Dominant Clonotypes within HIV-Specific T Cell Responses Are Programmed Death-1 high and CD127 low and Display Reduced Variant Cross-Reactivity


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Dominant Clonotypes within HIV-Specific T Cell Responses Are Programmed Death-1\textsuperscript{high} and CD127\textsuperscript{low} and Display Reduced Variant Cross-Reactivity

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HIV epitope-specific T cell responses are often comprised of clonotypic expansions with distinct functional properties. In HIV\textsuperscript{+} individuals, we measured programmed death-1 (PD-1) and IL-7R\textsuperscript{a} expression, MHC class I tetramer binding, cytokine production, and proliferation profiles of dominant and subdominant TCR clonotypes to evaluate the relationship between the composition of the HIV-specific T cell repertoire and clonotypic phenotype and function. Dominant clonotypes are characterized by higher PD-1 expression and lower C127 expression compared with subdominant clonotypes, and TCR avidity positively correlates with PD-1 expression. At low peptide concentrations, dominant clonotypes fail to survive in culture. In response to stimulation with peptides representing variant epitopes, subdominant clonotypes produce higher relative levels of cytokines and display greater capacity for cross-recognition compared with dominant clonotypes. These data indicate that dominant clonotypes within HIV-specific T cell responses display a phenotype consistent with ongoing exposure to cognate viral epitopes and suggest that cross-reactive, subdominant clonotypes may retain greater capacity to suppress replication of viral variants as well as to survive in the absence of strong antigenic signaling.

The online version of this article contains supplemental material.

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The online version of this article contains supplemental material.

Abbreviations used in this article: LCMV, lymphocytic choriomeningitis virus; MFI, mean fluorescence intensity; MHC-I, MHC class I; PD-1, programmed death-1; RCC, relative cytokine capacity; TRBJ, TCR \textit{\beta}-chain J region; TRBV, TCR \textit{\beta}-chain V region.

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clearly defined in model systems or natural infections. We found that dominant clonotypes express relatively higher levels of PD-1 and relatively lower levels of CD127 in comparison with corresponding subdominant clonotypes. PD-1 expression correlated strongly with the ability of clonotypes to bind MHC class I (MHC-I) tetramers, and although dominant and subdominant clonotypes were able to respond to stimulation with HIV peptide epitopes matching circulating sequence, subdominant clonotypes were more cross-reactive in response to common variant peptide epitopes. Additionally, dominant clonotypes displayed an impaired ability to survive in culture at low levels of Ag stimulation. These data provide insight into the relationships between the structural composition of HIV-specific CD8+ T cell responses, the relative Ag exposure of clonotypes within the epitope-specific TCR repertoire, and the functional capacity of these clonotypes in ongoing HIV infection.

Materials and Methods

Individual cohort and HLA typing

This cohort was organized within the Vanderbilt–Meharry Center for AIDS Research and was comprised of anti-retroviral therapy naive patients recruited through the Comprehensive Care Center (Nashville, TN). All individuals were typed for HLA class I by the DCI Tissue Typing Laboratory (Nashville, TN). This study was approved by the Institutional Review Board at Vanderbilt University, and all participating individuals provided written informed consent.

Flow cytometric evaluation of lymphocyte surface molecules

Gating strategy is shown in Supplemental Fig. 1. Lymphocyte subsets were evaluated using fresh and cryopreserved PBMCs and a combination of mAbs, including CD3-Alexa Fluor 700 (BD Biosciences), CD4-PE-Texas Red (Caltag Laboratories), CD8-Pacific Orange (Caltag Laboratories), CD14-PerCP (BD Biosciences), CD19-PerCP (BD Biosciences), CD56-PE-Cy5 (BD Biosciences), Via-Probe (BD Biosciences), CD127-biotin (eBioscience), streptavidin-allophycocyanin-Cy7 (BD Biosciences), purified PD-1 (mouse IgG1, clone EH12:2H7; BioLegend), goat-anti-mouse IgG-Pacific Blue (Molecular Probes), anti-TCR β-chain V region (TRBV)-PE/FITC (Beckman Coulter), and MHC-I tetramers-PE/allophycocyanin. MHC-I tetramers included HLA-A*08-01 (EYIKRQW), HLA-B*08-01 (FLKEKGGG), HLA-B*15-01 (GLNKIVRMY), HLA-B*15-01 (TGGYFPDQWNY), HLA-B*27-01 (KRWILGLNK), which were synthesized by the National Institutes of Health Tetrramer Core Facility (Atlanta, GA), and HLA-B*57-01 (KAESPFPIMPD), HLA-B*57-02 (ISPRTLNAW), and HLA-B*57-02 (QASQEVKNW), which were synthesized by Beckman Coulter.

Cells were labeled with MHC-I tetramers at 21°C for 10 min. Anti-CD14-PerCP was added to the suspension and incubated for a further 20 min. Cells were washed and labeled in separate steps with intervening washes with FACS buffer, resuspended, aliquoted, and labeled with the following direct conjugated surface Abs listed above.

