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# Restoration of CD4<sup>+</sup> Responses to Copathogens in HIV-Infected Individuals on Antiretroviral Therapy Is Dependent on T Cell Memory Phenotype

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Antiretroviral therapy (ART) induces rapid suppression of viral replication and a progressive replenishment of CD4<sup>+</sup> T cells in HIV-infected individuals. However, the effect of ART on restoring pre-existing memory CD4<sup>+</sup> T cells specific for common copathogens is still unclear. To better understand the dynamics of Ag-specific CD4<sup>+</sup> T cells during ART, we assessed the frequency, functional capacity, and memory profile of CD4<sup>+</sup> T cells specific for *Mycobacterium tuberculosis* and CMV in 15 HIV-infected individuals before and 1 y after ART initiation. After ART initiation, the frequency of *M. tuberculosis*-specific CD4<sup>+</sup> T cells showed little change, whereas CMV-specific CD4<sup>+</sup> T cells were significantly lower ( $p = 0.003$ ). There was no difference in the polyfunctional or memory profile of Ag-specific CD4<sup>+</sup> T cells before and after ART. The replenishment of Ag-specific CD4<sup>+</sup> T cells correlated with the memory differentiation profile of these cells prior to ART. Pathogen-specific CD4<sup>+</sup> T cells exhibiting a late differentiated profile (CD45RO<sup>+</sup>CD27<sup>-</sup>) had a lower capacity to replenish ( $p = 0.019$ ;  $r = -0.5$ ) compared with cells with an early differentiated profile (CD45RO<sup>+</sup>CD27<sup>+</sup>;  $p = 0.04$ ;  $r = 0.45$ ). In conclusion, restoration of copathogen-specific memory CD4<sup>+</sup> T cells during treated HIV infection is related to their memory phenotype, in which early differentiated cells (such as most *M. tuberculosis*-specific cells) have a higher replenishment capacity compared with late differentiated cells (such as most CMV-specific cells). These data identify an important, hitherto unrecognized, factor that may limit restoration of copathogen immunity in HIV-infected individuals on ART. *The Journal of Immunology*, 2015, 195: 2273–2281.

The hallmarks of untreated HIV infection are a progressive loss of CD4<sup>+</sup> T cells, sustained cellular activation, and chronic inflammation (1–3). In addition to the numerical depletion of CD4<sup>+</sup> T cells, HIV can also alter the functional ca-

capacity of these cells, impairing their proliferative potential, altering their cytokine secretion profiles, and changing their phenotypic characteristics in response to HIV Ags as well as various copathogens (4–7). Both of these quantitative and qualitative alterations can lead to increased susceptibility to opportunistic infections, including tuberculosis (TB), Candidiasis, and human papilloma virus infection (8). Indeed, HIV is the best-recognized risk factor for TB disease even before profound CD4<sup>+</sup> T cell deficiency (9, 10). The introduction of antiretroviral therapy (ART) has drastically decreased morbidity and mortality in HIV-infected individuals (11), inducing a rapid reduction of plasma viral load and a progressive repletion of CD4<sup>+</sup> T cells (12). Although the clinical benefit of ART is undeniable, the extent to which ART can fully normalize functional immunity remains unclear (13). HIV-infected individuals on ART exhibit a differential degree of recovery of copathogen-specific CD4<sup>+</sup> T cell responses, depending on the pathogen they target (14–20). For example, it has been shown that the restoration of CMV-specific CD4<sup>+</sup> T cells occurs early after ART (19), but appears to be short-lived (15). Conversely, *Candida*-specific CD4 responses recover slowly (16). Contrasting data exist on the degree of recovery of *Mycobacterium tuberculosis*-specific CD4<sup>+</sup> T cell responses upon ART. Jambo et al. (20) showed in a cross-sectional study that the frequency and polyfunctional profile of *M. tuberculosis*-specific CD4<sup>+</sup> T cell responses were similar in ART-naive or treated individuals, whereas Sutherland et al. (18) reported that ART increases the polyfunctional capacity of these cells. Other studies described only a partial reconstitution of *M. tuberculosis*-specific CD4<sup>+</sup> T cell responses after ART (14, 17).

It is of particular importance to define the factors that associate with successful pathogen-specific CD4<sup>+</sup> T cell recovery upon ART, as limited “normalization” of functional CD4<sup>+</sup> T cell responses could account for sustained incidence of opportunistic infections.

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The online version of this article contains supplemental material.

Abbreviations used in this article: ART, antiretroviral therapy; ED, early differentiated; IQR, interquartile range; LD, late differentiated; LDL, lower detection limit; SEB, staphylococcal enterotoxin B; TB, tuberculosis.

