D^d-PI10 (B), harvested from the spleens of immunized mice at day 14 postboost (Fig. 3C), were analyzed by flow cytometry. Representative data are shown in A and B. The overall phenotypic distribution of the CD8^+ T cell population is shown in the second column; the fourth column shows the same analysis for the tetramer-positive events. Both sets of data are combined in the third column, which shows the tetramer-positive events (yellow) superimposed on the total CD8^+ T cell population (gray density cloud) in a bivariate plot of CD127 vs CD62L. C, Shows the phenotypic subset distribution of D^d-PA9-specific and D^d-PI10-specific CD8^+ T cells elicited by DNA/rAd (upper panels) and rBCG/rAd (lower panels) in each animal gated according to standard definitions. CM, CD44^highCD127^highCD62L^high; EM, CD44^highCD127^lowCD62L^low; E (effector), CD44^highCD127^lowCD62L^low. The CM cells were further defined to be CCR7^-KLRG-1int (data not shown).
variation in the remaining residues. The complex with PA9 (Fig. 6, A, D, G, and J) shows exposure of peptide residues F7 and Y8, with the C-terminal A9 buried in the F pocket. With the additional residue found in the 10-mers, PI10 (Fig. 6, B, E, H, and K) and P18I10 (Fig. 6, C, F, and I), residues 6, 7, 8, and 9 buckle out. PI10 forms an aromatic stacking interaction between F7 and Y8, hiding much of Y8 from exposure to the TCR. P18I10, with the added flexibility of the additional residue, but lacking the potential for the aromatic stacking found in PI10, thrusts F7 back toward the helix. Calculations of solvent-exposed surface area per peptide residue (Fig. S3) are consistent with the visual impression: PA9 exposes residues G4, A6, and Y8; PI10 exposes G4, A6, F7, and T9; and P18I10 exposes G4, A6, F7, and T9. These conformational differences may explain the difference in the binding of D²-P10, D²-PA9, and D²-P18I10 tetramers to specific CD8⁸⁺ T cells, and the differences in exposure of peptide residues 7, 8, and 9 between PA9 and PI10 offer a conceptual framework for possible differences in priming between PT9 and PT10 (see Discussion).

Discussion

Virus-specific CD8⁸⁺ T cells that arise after MHC-I-restricted presentation of peptides derived from the Env play a key role in the recognition of HIV-1-infected cells and in the control of virus replication. Although most viral proteins contain a large number of potential MHC-binding peptide epitopes, in general only a few of these epitopes evoke significant CD8⁸⁺ T cell responses. In a number of mouse strains, and also among human CD8⁸⁺ T cell responses, epitopes derived from the V3 loop of the HIV envelope are overrepresented (19, 33). In this study, we examined CD8⁸⁺ T cells elicited by different HIV-1BaL vector vaccines using different H-2Dd/peptide tetramers. We suspected that the predicted immunodominant peptide of this isolate, PT10 (IGPGRAFYTT), or possibly the nonamer contained within, PT9 (IGPGRAFYT), might bind H-2Dd with low affinity. Therefore, we explored the endogenous peptides as well as a set of related peptide variants for their ability to bind H-2Dd (Fig. 1B) and elicit potent functional responses after immunization with HIV-1 BaL vector vaccines (Fig. S1).

What is the difference between these tetramer-specific CD8⁸⁺ T cell subsets? First, differences among these MHC-I/peptide complexes in the size of the exposed peptide loop as well as in the conformation of the exposed peptide present alternative targets for
TCR engagement. The difference in the reactivity of CD8⁺ T cells specific for D₄-P10 and D₄-PT10 is more subtle, but the finding that PT10 binds H-2D⁺ with significantly lower affinity supports the view that it binds differently than P10, and leads to the conclusion that the PT10 peptide is presented in a distinct conformation from P10. Second, clear differences were apparent in the kinetics with which CD8⁺ T cells specific for D₄-P10 appeared relative to D₄-PA9-reactive CD8⁺ T cells; these distinct mobilization kinetics confirm differential Ag recognition by D₄-P10-specific and D₄-PA9-specific CD8⁺ T cell populations. Several explanations are possible for these differential kinetics. One is that the proportion of D₄-P10-reactive cells after priming is greater and that these expand more prominently following the boost. Another is that the D₄-PA9-reactive cells are of higher avidity for their cognate MHC/peptide complex, and, as shown in another model system (34–36), may eventually outcompete their lower avidity counterparts (37, 38). It is also possible that both mechanisms play a role in our immunization scheme, but additional experiments will be needed to assess both the relative proportion of reactive T cells as well as the relative avidity of these different CD8⁺ T cell populations for their respective Ags.

The maturation and differentiation status (39, 40) of the two V₃-specific tetramer-positive CD8⁺ T cell populations was clearly different at the peak of the immune responses. D₄-P10-specific CD8⁺ splenocytes were CM rich, whereas the corresponding D₄-PA9-specific cells behaved much more like a primary population with higher peak effector cell numbers, thereby suggesting a greater degree of maturation and expansion. Together with the tetramer-specific double-staining profiles discussed above, it is apparent that HIV-1 Env-specific immunodominant CD8⁺ T cell responses are mediated by distinct V₃ epitope-specific subsets, each with a different phenotype. It is noteworthy in this context that the ability of rAd vectors to generate CM seems to be important. In this mouse study, modeling the events that might be occurring in vaccinated humans, boosting DNA-prime animals with rAd induced more notable maturation of dominant D₄-PA9-specific CD8⁺ T cells into effector cells. In contrast to this effector cell differentiation, generation of CM was significantly decreased in this immunization regimen. Lately, EM has been reported to be responsible for protection of animals from SIV challenge (36). Because low-dose rAd boosting increased the proportion of CM (data not shown) and because CM have been shown to expand in vivo and mediate protective immunity against pathogenic virus (39, 41), both low- and high-dose immunizations with rAd might be worth testing to determine whether one regimen will be preferable in generating more effective protective T cell immunity. In general, further studies in nonhuman primate challenge models will be needed to permit evaluation of the character of the T cell response that contributes to protection, and such studies have been initiated.

