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Increased Memory Differentiation Is Associated with Decreased Polyfunctionality for HIV but Not for Cytomegalovirus-Specific CD8+ T Cells

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The generation of polyfunctional CD8+ T cells, in response to vaccination or natural infection, has been associated with improved protective immunity. However, it is unclear whether the maintenance of polyfunctionality is related to particular cellular phenotypic characteristics. To determine whether the cytokine expression profile is linked to the memory differentiation stage, we analyzed the degree of polyfunctionality of HIV-specific CD8+ T cells within different memory subpopulations in 20 antiretroviral therapy-naive HIV-1-infected individuals at ~34 wk postinfection. These profiles were compared with CMV-specific CD8+ T cell responses in HIV-uninfected control subjects and in individuals chronically infected with HIV. Our results showed that the polyfunctional abilities of HIV-specific CD8+ T cells differed according to their memory phenotype. Early-differentiated cells (CD45RO+CD27+) exhibited a higher proportion of cells positive for three or four functions (p < 0.001), and a lower proportion of monofunctional cells (p < 0.001) compared with terminally differentiated (TD; CD45RO-CD27-) HIV-specific CD8+ T cells. The majority of TD HIV-specific CD8+ T cells were monofunctional (median 69% [interquartile range: 57–83]), producing predominantly CD107a or MIP1β. Moreover, proportions of HIV-specific monofunctional CD8+ T cells positively associated with proportions of TD HIV-specific CD8+ T cells (p = 0.019, r = 0.54). In contrast, CMV-specific CD8+ T cell polyfunctional capacities were similar across all memory subpopulations, with terminally and early-differentiated cells endowed with comparable polyfunctionality. Overall, these data show that the polyfunctional abilities of HIV-specific CD8+ T cells are influenced by the stage of memory differentiation, which is not the case for CMV-specific responses. The Journal of Immunology, 2012, 189: 000–000.

Increasing body of evidence suggests that HIV-1 replication can be partially controlled by T cell immune responses (1–3). However, progression to AIDS occurs in almost all untreated individuals, reflecting the inability of the immune system to mount effective, sustained responses. It has been clearly demonstrated that the overall magnitude of IFN-γ-producing, HIV-specific CD8+ T cells does not associate with viral control or the establishment of the viral set point (4–8). However, Betts and others (9–12) have reported that long-term nonprogressors were characterized by having higher frequencies of polyfunctional HIV-specific CD8+ T cells compared with noncontrollers, introducing the concept that the quality, rather than the quantity, of Ag-specific T cell responses may dictate T cell antiviral capacity and play a role in controlling viral replication.

In addition to the range of functional abilities that Ag-specific T cells exhibit, there is enormous phenotypic heterogeneity within the memory T cell compartment. Tremendous effort has been deployed to decipher the phenotypic characteristics of HIV-specific T cells that correlate with viral control. HIV-specific CD8+ T cells possess a distinct memory differentiation profile when compared with other virus-specific CD8+ T cells such as EBV, CMV, and HCV (13), exhibiting mainly a transitional memory phenotype (i.e., CD45RO+CD27+CCR7+) (14–16). Moreover, the distribution of HIV-specific CD8+ memory T cells in early infection influences the subsequent viral

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*Abbreviations used in this article: ART, antiretroviral therapy; CAPRISA, Centre for AIDS Program of Research in South Africa; ED, early-differentiated; Inter, intermediate cell; IQR, interquartile range; LD, late-differentiated; TD, terminally differentiated.
HIV- and CMV-specific CD8+ T cell responses. Hence for CMV-specific participation in the study. The University of Witwatersrand Research Ethics Committee approved the study, and all participants provided written informed consent for participation in the study.

Distinct memory T cell subsets have different proliferation, survival, and homing capacities (19–21), so it is therefore plausible that cell maturation could impact on the functional abilities of Ag-specific CD8+ T cells. Indeed, different CD8+ T cell memory subsets have variable capacities to produce cytokines such as IFN-γ or IL-2 in response to stimulation with PMA/ionomycin (22), establishing that distinct memory subpopulations have differing inherent functional abilities. However, the relationship between the differentiation stage of a T cell and its polyfunctional profile appears to be more complex, because Ag-specific CD8+ T cells exhibit unique memory maturation profiles depending on their viral specificity (13, 23). Thus, it is likely that Ag load, Ag persistence, quality of costimulation, as well as the cytokine milieu are all contributing factors controlling both cell differentiation and the polyfunctional profile of Ag-specific CD8+ T cells (24–27). However, it is still unclear whether the stage of memory maturation defines the polyfunctional capacity of CD8+ T cell after Ag restimulation. In this study, we sought to examine the link between memory phenotype and the polyfunctional ability of Ag-specific CD8+ T cells by assessing the degree of polyfunctionality of HIV-specific CD8+ T cells within distinct memory subsets in 20 antiretroviral therapy (ART)-naive HIV-1–infected individuals and comparing them with CMV-specific CD8+ T cell responses.