Identification of dominant and subdominant clonotypes and TRBV populations

The phenotype of T cell clonotypes was determined by a combination of labeling with tetramer, anti-TRBV Abs, and Abs to cell surface markers. Single TCR clonotypes identified by sequencing, and which comprised >50% of the epitope-specific population, were considered dominant. In TCR repertoire analyses in which no clonotype comprised >50% of the total, the largest population was considered dominant, and the remaining populations were considered subdominant. mAbs are not available to label TRBV7, and thus in the five cases in which the dominant TRBV7 clonotype was not directly labeled, TCR β-chain sequence data informed the identification of subdominant populations that were directly labeled. In these cases the unlabelled population of tetramer+ cells represented the dominant clonotype. We determined TRBV7 repertoire for 11 epitopes in this study by using TRBV Ab panels (IOTest Beta Mark, TCR Vβ repertoire kit; Beckman Coulter). Dominant TRBV7 populations were definitively labeled within these responses, and subdominant populations were defined as tetramer+/TRBV7-.

cDNA synthesis and TCR sequencing

Epitope-specific T cells were labeled with appropriate MHC-I tetramers and sorted by FACS to >95% purity on a FACSARia cell sorter (BD Biosciences). RNA was extracted from sorted cells, and anchored RT-PCR was performed from total RNA as previously described (28). PCR product was cloned into Escherichia coli and sequenced on an ABI 3130xl automated sequencer (PE Applied Biosystems, Norwalk, CT). After editing and alignment using Sequencer (Gene Codes, Ann Arbor, MI), TRBV/TCR β-chain J region (TRBJ) usage was determined using the human TCR gene database (http://www.tigem.cines.fr). The TCR V region classification system of the ImmunoGeneTics database is used throughout this article.

Sequencing of autologous virus

Population viral sequence was obtained using viral RNA isolated from plasma (Qiagen) and reverse transcribed in one step (Qiagen) using HIV Gag- and HIV Nef-specific primers. DNA was amplified by PCR with the following primers: 5′ gag 3′-28, 5′-GCC AGA GCG TCA GTA TTA AGC G-3′; 3′ gag 5′-1683-1693, 5′-TCT GAG GGA AAC TAA AGG ATC TT-3′; 3′ gag 5′-1420-1539, 5′-AAA ATT AGC CTG TCT CCT CCC AT-3′; nefV-3, 5′-ATG GTT GGC AAC TGG TCA A-3′; nefV-3g, 5′-TGC TAG GCG GTC GTC AAA-3′. Resulting PCR fragments were gel-purified (QIAquick), sequenced bidirectionally on an ABI 3130xl automated sequencer using the same primers. Sequencer (Gene Codes) was used to edit and align sequences, and identification was made using the Los Alamos HIV Sequence Database (http://www.hiv.lanl.gov).

Intracellular cytokine staining

Intracellular cytokine staining assays were performed using 10 μg/ml indicated peptide, anti-CD28 and anti-CD49d mAbs (1 μg/ml each; BD Biosciences), and GolgiPlating at 1 μg/ml (BD Biosciences). Cells were stimulated for 6 h and labeled with surface and intracellular Abs. Surface staining panels included CD3 (Alexa Fluor 700; BD Biosciences), CD4 (PE-Texas Red; Caltag Laboratories), CD8 (Pacific Blue; BD Biosciences), CD14 (PerCP; BD Biosciences), and FoxP3 (eBioscience), and Fixable Live-Dead Aqua (Invitrogen). Intracellular cytokine production included IFN-γ (PE-Cy7; BD Biosciences) and TNF-α (allophycocyanin; BD Biosciences). Positive controls were included for each individual. Reported cytokine production was subtracted from negative control values. Epitope variant panels (delineated with lowercase boldface letters) were B*08-01 (FLKEKGGG; variant 1, FLLRERGGG; variant 2, FLKDKEGGG); B*08-01 (EYIKRQWI; variant 1, DYIKRQWI; variant 2, EYIKRQWI); B*27-01 (KRWILGLNK; variant 1, KRWILGLNK; variant 2, KRWILGLNK; variant 3, KRWILGLNK; variant 4, KRWILGLNK; variant 5, QASQEVKNW; variant 1, QARIQDKVN; variant 2, QAQVEKVN; variant 3, peptide synthesis by Gene- Meds Synthesis, South San Francisco, CA).

Tetramer binding analysis

PBMCs were washed in FACS buffer, resuspended, aliquoted, and labeled for 30 min at room temperature with tetramer (allophycocyanin-conjugated) at the following dilutions from manufactured stock: 1:25, 1:50, 1:100, 1:200, 1:400 final concentrations (PE-Cy7; BD Biosciences). At 1:200 final concentration, anti-CD28 and anti-CD49d mAbs (1 μg/ml each; BD Biosciences) were added to each aliquot of PBMCs. At 30 min, labeled cells were immediately washed with PBS and resuspended. Cells were fixed with 2% paraformaldehyde and washed in PBS. Fixed PBMCs were first labeled with anti-TRBV-FITC–conjugated Abs and subsequently with Abs to surface markers CD3, CD4, CD8, and CD14/19/56 (fluorescent Abs and manufacturers as detailed above) for 30 min at room temperature. Surface Abs were fixed to cells a final time and analyzed immediately.

In vitro culture and proliferation

PBMCs were labeled with CFSE buffer, resuspended, aliquoted, and labeled for 30 min at room temperature with tetramer (allophycocyanin-conjugated) at the following dilutions from manufactured stock: 1:25, 1:50, 1:100, 1:200, 1:400 final concentrations (~1:4–6 μM). With 5 min remaining for tetramer incubation, fixable Live/Dead Aqua dead cell stain (Invitrogen) was added to each aliquot of PBMCs. At 30 min, labeled cells were immediately washed with PBS and resuspended. Cells were fixed with 2% paraformaldehyde and washed in PBS. Fixed PBMCs were first labeled with anti-TRBV-FITC–conjugated Abs and subsequently with Abs to surface markers CD3, CD4, CD8, and CD14/19/56 (fluorescent Abs and manufacturers as detailed above) for 30 min at room temperature. Surface Abs were fixed to cells a final time and analyzed immediately.

Flow cytometry

All samples were sorted and data acquired on a FACSARia (BD Biosciences) cell sorter. Data were analyzed using FACSData software (BD Biosciences). Phenotypic expression using log10 fluorescence; histograms are log10 fluorescence versus count.