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Several parameters influence the degree and dynamics of recovery of the overall CD4<sup>+</sup> T cell compartment in response to ART, such as age, CD4 count at the time of treatment initiation, and timing of ART initiation after HIV infection (21–23). However, it is still unclear why CD4<sup>+</sup> T cells of different pathogen specificities have different profiles of restoration, and the mechanisms mediating this variable recovery of memory CD4<sup>+</sup> T cells to copathogens are still incompletely understood. Thus, to better understand the effect of successful ART on the dynamics of recovery of copathogen-specific CD4<sup>+</sup> T cells, we compared the magnitude, functional capacity, and memory differentiation profiles of *M. tuberculosis*- and CMV-specific CD4<sup>+</sup> T cells before and 1 y after ART initiation in a cohort of HIV-infected individuals and HIV-uninfected controls.

## Materials and Methods

### Study participants

Blood samples were collected from 15 women participating in the CAPRISA 002 study, a cohort study following HIV-infected women from HIV seroconversion until 5 y on treatment. The cohort is situated in KwaZulu-Natal, South Africa, and has been previously described (24, 25). Participants were selected based on sample availability. Blood samples from nine HIV-uninfected participants were provided from the CAPRISA 004 vaginal microbicide (1% tenofovir) gel trial (26). An additional 14 HIV-uninfected participants from CAPRISA 004 were studied for immune activation. HIV-uninfected participants were from the same community as the HIV-infected individuals and age-matched; they were either in the preintervention phase or the placebo arm of the trial. For HIV-infected individuals, the time of infection was estimated either as the same date as a prospective RNA-positive/Ab-negative measurement or taken as the midpoint between the last Ab negative test and first Ab-positive ELISA test. Participants in the cohort were offered ART according to South African national HIV treatment guidelines (at a CD4 count of <200 cells/mm<sup>3</sup> prior to October 2012; <350 cells/mm<sup>3</sup> until the present). Eight of the 15 participants were taking standard first-line therapy (TDF/3TC/EFV), 3 were on ddI-EC/3TC/EFV, and 1 each on d4T/3TC/EFV, TDF/3TC/NVP, d4T/3TC/NVP, and AZT/3TC/LPV/r. Two participants switched drug regimens while on study, namely CAP200 (EFV/3TC/ddI-EC to EFV/3TC/TDF at month 11) and CAP255 (EFV/3TC/d4T to EFV/3TC/AZT at month 10). No participants had active TB during the study period or exhibited any immune reconstitution disease upon HIV treatment. Ethical approval for the study was obtained from the University of KwaZulu-Natal and University of Cape Town Research Ethics Committees. All participants provided written informed consent to participate in the study.

### Determination of plasma viral load and CD4 counts

Plasma HIV viral loads and CD4 counts were quantified at each study visit. Over the course of the study, the viral load PCR assay switched from Roche AMPLICOR HIV-1 monitor test version 1.5 (lower detection limit [LDL] of 400 RNA copies/ml), to Roche TaqMan version 1.0 on June 1, 2010 (LDL 40 RNA copies/ml), and then to Roche TaqMan version 2.0 on January 9, 2012 (LDL 20 RNA copies/ml). Absolute blood CD4 and CD8 T cell counts were measured using the FACSCalibur TruCOUNT method (BD Biosciences) and expressed as cells/mm<sup>3</sup>. Plasma samples matching the visits where PBMC were studied were tested for Abs to CMV and CMV DNA. Seropositivity was determined using the Cobas CMV IgG Assay (Roche), and CMV viral load was detected and quantified using a CMV R-gene PCR kit (Argene), with a detection limit of 30 copies/ml.

### Cell preparation

PBMC were isolated by standard Ficoll-Hypaque density gradient centrifugation (Amersham Pharmacia) and cryopreserved in 90% heat-inactivated FCS (Invitrogen) plus 10% DMSO and stored in liquid nitrogen until needed. Cryopreserved PBMC were thawed and rested in R10 (RPMI 1640 containing 10% heat-inactivated FCS [Sigma-Aldrich] and 50 U/ml of penicillin-streptomycin) at 37°C and 5% CO<sub>2</sub> for 8 h prior to Ag stimulation.