Materials and Methods

Study participants

For HIV-specific CD8+ T cell responses, a subset of 20 individuals from the Centre for AIDS Program of Research in South Africa (CAPRISA) HIV-1 acute infection cohort were analyzed. This cohort, located in Durban, South Africa, has been described previously (28, 29). The time postinfection was estimated either by a prospective RNA/Ab measurement or taken as the midpoint between the last Ab+ test and first Ab+ ELISA test. Study participants were followed for 12 mo, and follow-up is ongoing. Data from samples collected before day 3 postinfection (median 34 days ranging from 22 to 42) are reported in this article. All studied subjects were ART naive. The University of KwaZulu-Natal, University of Witwatersrand, and University of Cape Town Research Ethics Committees approved the study, and all participants provided written informed consent for participation in the study.

Because no data were collected on CMV-specific responses in the CAPRISA cohort, we were obliged to use a different cohort to compare HIV- and CMV-specific CD8+ T cell responses. Hence for CMV-specific CD8+ T cell responses, 12 individuals from another cohort (Canadian/African Prevention Trial) recruited from the Perinatal HIV Research Unit, Johannesburg, South Africa, were analyzed. Six individuals were not HIV infected, and the remaining six were chronically infected with HIV-1 and were ART untreated. The University of Witwatersrand Research Ethics Committee approved the study, and all participants provided written informed consent for participation in the study.

Plasma viral load and CD4 T cell count determination

Plasma HIV-1 RNA levels were quantified using the COBAS AMPLICOR HIV-1 monitor test version 1.5 (Roche Diagnostics). Absolute blood CD4+ and CD8+ T cell counts were measured using a FACSCalibur flow cytometer and expressed as cells per cubic millimeter. For the CAPRISA cohort (n = 20), the median plasma viral load, at the time point studied, was 26,550 HIV RNA copies/ml (ranging from 400 to 425,000), and the median CD4+ count was 484 cells/mm3 (ranging from 342 to 1411). For the HIV-infected subjects (n = 6) from the Canadian/African Prevention Trial cohort, the median viral load was 35,625 HIV RNA copies/ml (ranging from 440 to 281,000), and the median CD4 count was 542 cells/mm3 (ranging from 391 to 713). Of note, there were no statistical differences in the absolute CD4 count and viral load between the two HIV-infected groups.

Synthetic peptides

A set of 432 synthetic overlapping peptides spanning the entire expressed HIV-1 clade C proteome corresponding to gene products from the HIV-1 consensus C (Gag), isolate DU151 (Pol and Nef), and isolate DU179 (gp160 Env) was synthesized using 9-fluorenylmethoxy carbonyl chemistry and standard-based solid-phase techniques (Natural and Medical Sciences Institute, University of Tubingen, Tubingen, Germany). The nonconsensus synthesized peptides were based on sequences from isolates used for manufacturing of a clade C vaccine (30). The CMV peptide pool, consisting of a set of 138 peptides (15 mer overlapping by 11 aa) corresponding to human CMV pp65, was obtained from the National Institutes of Health AIDS Research and Reference Reagent Program. Pooled peptides were used at a final concentration of 1 μg/ml.

In three individuals, HIV-specific CD8+ T cell polyfunctional profiles were also measured in response to autologous T cell lines. For CAP210, p24-Gag(464–472), Gag-YL9 (164YVDRFFKTL172) and Vif-W19 (WHLHGHGVS1) peptides were used. For CAP228, EnvV138 (Env-DV9; sDACKAYEREVS) peptide was used, and for CAP239, Nef82–90 (Nef-KP9; qKAAVDLSEFq) was used. Each peptide was used at a final concentration of 2 μg/ml. Viral sequencing was carried out as previously described (31).

The estimated purity of peptides was >80% as measured by HPLC and mass spectrometry. Individual peptides were diluted in DMSO (Sigma-Aldrich) and prepared as previously described (6). All prepared individual peptides or peptide pools were stored at −80°C before use.

Cell preparation

PBMCs were isolated by standard Ficoll-Hypaque density gradient centrifugation (Amersham Pharmacia) and cryopreserved in 90% heat-inactivated FBS (Invitrogen) with 10% DMSO in liquid nitrogen until needed. Frozen PBMCs were thawed and rested in RPMI 1640 (Invitrogen) containing 10% heat-inactivated FBS and 50 μg/ml gentamicin (Invitrogen) at 37°C and 5% CO2 for 18 h before use in intracellular cytokine staining assays.