Statistical analysis

Comparisons between whole CD4+, CD8+, and epitope-specific T cell populations were performed using Mann–Whitney tests. All paired comparisons were made using a Wilcoxon matched pairs test. A Fisher exact
test for proportions was used to determine significance between PD-1 and CD127 expression on dominant and subdominant populations. Spearman rank correlation was used to test for the relationship between PD-1 expression and avidity for tetramer. All statistics were calculated using GraphPad Prism v5.01.

Results

**Epitope-specific T cell populations express high levels of PD-1**

We evaluated the degree of PD-1 expression on total CD4⁺, CD8⁺, and HIV-specific CD8⁺ T cell populations in 22 chronic HIV⁺ patients off anti-retroviral therapy (Fig. 1). These individuals had varying levels of disease progression (Table I; median viral load, 2,474 copies/ml; range, ≤50–382,000; median CD4, 688; range, 132–1,374). PD-1 expression (mean fluorescence intensity [MFI]) was measured on CD4⁺, CD8⁺, and 35 CD8⁺, HIV epitope-specific T cell populations identified by MHC-I tetramers (Table I; mean, 1.6 epitopes/individual; range, one to five epitopes per individual). As has been observed by other groups (11, 12), we found PD-1 expression to be higher on HIV-specific CD8⁺ T cell populations when compared with total CD4⁺ (p = 0.007; mean, 2.4-fold higher) and CD8⁺ (p = 0.0003; mean, 1.9-fold higher) T cell populations (Fig. 1A, 1B).

PD-1 expression on CD4⁺, CD8⁺, and HIV-specific CD8⁺ T cell populations was often bimodal, and we were able to measure the percentage of PD-1high cells within a given T cell population. Tetramer⁺, HIV-specific populations have a larger fraction of PD-1high cells than do CD4⁺ or parent CD8⁺ T cell populations (p = 0.0001 and p < 0.0001, respectively; Fig. 1A, 1C). Despite overall high levels of PD-1 expression on epitope-specific T cells, we observed PD-1 expression as low as 40% on some epitope-specific populations, which may represent a subset of epitope-specific responses (Fig. 2). The percentage of PD-1high cells within a given T cell population.

**Dominant TRBV populations within HIV-specific T cell responses are predominantly clonotypic and express higher levels of PD-1 and lower levels of CD127 compared with subdominant TRBV populations**

We next evaluated TRBV usage and clonotypic composition within HIV-specific PD-1high and PD1low populations. To identify TCR usage within HIV-specific CD8⁺ T cell populations, we sequenced FACS-isolated HIV-specific CD8⁺ T cells in combination with direct staining of PBMCs with HIV epitope-specific MHC-I tetramers and an anti-TRBV mAb panel as previously described (25, 27). Twenty-one of 35 HIV-specific CD8⁺ T cell responses were sequenced to determine TRBV, CDR3, and corresponding TRBJ regions (Table II), with subsequent repertoire confirmation using monoclonal anti-TRBV Abs. Within each epitope-specific TCR repertoire, we identified a single, dominant CDR3 clonotype, although sometimes this dominant clonotype was found with other clonotypes within a single TRBV family (Table II). For example, subject 10002 recognized the HLA B*5701-restricted epitope IW9. Although we identified eight clonotypes responding to this epitope, one TRBV27-TR27 clonotype comprises 64% of the sequences. Two other clonotypes also use TRBV27, but combined, they only contribute to 6% of the total sequences. In this case, staining with anti-TRBV27 Ab was used to identify the dominant T cell clonotype for phenotypic analysis. We noted highly significant concordance between these two methods used to identify clonotypes within the TCR repertoire (Supplemental Fig. 2).

We show representative plots of PD-1 expression corresponding dominant and subdominant TRBV populations in a single HIV epitope-specific T cell response (Fig. 2A). Within HIV epitope-specific responses, PD-1 expression is higher on dominant TRBV populations compared with subdominant TRBV populations when measured by MFI (p = 0.001; Fig. 2B) or frequency of PD-1high cells (p = 0.0001; Fig. 2C). We evaluated multiple HIV epitope-specific populations in 9 of 22 individuals studied (range, two to five epitopes per individual; Tables I, III). We did not find a correlation between the degree of dominance within the repertoire and the degree of PD-1 expression on dominant and subdominant clonotypes within epitopes, suggesting that the magnitude of expansion within a parent population is not the sole determinant of PD-1 expression.

If we limit our phenotypic analysis to those epitopes for which we have sequence confirmation that the dominant TRBV population is monoclonal, the relationships we highlight between clonotypic dominance and PD-1 and CD127 expression remain

**FIGURE 1.** PD-1 is highly expressed in a bimodal pattern on epitope-specific T cells in HIV⁺ individuals. Histograms show PD-1 expression on T cell populations in a single individual; PD-1 MFI and percentage PD-1high values are provided in the corner of each histogram; CD8⁺ (top panel, light gray), CD4⁺ (top panel, dark gray), and HIV-specific, tetramer⁺ (bottom panel) values are also shown (A). PD-1 MFI is higher on tetramer⁺ cells compared with CD4⁺ T cells (p = 0.007) and CD8⁺ T cells (p = 0.0003) (B). The percentage of tetramer⁺PD-1high cells is higher than the percentage CD4⁺PD-1high T cells (p = 0.0001) and CD8⁺PD-1high T cells (p < 0.0001) (C). Epitope-specific responses (n = 35) in 22 HIV⁺ individuals are shown. 

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statistically significant (PD-1 MFI, \( p = 0.0398 \); CD127 MFI, \( p = 0.0342 \)). Additionally, there are several ways to define clonotypic dominance within epitope-specific TCR repertoires in the absence of a single, highly dominant clonotype; however, even using a more stringent criterion that dominant clonotypes must comprise >70% of the TRBV repertoire (19 epitope-specific responses in 14 individuals fit this criteria), comparison between dominant and subdominant clonotypes yields significant relationships for MFI and percentage PD-1 high (MFI, \( p = 0.03 \); percentage PD-1 high, \( p = 0.001 \); Supplemental Fig. 2B, 2C). These data support our observations that dominant clonotypes express higher levels of PD-1 despite relative differences in dominance within the clonotypic repertoire.