Of particular interest is the observation that the different Env vaccine/immunization regimens elicited CD8⁺ T cell populations with different fine specificities and clonotype usage. The x-ray crystallographic analysis of the D₄-PA9 and D₄-PT10 complexes revealed a structural basis for these differences in peptide specificity, suggesting that alternative vaccination regimens lead to differences in Ag processing and presentation that in turn elicit distinct populations of Ag-specific T cells. Structural differences in the size of the exposed peptide loop (nonamer vs decamer) and in the conformation of the exposed peptide were apparent. Although we have been unable to obtain crystals of either D₄-PT9 or D₄-PT10, we speculate that D₄-PT9 has a stable peptide conformation very similar to that of D₄-PA9. D₄-PT10, however, lacking the strong p10 isoleucine anchor residue, may have at least two distinct conformations of the bound peptide, one with the position 9T down in the F pocket and the position 10T up exposed to solvent extending beyond the peptide-binding groove, and a second with the position 10T down in the F pocket. Such conformational dimorphism of MHC-I-bound peptides has been suggested by two different high resolution structures of HLA-B*2705, complexed with either pVIPR (RRKWRWRWHL) (42) or pGR (RRRWRHR WRL) (43). These two peptides have been reported to induce cross-reactive CD8⁺ T cells.

The observation that distinct populations of T cells that recognize D₄-PA9, D₄-PT9, D₄-PT10, and D₄-P10 are differentially elicited by a single protein immunogen is reminiscent of the two different classes (types A and B) of hen egg lysozyme-specific CD4⁺ T cells observed by Unanue and colleagues (44). Type A conventional T cells recognize a distinct conformation of the peptide/I-Aabcd complex generated in the presence of H₂-DM in late endocytic vesicles, whereas type B cells identify a distinct conformation of the same peptide/I-Aabcd complex produced by peptide exchange in the absence of H₂-DM. There clearly are differences in the MHC-II processing and presentation systems observed for hen egg lysozyme as compared with the Env vaccine systems that we have studied. However, the distinct T cell populations that we observe may be indicative of different conformations of peptide/MHC-I complexes generated by processing and presentation from different types of APC, by a cellular mechanism similar to that observed for hen egg lysozyme/I-Aabcd. Complex mechanisms of Ag processing and presentation, as well as variations in the T cell repertoire, play important roles in the variability and specificity of the Ag-specific T cell response (45, 46). The differences in Ag-specific CD8⁺ T cell induction between the two prime-boost regimens are most likely due to differences in the priming Ags. The i.m. injection of plasmid DNA-encoding Env results in cellular gene expression, which typically leads to Ag processing through a proteasomal pathway (47). In contrast, the injected BCG must enter an endosomal/lysosomal pathway to generate peptides for presentation by MHC-I (48). Thus, endogenously generated PT9 and PT10 may be presented differently in the context of H-2D⁺ in different APC, and moreover, PT10 may be displayed in two distinct conformations (Fig. 7). Furthermore, this mechanistic explanation enables an understanding of the relative immunodominance profiles between D₄-P10-specific and D₄-PA9-specific CD8⁺ T cell populations. Differences in epitope abundance and compartmentalization (49), both temporally and between the different vaccination regimens, could explain the observed differential induction of CD8⁺ T cells specific for D₄-P10 and D₄-PA9.

Immune elicitation of protective T cell responses offers a reasonable strategy for design of vaccines against a variety of infectious diseases (50, 51), and MHC-I/peptide tetramers can be effective for evaluating not only the extent, but also the specificity of T cell immunity. The general approach to evaluating CD8⁺ T cell-inducing vaccines in this study relied on several experimental strategies. First, we focused on the region of the immunizing envelope Ag known to elicit the immunodominant response. Second, we empirically tested a set of clade-specific and synthetic variant peptides for their ability to bind the known MHC-I-presenting molecule. Using both the optimal binding peptides and those that are putatively endogenously generated to produce H-2D⁺ tetramers, we detected different CD8⁺ T cell subsets specific for P10 and PA9. Structural understanding of the MHC-I/peptide complex may facilitate our ability to identify Ag-specific CD8⁺ T cell activation in vivo in response to vector-based vaccines. Such an approach toward exploring variant peptides for MHC-I binding and for tetramer production may allow broader detection of T cell responses to HIV-1.
In summary, we have shown that different prime-boost vaccination regimens can elicit CD8+ T cell responses with distinct specificity, cross-reactivity, clonotypic structure, and maturation. The immunodominant HIV Env V3 responses were mediated by two distinct tetramer-specific CD8+ T cell subpopulations. Together, these findings suggest the importance of an understanding of MHC-peptide binding and structure for the rational design of effective vaccines.

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Disclosures

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References