Surface phenotypic and intracellular cytokine staining using flow cytometry

Flow cytometric detection of phenotypic and functional markers was performed as described by Lamoreaux and colleagues (32). The following Abs and fluorophores were used: CD3-allophycocyanin-Cy7, CD45RO-PE, Texas Red, CD27-PE-Cy5, IFN-γ–FITC, and TNF-α–PE–Cy7 (all Beckman Coulter); CD4-PE-Cy5.5 (Caltag); CD8-QD705 (Invitrogen); IL-2–PE–Cy5 (BD Biosciences); CD4–PE-Cy5.5 (Caltag); CD45RA–PE–Cy7 (BD Biosciences); and CD14-PE–Cy7 (Caltag). IL-2–allophycocyanin and CD107a–Alexa 680 (eBioscience); MIP1β–PE (R&D Systems); CD4–Pacific Blue and CD19–Pacific Blue (both BioLegend); and the violet amine reactive dye “Vivid” (Molecular Probes). All Abs were pretreated to optimal concentrations. In brief, PBMCs were stimulated with anti-CD28 and anti-CD49d (1 μg/ml) and one to four peptide pools (Gag, Pol, Nef, and Env), autologous peptides, or the CMV peptide pool for 6 h in the presence of brefeldin A (10 μg/ml; Sigma), monensin (0.7 μg/ml; BD Biosciences), and CD107a Abs. Cells were first stained with Vivid for 10 min and washed once with PBS. Cells were then surface stained with CD4, CD8, CD45RO, CD27, CD14, and CD19 Abs. Cells were fixed and permeabilized using Cytofix/Cytoperm buffer (BD Biosciences) and stained intracellularly with CD3, MIP1β, TNF-α, and IFN-γ and IL-2. After washing, cells were resuspended in 1% paraformaldehyde (Electron Microscopy Solutions). Approximately 500,000 events were acquired per stimulation on an LSRII flow cytometry (BD Biosciences), and analysis was performed using FlowJo (v9.4.3; Tree Star). Dead cells (Vivid+), monocytes (CD14+), and B cells (CD19+) were excluded from the analysis. Cells were gated on singlets, live CD3+, small lymphocytes, and CD8+ cells. Naive CD8+ T cells (CD45RO+ CD27+) were identified to total CD8+ Ag-experienced T cells (hereafter referred to as “memory” cells). Different memory subsets were identified using CD45RO and CD27 expression, and HIV- or CMV-specific CD8+ T cells were identified based on CD107a, MIP1β, TNF-α, and/or IFN-γ expression. We do not report on IL-2 production because it was absent or below our cutoff for a positive response (see later) for HIV- and CMV-specific CD8+ T cells. Memory subsets and cytokine-producing CD8+ T cells are expressed as a percentage of total CD8+ memory cells. The gating strategy is provided in Supplemental Fig. 1. A positive cytokine response was defined as at least twice the background (no Ag, only costimulatory Abs), >0.05% of total memory T cells, and at least 40 events. The latter criterion was introduced to minimize the possibility of error caused by a low number of
events when further subdividing these cells into the four memory subsets. Polyfunctionality of Ag-specific cells was analyzed using a boolean gating strategy and represented visually using Pestle (v1.6.2) and Spice (v5.1) software (provided by the National Institutes of Health) (33).

Statistical analysis

Statistical analysis and graphical presentation were performed using InStat and Prism software (v5; GraphPad). Data are expressed as median values with interquartile ranges (IQRs) and analyzed by the use of nonparametric statistics. Statistical analysis of significance was calculated using either Mann–Whitney or Kruskal–Wallis ANOVA using Dunn’s test for multiple comparisons. All tests were two-tailed, and a p value <0.05 was considered statistically significant. The relationship between memory populations was analyzed using Spearman rank correlations.

Results

Polyfunctional and memory maturation profiles of HIV-specific CD8+ T cell responses at 8 mo postinfection