We also evaluated CD127 expression on dominant and subdominant TRBV populations in a subcohort of 12 individuals, which included analysis of 19 epitope-specific responses (noted in Table I). In contrast to higher PD-1 expression observed on dominant TRBV populations, CD127 expression was lower in corresponding subdominant TRBV populations. The PD-1 expression pattern described above on dominant and subdominant TRBV populations remains intact in this smaller cohort (PD-1 MFI, \( p = 0.006 \)).

Within this subcohort of epitopes labeled with PD-1 and CD127, 15 of 19 dominant TRBV populations displayed a PD-1\textsuperscript{high}/CD127\textsuperscript{low} phenotype and 15 of 19 displayed a CD127\textsuperscript{low} phenotype compared with their corresponding subdominant population. However, there was not complete concordance between these populations. Most dominant clonotypes (11 of 19) displayed the combination of higher PD-1 expression and lower CD127 expression. In contrast, there were no instances (0 of 19) in which the subdominant clonotype had both higher PD-1 expression and lower CD127 expression (\( p < 0.0001 \)). In summary, our data indicate that clonotypic dominance within the epitope-specific TCR repertoire is associated with a PD-1\textsuperscript{high}/CD127\textsuperscript{low} phenotype.

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\( ^{a} \)Subjects in CD127 cohort.

\( ^{b} \)Subjects followed longitudinally.

AA, African American; F, female; M, male; NA, not available; W, white.

PD-1\textsuperscript{high}/CD127\textsuperscript{low} phenotype on dominant clonotypes in HIV-specific responses is stable over time

To characterize the stability of PD-1 and CD127 expression on dominant and subdominant TRBV populations, we performed a longitudinal analysis of HIV-specific responses from three individuals. Fig. 4 details longitudinal viral load and CD4\textsuperscript{+} and CD8\textsuperscript{+} T cell number (Fig. 4A), epitope-specific CD8\textsuperscript{+} T cell frequency and corresponding TRBV repertoire composition (Fig. 4B), and PD-1 and CD127 expression (Fig. 4C, 4D) on TRBV populations for the dominant clonotype within the HLA-B*08-FL8 response in 10022, the dominant TRBV population within the HLA-B*57-QW9 response in 10027 (this epitope-specific T cell population was not sequenced), and the dominant clonotype within the HLA-B*08-FL8 response in 10071 for the most recent 6 y of their infections (duration of infection, 16, 16, and 15 y, respectively). Subjects 10022 and 10071 are long-term controllers with stable viral loads and CD4\textsuperscript{+} T cell counts, and 10027 is a chronically infected individual with progressive disease (increasing viral load and decreasing CD4\textsuperscript{+} T cell counts).

Although expression levels of PD-1 and CD127 on the TRBV clonotypes within these HIV-specific responses are dynamic, the association of higher PD-1 expression and lower CD127 expression with TRBV dominance remained consistent during the 6 y of our analysis. The B*08-FL8-specific TRBV repertoire in 10022 was relatively stable over time. The dominant TRBV2 population in this individual maintained higher PD-1 expression over time, whereas the subdominant TRBV populations had higher and increasing CD127 levels during the same period. The B*57-KF11-specific TRBV repertoire in 10071 was characterized by an increasingly dominant TRBV5 population and a corresponding increase in PD-1 expression compared with the subdominant TRBV7 population. In 10027, the B*57-QW9–specific TCR repertoire fluctuated early in our observations and as the TRBV28 population became dominant, its PD-1 expression levels increased. During this period, 10027 experienced declining T cell counts and increasing viral load with an overall increase in PD-1
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Dark shading identifies dominant clonotypes. Light shading identifies subdominant populations used for comparisons (clonotypes without shading were unlabeled). TRBV populations directly labeled with Abs are set off with borders. Note that ImMunoGeneTics nomenclature for TRBV designations is used throughout the table.

NA, not available.
expression on CD8+ T cells. The dominant circulating viral sequence in 10022 and 10027 was determined for the B*27-FL8 and B*57-QW9 epitopes at a midpoint in this analysis and corresponded to the peptide sequence within the tetramers in each case. Subject 10071 maintained viral loads of <50 copies/ml during this study, and we were unable to generate viral sequences from this individual.

We also evaluated mean PD-1 and CD127 expression levels at early and late time points on 10 additional epitope-specific responses and determined a similar and statistically significant expression pattern on dominant and subdominant TRBV populations (Supplemental Fig. 3). These longitudinal data suggest that dominance within the epitope-specific TRBV repertoire is associated with a more pronounced PD-1 high/CD127low phenotype over time and may be related to the course of disease.

Tetramer binding characteristics of TRBV populations correlate with PD-1 expression but are not directly related to dominance within the epitope-specific TCR repertoire

We next investigated whether differences in tetramer binding characteristics were related to dominance within the TRBV repertoire. Our group and others have previously described differential tetramer binding on epitope-specific T cell clonotypes (23, 29) and we observed a similar phenomenon in this study (Fig. 5A). Several groups have previously used differential levels of tetramer binding to define TCR avidity (29, 30), and so we measured tetramer binding (MFI) on TRBV populations over a 16-fold range of tetramer concentration and determined tetramer-binding curves for dominant and subdominant clonotypes of nine epitope-specific responses in four individuals (Fig. 5B, Supplemental Table I). Nonlinear regression analysis indicated that TRBV populations with lower half-maximal values have higher maximal binding values in eight of the nine epitopes tested. Thus, we used tetramer MFI on labeled TRBV populations as a surrogate measure of TCR avidity for tetramer complexes.