To investigate the relationship between the polyfunctional repertoire of HIV-specific CD8+ T cells and their maturation profiles, we selected positive responders to Gag, Env, Nef, and Pol peptide pools among HIV-1 subtype C-infected individuals. Twenty individuals were tested for Gag responses, 16 for Env and Nef responses, and 8 for Pol responses. Clinical characteristics of study participants are reported in Materials and Methods. PBMCs, collected at a median of 34 wk postinfection, were stimulated with the relevant peptide pools and labeled with a panel of Abs to assess four different functions and four distinct memory subsets. The functions measured were the cytokines IFN-γ and TNF-α, the chemokine MIP1β, and CD107a, used as a surrogate marker of degranulation. The four memory subsets were early-differentiated (ED) cells (CD45RO+CD27+), late-differentiated (LD) cells (CD45RO+CD27−), intermediate cells (Inter; CD45RO+CD27dim), and TD cells (CD45RO−CD27−). The average magnitude of total HIV-specific CD8+ T cells in responding individuals was 1.8% (ranging from 0.15 to 11.2% of total memory CD8+ T cells; Fig. 1A). No statistically significant differences were observed between the frequencies of Gag, Env, Nef, or Pol responses.
responses. Of note, the frequency of HIV-specific CD8+ T cells strongly associated with the absolute number of HIV-specific CD8+ T cells (calculated based on absolute CD8+ count, \( r = 0.84, p < 0.0001 \), data not shown). Further assessment of the combination of functions exhibited by HIV-specific CD8+ T cell responses revealed that \( \sim 40\% \) of the total responses consisted of cells positive for three or four functions, with no significant differences in the polyfunctional abilities of cells between the different HIV peptide pools tested (Fig. 1B). Regardless of the specificity of the CD8+ T cell responses against the HIV peptide pools tested, the most prevalent population observed consisted of cells simultaneously producing IFN-\( \gamma \), MIP1ß, and degranulating (IFN-\( \gamma \)-MIP1ßCD107a+; median 25% of total response [IQR: 11–34]). HIV-specific CD8+ T cells endowed with two functions were mostly distributed among the CD107a*MIP1ß+, IFN-\( \gamma \)-MIP1ß+, and CD107aIFN-\( \gamma \)+ cells (median 11% [IQR: 7–17], 8% [IQR: 4–9], and 6% [IQR: 1.6–7] of total response, respectively). Monofunctional HIV-specific CD8+ T cells were predominantly CD107a+ or MIP1ß+ (median 18% [IQR: 10–29] and 14% [IQR: 8–23] of total response, respectively). Of note, the proportion of cells producing only IFN-\( \gamma \)- \( \alpha \) was marginal, representing \( \sim 3\% \) of the total measured response, and TNF-\( \alpha \) was detected only in cells endowed with four functions (Fig. 1B). These profiles are consistent with previous reports on the functional characteristics of HIV-specific CD8+ T cells (9, 34–36).

We next assessed the memory maturation profiles of HIV-specific CD8+ T cells based on the expression of CD45RO and CD27. A representative dot plot of the memory profile of HIV-specific CD8+ T cells is presented in Fig. 1C. Total HIV-specific CD8+ T cells exhibited predominantly an ED memory phenotype (CD45RO+CD27+); however, the frequencies of ED HIV-specific CD8+ T cells were variable among the individuals analyzed (median 57%, ranging from 7 to 88%; Fig. 1D). The percentage of ED HIV-specific CD8+ T cells was negatively correlated with viral load \( (p < 0.0001, r = -0.6, \text{data not shown}) \), suggesting that these variations likely reflect the differences in viral replication levels in these individuals. This is in accordance with data previously reported from the same cohort (15). Of note, the memory maturation profiles did not differ significantly among Gag-, Env-, Nef-, and Pol-specific responses (Supplemental Fig. 2A).

In summary, our results show that Gag-, Env-, Nef- and Pol-responsive CD8+ T cells were quantitatively and qualitatively similar in our cohort, and HIV-specific CD8+ T cells exhibited mainly an ED memory phenotype and moderate polyfunctionality.

**Functionality of HIV-specific CD8+ T cells within distinct memory subsets**

To determine whether the polyfunctional properties exhibited by Ag-specific CD8+ T cells were dictated by their differentiation stage, we next compared the functional profiles of HIV-specific CD8+ T cells within different memory subsets. Because the functions and phenotypes of CD8+ T cells specific for Gag, Env, Nef, and Pol were similar (Supplemental Fig. 2B), we combined all HIV-specific responses for these analyses, and report on 43 peptide pool responses in 20 individuals. In brief, total HIV-specific CD8+ T cells were gated on distinct memory subsets (ED, Inter, LD, and TD), and we then measured the distribution of HIV-specific CD8+ T cells expressing four, three, two, or one function in each memory subpopulation. Fig. 2A shows representative dot plots of memory profiles of HIV-specific T cells positive for four, three, two, or one function, from two individuals with a high- and a low-magnitude Gag-specific CD8+ T cell response. Depending on their memory profiles, HIV-specific CD8+ T cells had distinct polyfunctional abilities, with decreasing polyfunctionality coinciding with an increase in differentiation from ED to TD memory subsets (Fig. 2B). HIV-specific CD8+ T cells with a TD phenotype were primarily monofunctional (median 69% [IQR: 57–83]), whereas ED CD8+ T cells had a significantly lower proportion of monofunctional cells (median 27% [IQR: 16–38]; \( p < 0.001 \)) and a significantly higher proportion of cells positive for 4 and 3 functions \( (p < 0.001) \) compared with TD CD8+ T cells. Inter and LD HIV-specific CD8+ T cells showed an intermediate polyfunctional profile consisting of: 1) significantly more monofunctional cells compared with the ED subset (median 51% [IQR: 22–61], \( p < 0.01 \), and 43% [IQR: 30–54], \( p < 0.01\) respectively); and 2) significantly more cells positive for 4 and 3 functions compared with the TD subset (Fig. 2B). It is worth noting that although the TD subset had the highest proportion of monofunctional cells (Fig. 2B), they contributed only \( \sim 20\% \) to the absolute number of HIV-specific monofunctional CD8+ T cells (data not shown), because the TD subset itself represents only a median of 14% of all HIV-specific CD8+ T cells (as shown in Fig. 1D). Consequently, even though there was a lower proportion of monofunctional CD8+ HIV-specific cells within the ED subset compared with the TD subset, in absolute numbers, there were more circulating monofunctional ED cells than monofunctional TD cells, because ED cells make up almost 60% of total HIV-specific CD8+ T cells.

To determine whether differences in HIV-specific CD8+ T cell polyfunctional profiles observed within each memory subset could also be identified using single peptides, and were not an artifact of heterogeneous specificities in the pools, we assessed the degree of polyfunctionality in each memory subpopulation in response to stimulation with optimal autologous 9mer peptides. Fig. 2C shows the polyfunctional profile of Gag-VL9-specific CD8+ T cells within the different memory subsets in one individual (CAP210). We found that increasing cell differentiation from ED to TD subsets resulted in a decreased proportion of epitope-specific cells exhibiting three and four functions, and an elevated proportion of monofunctional epitope-specific cells. Four autologous peptide responses (Gag-VL9, Vif-W19, Env-DV9, and Nef-KP9) were measured in three individuals (CAP210, CAP228, and CAP239). As demonstrated with peptide pools, epitope-specific CD8+ responses displayed greater polyfunctionality in the ED subset when compared with the TD subset \( (p < 0.05; \text{Fig. 2D}) \).

To assess the specific cytokines that made up the different polyfunctional subsets, we next compared the proportions of the eight most prevalent combinations of functions detected within each HIV-specific CD8+ T cell memory subset (Fig. 3A). Both CD107a+ and MIP1ß+ cells accounted for the increased proportion of monofunctional cells in the TD memory subset; the proportion of CD107a+ cells were almost 3-fold higher in ED compared with TD subsets, from a median of 13% [IQR: 6–17] to 33% [IQR: 16–45; \( p < 0.0001 \)), whereas the proportion of CD8+ T cells producing MIP1ß alone was a median of 13% [IQR: 5–15] in ED HIV-specific cells compared with 34% [IQR: 19–43] in TD HIV-specific cells \( (p < 0.0001; \text{Fig. 3A}) \). Interestingly, no significant change in the proportion of cells expressing IFN-\( \gamma \)-only was observed between the different memory subpopulations. Fig. 3B summarizes the mean distribution of detectable cytokine combinations within each HIV-specific CD8+ T cell memory subset, illustrating the progressive increase in the proportion of CD107a+ and MIP1ß+ monofunctional cells and the decrease in the proportion of cells endowed with three (CD107a*MIP1ß*IFN-\( \gamma \)) and four functions in HIV-specific cells from early to terminal differentiation. Importantly, the degree of polyfunctionality of HIV-specific CD8+ T cells in each memory subset was similar between individuals with low \( (<3000 \text{copies/ml}) \) or high viral
FIGURE 2. Polyfunctional profile of HIV-specific CD8+ T cells in defined memory subsets. (A) Representative density and overlay dot plots of memory maturation in total CD8+ T cells (density) and Gag-specific CD8+ T cells endowed with four (red), three (orange), two (green), or one (blue) function for two study individuals with a high and low Gag response. (B) Proportions of HIV-specific CD8+ T cells, exhibiting four, three, two, or one function, across distinct memory subsets. Responses to Gag, Env, Nef, and Pol have been pooled (n = 43 in 20 individuals). Results are shown as box and whisker (10–90 percentile) plots, with outliers depicted with black dots. (C) Representative example of memory profile of total Gag-YL9–specific CD8+ T cells (producing CD107a, MIP1b, IFN-γ, or TNF-α). The pie chart inlaid within the dot plot corresponds to the polyfunctional profile of total Gag-YL9–specific CD8+ T cells. Adjacent pies represent the degree of polyfunctionality in Gag-YL9–specific CD8+ T cells according to their maturation phenotype. (D) Proportion of HIV-specific CD8+ T cells expressing four, three, two, and one function across distinct memory subsets (n = 4 responses in 3 individuals). Each symbol corresponds to an autologous peptide response. ○, Gag (YL9)-specific response in CAP210; •, Vif (W9) response in CAP210; ×, Env (DV9)-specific response in CAP228; ■, Nef (KF9)-specific response in CAP239. The median and IQRs are shown for each group. The p values were calculated using one-way ANOVA nonparametric Kruskal–Wallis test; *p < 0.05, **p < 0.01, ***p < 0.001.
load (>100,000 copies/ml; Supplemental Fig. 3A), indicating that the distribution of polyfunctional cells within each memory subset was independent of viremia.