We compared tetramer binding levels on corresponding dominant and subdominant TRBV populations. Although there was a trend suggesting that dominant TRBV populations have higher avidity for tetramer than do corresponding subdominant populations, this pairing was not statistically significant (p = 0.09; Fig. 5C). We found a positive and significant correlation between clonotypic avidity for tetramer and clonotypic PD-1 expression (r = 0.34, p = 0.004; Fig. 5D). These data indicate that while clonotypic avidity for tetramer does not strictly govern dominance within the repertoire, it may influence the degree of PD-1 expression.

Subdominant TRBV populations display greater cytokine production capacity and cross-recognition in responses to epitope variant peptides

We assessed the capacity of dominant and subdominant TRBV populations to produce cytokines after stimulation with consensus and variant peptides. Two common viral sequence variants for each of four HIV epitopes were tested in seven individuals. We performed viral sequencing in these individuals and found that circulating viral sequence matched the consensus epitopes used in the tetramer reagents in each individual except for 10094, who harbored a circulating sequence variant at the FL8 epitope that matched the FL1KdKGGL variant we used in our functional assay. Taking our analysis of the B*27-KK10 response in 10022 as an example, the dominant TRBV12 clonotype comprises 45% of the B*27-KK10 response, which is 19% of total CD8+ T cells. The

FIGURE 2. PD-1 expression is higher on dominant TRBV compared with subdominant TRBV populations within epitope-specific responses. Dot plot and histogram show PD-1 expression on dominant (blue) and subdominant (green) TRBV populations in a single epitope-specific response. PD-1 MFI and percentage PD-1 high values are provided in the upper corner histogram for the dominant and subdominant TRBV populations (A). PD-1 expression is higher on dominant TRBV compared with subdominant TRBV as measured by MFI (B), p = 0.001; for percentage PD-1 high (C), p = 0.0001. Epitope-specific responses (n = 35) in 22 HIV+ individuals are shown.

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by guest on April 30, 2017 http://www.jimmunol.org/ Downloaded from
maximal possible cytokine production by the TRBV12 clonotype is therefore 8.6% of total CD8+ T cells. Likewise, maximal cytokine production for the subdominant clonotypes (TRBV6-5 and TRBV20-1, together 55% of the KK10-tetramer+ population) is 10.4% of total CD8+ T cells. We determined the relative cytokine production for the subdominant clonotypes (TRBV6-5 and TRBV20-1, together 55% of the KK10-tetramer+ population) is 8.6% of total CD8 + T cells. Likewise, maximal cytokine production potential without regard to its absolute percentage within the TCR repertoire.

Representative plots are shown in Fig. 6A detailing cytokine production (IFN-γ, upper plots; TNF-α, lower plots) by the dominant TRBV12 clonotype and the subdominant clonotypes in response to stimulation with consensus and variant peptides for the HLA-B*27-KK10 epitope. In response to stimulation with consensus peptide, the TRBV12 clonotype reached absolute cytokine production levels of 8.1 (IFN-γ) and 3.4% (TNF-α) of total CD8+ T cells, and the subdominant clonotypes reached cytokine production levels of 8.1 (IFN-γ) and 3.3% (TNF-α) of total CD8+ T cells. TRBV12 RCC values are 95 (IFN-γ) and 40% (TNF-α). The subdominant clonotypes together comprise a larger part of the TCR repertoire than does the dominant TRBV12 clonotype, and so despite similar levels of absolute cytokine production, their corresponding RCC values are lower at 78 (IFN-γ) and 31% (TNF-α). The strong cytokine response and high RCC values for the dominant TRBV12 clonotype suggest that these cells recognize consensus peptide more effectively than do the subdominant clonotypes.

Comparison of RCC values for the clonotypic cytokine responses in a further 8 epitopes from six additional individuals (total, 10 epitopes in seven individuals; Fig. 6C, 6D) reveals that both dominant and subdominant TRBV populations are capable of cytokine production to consensus peptides (Fig. 6C; p > 0.05 for IFN-γ and TNF-α production). In response to stimulation with common variant peptides, subdominant TRBV populations have higher RCC ratios for IFN-γ production (p = 0.04; Fig. 6D) with a trend toward higher subdominant RCC ratios for TNF-α production as well (p = 0.08; Fig. 6D). These results indicate that although dominant and subdominant clonotypes are capable of producing cytokines in response to stimulation with consensus and

### Table III. TRBV repertoire data

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The TRBV repertoire was determined by Ab labeling. For sort sequence designations, tetramer+, epitope-specific cells were sorted and subjected to TCR sequence analysis before costaining with anti-TRBV Abs. For TRBV designations, tetramer+, epitope-specific cells were costained with anti-TRBV Abs.
variant peptide epitopes, subdominant clonotypes seem to retain greater capacity for cross-recognition and secretion of cytokines in response to the common viral epitope variants we tested.

**Dominant TRBV populations display a survival defect in culture**

Proliferation upon Ag exposure is an important measure of T cell function and has been associated with improved control of viral replication (21). We labeled T cells with CFSE and cultured them with varying concentrations of peptide for 4 d to assess changes to the epitope-specific TRBV repertoire and capacity for proliferation of dominant and subdominant TRBV populations. The ex vivo epitope-specific response and its clonotypic repertoire are shown for the B*57-restricted–QW9 response in 10002 (Fig. 7A). Representative plots are shown to illustrate epitope-specific responses (Fig. 7B) and CFSE dilution (Fig. 7C) for the dominant and subdominant clonotypes after 4 d culture with low (0.2 ng/ml) and high (200 ng/ml) concentrations of optimal peptide Ag. At the 200 ng/ml peptide concentration, the dominant TRBV27 clonotype made up 91% of the total repertoire at the end of the 4-d stimulation period, reflecting the ex vivo repertoire. However, at the 200 pg/ml concentration, the TRBV27 clonotype comprised 58% of the repertoire. Therefore, although both dominant and subdominant TRBV clonotypes proliferate well in response to stimulation with higher concentrations of consensus peptide, the dominant clonotype does not survive as well at lower peptide concentrations and therefore does not maintain the same degree of dominance in vitro. Moreover, as measured by the percentage of CFSE<sub>low</sub> cells, the dominant TRBV27 clonotype proliferates better than do the subdominant clonotypes in response to stimulation with consensus peptides, reflecting in vitro what happens naturally in vivo.

Aggregate data from 15 epitopes in seven subjects indicate that dominant TRBV populations fail to maintain their dominance at low concentrations of peptide (p = 0.0026; Fig. 7D). Conversely, dominant TRBV populations more effectively maintain their level of dominance at higher concentrations of peptide stimulation (p = 0.2078; Fig. 7D). In this series of experiments, the addition of Ab to block PD-1/PD-L1 interaction did not significantly alter the relative proliferative capacity of dominant and subdominant TRBV populations over the short duration of this assay (data not shown). These results suggest that although clonotypic constituents may not expand well at low concentrations of stimulation, subdominant clonotypic populations are better able to survive culture conditions with low levels of Ag.

**Discussion**

Several groups have observed enhanced global expression of PD-1 on T cells in HIV+ individuals, with the highest level of PD-1 expression found on HIV epitope-specific cells (11, 12, 14, 31). A detailed analysis by Day et al. (11) found that different epitope-specific responses, even within the same individual, had differing degrees of PD-1 expression. This has led to speculation that the degree of PD-1 expression could be linked to the efficacy of viral control for individual epitopes (11, 32). In this study, we evaluated constituent clonotypes within epitope-specific responses and determined that clonal dominance within epitope-specific responses is associated with a PD-1<sup>high</sup>/CD127<sup>low</sup> phenotype, that PD-1 expression correlates with clonotypic TCR avidity for tetramer, and that dominant clonotypes display defects in their ability to respond to variant peptide epitopes and survive in the absence of strong Ag signals.

We found that the most dominant clonotype within an epitope-specific response tended to have the highest level of PD-1 expression (p = 0.001) and the lowest level of CD127 expression (p = 0.007). We did not see a relationship between the overall magnitude of a response (or the degree of clonotypic expansion within a response) and PD-1 expression, suggesting that PD-1 expression may not be directly related to the level of T cell expansion or exhaustion, but could mark T cells that have recently been exposed to their cognate Ags (15, 33, 34). In LCMV infection downregulation of PD-1 and upregulation of CD127 occur after viral epitope escape (15), suggesting that ongoing Ag exposure is a key factor in pushing T cells toward a PD-1<sup>high</sup>/CD127<sup>low</sup> phenotype. Lichterfeld et al. (35) described progressive reductions in CD127 expression on high-avidity HIV epitope-specific clonotypes that were eventually deleted, and more recent work by Streeck et al. (4) found PD-1 expression on HIV-1 epitope-specific T cells decreased after in vivo selection for escape mutations. Although this recent work highlights the relationship between PD-1 and CD127 expression on epitope-specific responses (15, 18), the data we present in this study are the first, to our knowledge, that describe differential expression of these markers on individual T cell clonotypes and link dominance to specific differences in clonotypic function.

We have shown that epitope-specific T cell populations are often comprised of a single dominant and various subdominant clonotypic populations that can respond variably to changes in viremia (23), and that these clonotypes have differing abilities to recognize epitope variants (27). Our more recent work demonstrates a relationship between TCR use and memory phenotype (28). Thus, our new finding that dominant and subdominant T cell clonotypes
have phenotypic and functional characteristics linked to Ag sensing is yet another indicator that the fine specificity of individual T cell clones plays a role in the evolution of epitope-specific immune responses.

Most individuals we sequenced had dominant circulating sequences matching HIV clade B consensus, with the exception of 10094 (Supplemental Table I and data not shown), and this subject still preferentially recognized the consensus peptide over the circulating variant. Despite their PD-1 high/CD127 low phenotype, we present evidence that dominant T cell clonotypes able to recognize circulating viral sequences have the capacity to produce multiple cytokines after stimulation with consensus and variant peptide epitopes and that subdominant clonotypes have increased ability to recognize common HIV-1 epitope variants. Improved recognition of viral variants by subdominant clonotypes might also be influenced by the diversity of TCR clonotypes within these subdominant populations. Each of the epitope-specific responses we assessed is comprised of a single dominant clonotype and at least one, and in some cases more than one, subdominant clonotype. Effective recognition of variant epitopes may also be a reflection of increased diversity within subdominant TRBV populations.

Immune selection pressure mediated by CD8+ T cells can lead to viral mutation and epitope escape from immune recognition (36–38), and therefore the frequency of circulating epitope variants and the degree to which individual clonotypes are able to recognize these variants may also play a role in the development and maintenance of the epitope-specific TCR repertoire. A recent study from van Bockel et al. (39) offers insight into the relationship between clonal evolution within the TCR repertoire in HIV+ individuals and viral epitope variation. Their work highlights TCR repertoire remodeling within HLA-B*27–restricted responses to a viral epitope known to consistently undergo immune-mediated mutational escape (25). The authors in this study found that in the presence of epitopes that varied from consensus, dominant T cell clonotypes were maintained over time and expressed higher levels of CD127 compared with subdominant clonotypes. In contrast to van Bockel et al., we found dominant clonotypes to have lower levels of CD127 compared with subdominant clonotypes. Our study was different in that we evaluated 35 different epitope responses (representing eight discrete HIV epitopes) in 22 individuals, and in most cases the circulating viral sequence corresponded to the tetramer peptide sequence. These findings are