Overall, these data suggest that in HIV infection, the polyfunctional profile of HIV-specific cells is related to their differentiation stage, where ED cells exhibit a greater degree of polyfunctionality, and TD cells are more monofunctional.

**Maturation and functional characteristics of CMV-specific CD8+ T cells**

To investigate whether our observations linking the degree of polyfunctionality with the memory maturation profile in HIV infection holds true for another pathogen, we measured the polyfunctional attributes of distinct CMV-specific CD8+ T cell subsets in 12 participants, 6 of whom were not HIV infected and 6 chronically infected with HIV. All HIV-infected participants were ART naive. CMV-specific CD8+ T cells were highly polyfunctional, with ~75% (IQR: 63–85) of the total response consisting of cells positive for four functions (i.e., CD107a, MIP1β, IFN-γ, and TNF-α; Fig. 4A). No significant differences were detected between HIV-infected and uninfected individuals. Interestingly, the prevalent cytokine combinations observed in CMV-specific CD8+ T cells were distinct from HIV-specific responses, with CMV-specific cells positive for three functions consisting mostly of MIP1β+IFN-γ+TNF-α+ cells. CMV-specific cells endowed with two functions were present at low levels or were undetectable, and monofunctional CMV-specific cells expressing TNF-α only were detectable (Fig. 4A), a subset that was absent for HIV-specific responses. Representative examples of CMV-specific CD8+ T cells and their memory subset distribution are depicted in Fig. 4B.

When we compared the cell memory distribution for each Ag, we found that the proportion of TD cells was significantly higher in CMV-specific CD8+ T cells when compared with HIV-specific CD8+ T cells (median 31% [IQR: 24–40] and 14% [IQR: 9–20], respectively; p = 0.003), and this was true regardless of HIV infection status (Fig. 4C). Thus, CMV-specific responses exhibited a high degree of polyfunctionality and a differentiated phenotype, as previously described (13, 16, 34).

We next examined the distribution of polyfunctionality between different CMV-specific CD8+ T cell memory subsets, as we had done for HIV. Interestingly, and in contrast with HIV-specific CD8+ T cells, the polyfunctional capacity of CMV-specific CD8+ T cells was similar across all memory subsets (Fig. 5), suggesting that for
CMV-specific CD8+ T cell responses, polyfunctional profiles are neither linked to specific memory subsets nor enriched in particular memory subsets. Of note, the degree of polyfunctionality of CMV-specific CD8+ T cells within each subset was comparable in HIV-infected and uninfected individuals (Supplemental Fig. 3B). The difference between HIV- and CMV-specific CD8+ T cells was further emphasized when we compared the polyfunctional properties of each memory subset. Fig. 6A shows that ED HIV-specific CD8+ T cells exhibited a significantly higher proportion of cells positive for two or three functions and a lower proportion of four-function cells (p < 0.0001) compared with ED CMV-specific CD8+ T cells. Moreover, TD HIV-specific CD8+ T cells exhibited a significantly higher proportion of cells positive for one or two functions (p < 0.0001 and p = 0.0004, respectively) and a lower proportion of cells positive for three or four functions (p = 0.01 and p < 0.0001, respectively) compared with CMV-specific responses. Furthermore, there was a significant positive association between the proportion of HIV-specific CD8+ T cells endowed with one function and the proportion of TD HIV-specific CD8+ T cells (p = 0.019, r = 0.546), whereas the proportion of HIV-specific CD8+ T cells positive for three functions was inversely correlated with the proportion of TD HIV-specific CD8+ T cells (p = 0.01, r = −0.587; Fig. 6B, left panel). No such associations were observed for CMV-specific CD8+ T cells (Fig. 6B, right panel). Overall, these results show that for HIV-specific CD8+ T cells, polyfunctional properties were linked to differentiation levels, in contrast with CMV-specific CD8+ T cells, where polyfunctional capacities were similar across all memory subsets.

Discussion

Despite abundant literature on both the memory differentiation profiles and polyfunctional capacities of HIV-specific CD8+ T cells at different stages of infection (9–11, 13–15), less is known about how these T cell characteristics are associated with each other. In this study, we examined the relationship between the memory phenotype of CD8+ T cells and the polyfunctional responses of these cells, as measured by IFN-γ, TNF-α, MIP1β, and CD107a expression. We studied HIV-specific CD8+ T cell responses in 20 HIV-1–infected individuals at ~34 wk postinfection and compared them with CMV-specific T responses. We detected a higher proportion of polyfunctional CD8+ T cells specific for CMV compared