**FIGURE 4.** Longitudinal analysis of epitope-specific TCR repertoire dynamics and clonotypic PD-1 and CD127 expression. Absolute CD4+ (●) and CD8+ (■) T cell counts (left-hand axis) and viral load (dashed line, ▲; RNA copies per milliliter, right-hand axis) are shown (A). Epitope-specific responses as a percentage of total CD8+ parent population (▲, below y-axis split) and TRBV percentage of epitope-specific response (above y-axis split; dominant clonotype, solid line, ●; subdominant clonotype, dashed line, ■) are shown (B). PD-1 expression (C) and CD127 expression (D) on dominant (solid line, ●) and subdominant (dashed line, ■) TRBV populations within epitope-specific responses are shown.
broadly complementary to our own; both data sets indicate that dominant clonotypes are surprisingly persistent in vivo over time and support the notion that broad epitope-specific TCR repertoires may contain clonotypes capable of recognizing and suppressing viral sequence variants.

Although we cannot rule out the possibility that some HIV+ individuals in our cohort harbored viral variants not covered by the consensus or variant epitope sequences we selected, the recently reported associations between PD-1 expression and epitope escape (15) highlight the importance of this line of inquiry for future longitudinal in vivo and in vitro studies. The relationship between epitope exposure, recognition, escape, and corresponding epitope-specific T cell phenotype and functional capacity seems to be tightly related, although the effects of persistent exposure to Ag and viral escape on repertoire composition or clonotypic impairment have yet to be determined. In this cross-sectional study, we were unable to assess whether higher avidity clones had been deleted earlier in infection or whether circulating virus had already escaped immune control for all of the epitopes studied.

Despite the higher expression of PD-1 on dominant clonotypes, and the relative failure of these dominant clonotypes to survive at low peptide concentrations in vitro, blockade of the PD-1 signaling pathway could be an effective strategy to enhance the function of these clonotypes.

FIGURE 5. Tetramer binding correlates to PD-1 expression on epitope-specific T cell clonotypes. MHC-I tetramers were used to label epitope-specific T cell populations at a range of tetramer concentrations. Dot plots from highest and lowest tetramer concentrations show variable tetramer binding on clonotypes (A). Epitope-specific clonotypes were labeled at increasing tetramer concentrations from ~0 to 16 μM. Representative graphs are shown of tetramer binding curves for 10004-QW9, 10022-KK10, 10027-FL8, and 10071-FL8 (subject epitope) for whole epitope-specific populations (▲) and TRBV populations (dominant, ●; subdominant, ♦; sub-subdominant, □) (B). Comparison of tetramer binding levels (tetramer MFI) are depicted on dominant and subdominant TRBV populations for 35 epitopes and 22 individuals (p = 0.09) (C). Spearman correlations are shown of tetramer binding and PD-1 expression on dominant (○) and subdominant (●) clonotypes (r = 0.34, p = 0.004) (D).
pathway did not result in significant enhancement of clonotypic proliferation or survival. Studies evaluating the effect of PD-1 blockade on proliferative capacity have typically found modest increases in proliferation (6, 11, 14). The lack of enhanced proliferation we found may be due to the short duration of our assays, as well as to the inclusion of relatively healthy subjects with low viral loads. Future studies with combinations of PD-1/PD-L blockade and cytokine combinations may help us determine to what extent dominant clonotypes can be “rescued” in vitro.

Previous reports in mouse influenza models (40) and human EBV/CMV infection (29) indicate that T cell avidity for Ag is positively correlated with dominance in the epitope-specific TRBV repertoire. Our data support the notion that clonotypic TCR avidity is associated with higher expression of PD-1, but

FIGURE 6. Subdominant TRBV populations have high cytokine production potential in response to stimulation with variant peptides. Production of IFN-γ and TNF-α was assessed by intracellular cytokine staining on dominant and subdominant TRBV populations. Dot plots show dominant (solid box) and subdominant (dashed box) clonotypic cytokine production (IFN-γ, upper plots; TNF-α, lower plots) in response to stimulation with consensus and variant peptides (indicated above each column) for the B*27-KK10 (A) and B*08-FL8 (B) responses in 10022. Within each plot, absolute cytokine production for clonotypic populations as a percentage of total CD8+ T cells is shown to the right of each indicated population as well as RCC (in parentheses). Graphs representing cytokine production for each response are located below corresponding plots. Bars represent maximal cytokine production for dominant (unfilled) and subdominant (gray) clonotypes, absolute cytokine production (percentage of total CD8+ T cells) is represented by the hatched area within each bar, and RCC for each clonotype and condition is noted to the right of each bar (A, B). Comparisons are shown of clonotypic RCC ratios for IFN-γ and TNF-α production in response to stimulations using peptide matching consensus (C) (IFN-γ, p = 0.42; TNF-α, p = 0.38) and variants (IFN-γ, p = 0.04; TNF-α, p = 0.08) (D). Measurements are from 10 epitopes in seven HIV+ individuals.
they suggest that the association between overall TCR avidity and clonal dominance may be weaker in the setting of chronic HIV infection. Prior studies evaluated either acutely resolved or chronic viral infections with limited Ag variability and low levels of ongoing Ag exposure during chronic infection, and these conditions could account for the discrepancies between our study and this previously published work. It remains to be determined whether the associations between epitope-specific clonotypic dominance, phenotype, and function that we report in HIV infection also apply to other infections in humans and model systems.

T cell phenotype and function is determined not only by the fundamental interaction between TCR and p-MHC but represents a sum of inhibitory and stimulatory signals emanating from surface receptor molecules such as PD-1 and CD127. Recent work from Almeida et al. (26) suggests that a composite measure for T cell function such as “antigen sensitivity” might encompass not only avidity for Ag but a wide range of influential factors such as Ag receptor density, coreceptor-mediated signals, as well as activation status and expression of inhibitory signaling molecules. We suggest that the composition, phenotype, and functional profile of the clonotypic repertoire may be necessarily dynamic to respond to a highly variable pathogen such as HIV.

The following model accommodates our observations and experimental results: dominant clonotypes preferentially expand to circulating viral epitopes in vivo. Dominant clonotypes express a surface phenotype consistent with ongoing Ag exposure and activation. Continued exposure to cognate Ag may erode the capacity of dominant clonotypic responses as a result of accumulated PD-1 signal inhibition and a reduction in homeostatic turnover from reduced CD127 expression. Subdominant clonotypes expand suboptimally to circulating viral epitopes in vivo and express a phenotype consistent with reduced exposure to Ag. Subdominant populations may recognize noncirculating or low-level variants more effectively than do dominant populations and are exposed to relatively lower levels of their preferred cognate Ags, resulting in lower overall Ag exposure and concomitant activation. This sparing effect results in the maintenance of a population of cells better able to survive in the absence of strong antigenic signaling. These data also suggest that higher avidity clonotypes develop a relatively PD-1<sup>high</sup> phenotype compared with lower avidity clonotypes and are consistent with the observation that higher avidity responses

![Figure 7](http://www.jimmunol.org/)

**FIGURE 7.** Dominant epitope-specific TRBV populations display a survival defect at low peptide concentrations that is alleviated by increasing Ag stimulation. PBMCs were cultured in the presence of peptide Ag at the concentrations indicated above each set of plots, and TRBV repertoire composition was assessed by flow cytometry on day 4. Relevant percentages of parent are shown to the right of each population. Representative contour plots showing ex vivo T cell populations include: B*57-QW9<sup>+</sup> T cells (upper plot) as a percentage of CD8<sup>+</sup> T cells; B*57-QW9 epitope-specific population and its constituent dominant (blue) and subdominant (green) TRBV populations (lower plot) as a percentage of the epitope-specific population (A). MHC-I tetramer labeling after 4 d culture in the presence of different concentrations of peptide Ag (B). TRBV repertoire composition was determined by Ab labeling for the dominant TRBV as a part of the B*57-QW9<sup>+</sup> population. Dominant (blue) and subdominant (green) TRBV populations are indicated on each plot and their percentage composition of the B*57-QW9 response is shown at the right of each box. CFSE<sup>low</sup> percentages are shown for each population in the upper left corner of each box (C). Aggregate data were compiled and statistical comparisons were made between epitope-specific TRBV repertoire composition ex vivo and after 4 d proliferation in culture. Dominant populations fail to maintain dominance at low peptide concentrations (p = 0.0026), but repertoire composition is not significantly altered at higher concentrations (p = 0.2078) (D) (by Wilcoxon signed rank test). Measurements are from 15 epitope-specific populations in seven HIV<sup>+</sup> subjects.
are deleted early in infection (35). It remains to be determined whether TCR repertoire composition or clonotypic phenotype in HIV is significantly different in individuals with confirmed viral escape or in the absence of Ag, although data from LCMV infection and HIV infection suggest that this might be the case (4, 15, 39).

A diverse epitope-specific TCR repertoire comprised of clonotypes capable of recognizing and suppressing both circulating and variant epitopes would be a beneficial outcome from either prophylactic vaccine strategies or for strategies seeking to broaden existing immune responses in established HIV infections. Furthermore, manipulation of immunomodulatory surface proteins such as PD-1 or CD127 as a part of vaccination protocols could influence qualitative and quantitative aspects of the epitope-specific immune response including Ag sensitivity or clonotypic repertoire (9). Effective immunological strategies to control chronic infections such as HIV may require not only the generation or stimulation of Ag-specific cells but also a coordinated manipulation of inhibitory pathways.

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Disclosures
The authors have no financial conflicts of interest.

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Supplemental Figure 1 - Flow Cytometry Gating Strategy.

Supplemental Figure 1 - Flow Cytometry Gating Strategy. Lymphocytes were discriminated on the basis of cell size and granularity using forward- and side-scatter properties. A, CD3+ lymphocytes were gated from CD3- non-stable cells, CD4+ CD8+, and CD8+ cells. B, CD4+ and CD8+ T cell populations were selected. C, Epitope-specific Tetracer + and TRBV sub-populations were selected from the parent CD8+ population. D and E, Surface expression of PD-1 and CD127 was measured on parent CD4+ and CD8+ T cell populations as well as tetracer+ and constituent dominant and sub-dominant TRBV populations. F and G.
Supplemental Figure 2 - Correlation and alternative analysis of dominance. Epitope-specific TRBV repertoires were determined using two methodologies as described in Materials and Methods (sort/sequence and antibody labeling). Comparison of TRBV repertoire composition using these methodologies correlated strongly (r=0.86) and with high significance (p<0.0001, Spearman correlation). A, PD-1 expression was measured by MFI (upper charts) and percentage PD-1-high (lower charts) was compared between dominant and sub-dominant TRBV populations when dominant populations comprised more than 70%, B (n=19, p=0.03, p=0.001), or less than 70%, C (n=16, p=0.01 and p=0.03) of the overall epitope-specific repertoire (Wilcoxon matched pairs test was used for these comparisons).
Supplemental Figure 3 - PD-1high/CD127low phenotype on dominant TRBV populations is a stable relationship over time.

A

B

Supplemental Figure 3 – PD-1 high/CD127 low phenotype on dominant TRBV populations is a stable relationship over time. PD-1 and CD127 expression levels (MFI) were measured at multiple time points on dominant and sub-dominant TRBV populations and mean values were calculated for these populations over all time points. Dominant populations had higher average expression of PD-1 MFI (p=0.005), A, and lower average expression of CD127 MFI (p=0.003), B. Measurements from 13 (12 for the CD127 comparison) epitope-specific populations in 6 HIV+ individuals.