FIGURE 4. Polyfunctional and memory maturation profiles of CMV-specific CD8+ T cell responses. (A) Polyfunctional profiles of CMV-specific CD8+ T cells. All possible combinations of four functions (CD107a, MIP1β, IFN-γ, and TNF-α) produced by HIV-infected (n = 6) and HIV-uninfected (n = 6) individuals are shown on the x-axis. Box and whiskers indicate the median percentage and IQR of the total response contributed by CD8+ T cells. Functional profiles are grouped and color-coded according to number of functions and summarized in the pie charts. Each slice of the pie corresponds to the median production of four (red), three (orange), two (green), or one (blue) function. (B) Representative dot plots of the memory maturation profile of total CD8+ T cells (density) and CMV-specific total cytokine+ CD8+ T cells (red dots) in one HIV-infected and one HIV-uninfected individual. (C) Comparison of memory maturation profiles of CMV- and HIV-specific CD8+ T cells. Closed circles (○) correspond to HIV-infected individuals (n = 6), and open circles (●) represent HIV-uninfected individuals (n = 6). Horizontal lines indicate median values. The p values were calculated using a one-way ANOVA nonparametric Kruskal–Wallis test.

FIGURE 5. Polyfunctional profiles of CMV-specific CD8+ T cells in defined memory subsets. Proportion of CMV-specific CD8+ T cells exhibiting four, three, two, or one function across the different memory subsets. HIV-infected and −uninfected individuals have been pooled (n = 12). Results are shown as box and whisker (10–90 percentile) plots, and outliers are depicted with black dots. The p values were calculated using a one-way ANOVA nonparametric Kruskal–Wallis test; *p < 0.05.
with HIV, which agrees with previous observations (34). HIV-specific responses were enriched for cells with an ED phenotype, whereas CMV-specific cells were mainly of a TD phenotype, consistent with earlier findings (14, 37). Our novel observation was a distinct pattern of polyfunctionality between HIV- and CMV-specific CD8+ T cell memory subsets. For HIV-specific CD8+ T cells, ED memory cells (CD45RO+CD27+) were enriched for a polyfunctional response, and there was a significant reduction in the number of functions in TD cells (CD45RO-CD27-). In contrast, CMV-specific CD8+ T cells exhibited a polyfunctional profile that was similar across ED, LD, or TD memory subsets. Moreover, the CMV response was highly polyfunctional (80–90% of cells expressing three or four functions) in both HIV-infected and uninfected individuals, compared with HIV-specific CD8+ T cells (40–50%). This implies that there is not a global decrease in CD8+ T cell polyfunctionality in the background of HIV infection. Differences in Ag load or recurrence or CD4+ help may account for differential (poly)function between HIV-specific CD8+ T cells and cells of other specificities. Overall, this suggests that the hierarchical loss in the number of functions by an Ag-specific CD8+ T cell as memory differentiation proceeds is dependent on the infecting pathogen, and that memory phenotype and polyfunctional characteristics of CD8+ T cells can differ significantly.

Seminal work from Sallusto and colleagues (20) introduced the concept of Ag-specific cells being divided into memory subsets expressing different phenotypic markers with distinct homing and survival abilities, and also alluded to a functional distinction between different memory subsets. A number of factors have been shown to shape this functional and phenotypic heterogeneity of Ag-specific T cells. Costimulatory signals engaged during T cell priming, as well as cytokines such as IL-2 and IL-21, can modulate T cell functionality (38–41). Major determinants of CD8+ T cell polyfunctionality are Ag concentration (26, 42, 43) and TCR affinity (44). Work from murine models has proposed that the clonal expansion process can modulate the profile of secreted cytokines (45, 46). With regard to memory differentiation of CD8+ T cells, the degree of cell maturation is dependent on similar factors, namely, Ag load, costimulation signals, and the cytokine environment (reviewed in Ref. 47). Hence, being regulated by similar factors, it can be speculated that cell differentiation and polyfunctional potential could be codependent phenomena, where the generation and maintenance of late-stage differentiated effector cells, endowed with a rapid response to pathogens and a high degree of polyfunction, would be favorable to ensure viral control. Indeed, in certain well-controlled infections or successful vaccinations (such as CMV, vaccinia, or yellow fever vaccine),
Ag-specific CD8+ T cells that are generated are highly differentiated and highly polyfunctional (23, 48). In contrast, during uncontrolled HIV infection, HIV-specific CD8+ T cells exhibit mainly an ED memory phenotype, often regarded as an immature stage (14, 37). However, the relationship between maturation and polyfunction appears to be more complex, because Ag-experienced cells exhibit heterogeneous memory and cytokine secretion profiles dependent on different Ag specificities (reviewed in Ref. 21). Our data are in agreement with the latter observation, where we show that differentiation toward late memory for HIV-specific CD8+ T cells is accompanied by a progressive loss of polyfunctional capacities, whereas CMV-specific CD8+ T cells retain their polyfunctional capacities regardless of memory differentiation.

Although sustained HIV replication appears to play a predominant role in driving CD8+ T cells toward a late stage of memory maturation (15, 17, 18), we found no difference in the memory–polyfunction association between those with high and low HIV viral loads; that is, those controlling viral replication did not resemble the stable “polyfunctionality regardless of memory phenotype” pattern of CMV-specific CD8+ T cells. It would be of interest to determine whether this is also the case for HIV-specific cells from individuals on long-term, successful ART, where Ag load is reduced.

Our starting point for this study was that polyfunctional T cells, capable of carrying out a range of functions simultaneously, exhibit superior protective immunity (9, 49). We can speculate that measuring multiple functions on a per cell basis may more closely reflect the ability of CD8+ T cells to impart antiviral effects and may be more relevant than focusing only on one function, such as IFN-γ. However, the combination of specific functions rather than the number of functions per se may offer a more refined and accurate assessment of protective immunity (50). Upregulation of perforin has recently been highlighted as an important indicator of cytotoxic potential for control of HIV (51, 52). This is also a consideration in the question of how maturation profiles of CD8+ T cells and functional abilities are linked, because specific functions such as perforin and IL-2 may indeed be associated with particular memory phenotypes, whereas other functions may not be as tightly regulated. Loss of CD28 expression appears to be coupled to loss of IL-2 production, whereas T-bet expression correlates with perforin upregulation (53). In our study, we did not measure perforin nor could we detect IL-2 responses. Indeed, CMV-specific CD8+ T cells rarely produce IL-2 (53), and IL-2 production in the context of HIV infection is confined to long-term nonproducers (54, 55).

Excessive activation of the immune system can influence both the function and phenotype of CD8+ T cells. Several studies have described the upregulation of PD-1 and other inhibitory receptors such as CD160, 2B4, and LAG3 on CD8+ T cells in chronic viral infections, including HIV, which results in defective cytokine production and lack of polyfunctional responses. This can be partially reversed by blocking the interaction of these receptors with their ligands (56–58). Yamamoto and colleagues (37) recently described elevated levels and coexpression of several negative regulators on HIV-specific CD8+ T cells compared with CMV-specific cells. Coexpression of these inhibitory receptors correlated inversely with polyfunctionality, and PD-1 blockade restored cytokine production. It would thus be of interest to determine whether, in HIV infection, the memory maturation profile of HIV-specific CD8+ T cells coincides with their degree of functional inhibition.

In conclusion, our data show that the polyfunctional abilities of HIV-specific CD8+ T cells are influenced by the stage of memory differentiation, which is not the case for CMV-specific responses. This emphasizes that different pathogens generate CD8+ T cell responses with distinct polyfunctional–memory subset profiles; this may reflect the distinct life histories of pathogens and their interactions with the immune system. A better understanding of which memory–function combinations lead to superior and durable protective immunity against HIV is needed.

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Disclosures
The authors have no financial conflicts of interest.

References


Supplementary Figure S1: Description of the gating strategy. Singlets were identified in forward scatter plots. Dead cells, B cells and monocytes were excluded by Vivid, CD19 and CD14 labeling, and live lymphocytes were identified using CD3 staining. Total CD8+ T cells were gated taking into account potential CD8 down-regulation. Total CD8+ T cells were then separated by the expression of CD45RO and CD27, allowing us to discriminate five subpopulations (Naive: CD45RO-CD27+; Early-differentiated (ED): CD45RO+CD27+, Late differentiated (LD): CD45RO+CD27-, Intermediate (Inter): CD45RO-CD27dim) and Terminally-differentiated (TD): CD45RO-CD27-. An inverted gate was then applied to exclude naïve cells and identify total CD8+ antigen-experienced T cells. Gates applied for the identification of cytokine positive cells were defined according to the unstimulated samples for each individual.
Supplementary Figure S2: (A) Comparison of the memory differentiation profiles between total Gag, Env, Nef and Pol-specific CD8+ T cell responses. Bars represent the median values. (B) Comparison of the degree of polyfunctionality within each memory subset between total Gag, Env, Nef and Pol-specific CD8+ T cell responses. Bars represent the median values. ED: Early differentiated cells, Inter: Intermediate cells, LD: Late differentiated cells and TD: Terminally-differentiated cells.
Supplementary Figure S3: (A) Comparison of the proportion of HIV-specific CD8+ T cells producing 4, 3, 1 and 1 function across different memory subsets between individuals exhibiting low viral loads less than 3,000 HIV RNA copies/ml (Low VL, n = 15 HIV-specific responses from six individuals) and viral loads greater than 100,000 HIV RNA copies/ml (High VL, n = 15 HIV-specific responses from seven individuals). Results are shown as box and whisker (10-90 percentile) plots, with outliers depicted with black dots. Bars represent the median values. (B) Comparison of the proportion of CMV-specific CD8+ T cells producing 4, 3, 2 and 1 function within Early-differentiated (ED) and Terminally-differentiated (TD) memory subsets between HIV-infected individuals (●, n=6) and HIV-uninfected individuals (○, n=6). Bars represent the median values.