### Ag and cell stimulation

Following resting, PBMC were stimulated using *M. tuberculosis* Purified Protein Derivative (10 µg/ml; Statens Serum Institute), a pool of CMV peptides consisting of 138 peptides (15-mers overlapping by 11 aa) covering the entire HCMV pp65 protein (2 µg/ml; National Institutes of

Health AIDS Reagent Program) and staphylococcal enterotoxin B (SEB; 2.5 µg/ml; Sigma-Aldrich), used as a positive control. Of note, we found no major differences in the memory profile, cytokine secretion potential, and polyfunctional capacity between peptide and protein-stimulated PBMC (R. Bunjun, C. Riou, A.P. Soares, N. Thawer, T.L. Muller, A. Kiravu, Z. Ginbot, W. Hanekom, G. Walzl, R.J. Wilkinson, and W.A. Burgers, manuscript in preparation). Stimulations were performed in the presence of costimulatory Abs anti-CD28 and anti-CD49d (both at 1 µg/ml; BD Biosciences) at 37°C for 16 h. Brefeldin A (Sigma-Aldrich) was added after 1 h at a concentration of 10 µg/ml. Surface and intracellular cytokine staining was performed at the end of the incubation period. Sufficient cells were available for assessing CMV and SEB responses in only 12 out of 15 HIV-infected participants.

### Surface phenotypic and intracellular cytokine staining and flow cytometry

The following Abs were used for surface and intracellular staining: CD3-allophycocyanin-H7 (SK7; BD Biosciences), CD4-PE-Cy5.5 (S3.5; Invitrogen), CD8-Qdot 705 (3B5; Invitrogen), CD27-PE-Cy5 (1A4CD27; R&D Systems), CD45RO-ECD (UCHL1; R&D Systems), IFN-γ-Alexa Fluor 700 (B27; BD Biosciences), IL-2-APC (MQ1-17H12; BD Biosciences), TNF-α-PE-Cy7 (MAB11; eBioscience), CD14-Pacific Blue (Tük4; Invitrogen), CD19-Pacific Blue (SJ25-C1; Invitrogen), and a violet amine viability dye ("Vivid"; Molecular Probes). All Abs were titrated to obtain optimal concentrations prior to use. After stimulation, PBMC were first stained with Vivid for 20 min at room temperature. 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, IL-2, TNF-α, and IFN-γ. Cells were then resuspended in 1× CellFix (BD Biosciences). Samples were acquired on a BD Fortessa using FACSDiva software and analyzed using FlowJo (version 9.8.2; Tree Star). The gating strategy is presented in Supplemental Fig. 1. A positive cytokine response was defined as at least twice the background (in the presence of costimulatory Abs and no Ag). For phenotyping the memory profile of cells producing cytokines, a cutoff of 40 events was used. Polyfunctionality of Ag-specific cells was analyzed using Pestle (version 1.7) and Spice (version 5.35) software (27). Immune activation was measured by surface staining of unstimulated cells using the CD3, CD4, CD8, CD14, and CD19 Abs listed above, as well as CD38-APC (HIT2) and HLA-DR-allophycocyanin-Cy7 (L243) (both BD Biosciences), along with the inclusion of Vivid.

### Statistical analysis

Statistical analyses were performed using GraphPad Prism (version 5.0; San Diego, CA). Nonparametric statistical tests were used for all comparisons. The Mann-Whitney *U* test and Wilcoxon signed-rank test were used for unmatched and paired samples, respectively, and the Kruskal-Wallis ANOVA using Dunn's test for multiple comparisons. Correlations were performed using the Spearman rank test. A *p* value <0.05 was considered statistically significant.

## Results

### Effect of ART on the restoration of copathogen-specific CD4<sup>+</sup> T cells

In order to better understand the replenishment dynamics of CD4<sup>+</sup> T cells specific for copathogens in HIV-infected individuals on treatment, we studied 15 HIV-infected individuals before and after ART initiation. The cohort consisted of women infected with HIV-1 for a median of 4.9 y (interquartile range [IQR]: 3.8–5.8 y) at the time of treatment initiation (Table I). Pre-ART samples were obtained at a median of 1.7 mo (IQR: 0.8–2.5) prior to starting ART. The median plasma viral load was 21,955 HIV RNA copies/ml (IQR: 4,743–52,800), and the median CD4 count was 289 cells/mm<sup>3</sup> (IQR: 193–322). After a median of 12 mo of treatment (IQR: 11–12.3 mo), all individuals exhibited some degree of viral suppression (*p* < 0.0001, Supplemental Fig. 2A), which was below the limit of detection of the assay in 12 out of 15 individuals and <400 RNA copies/ml in the remainder. An increase in absolute CD4 count occurred in 13 out of 15 individuals over the period measured (*p* < 0.0001; Supplemental Fig. 2B). The median fold change in absolute CD4 count was 1.9 (IQR: 1.6–2.3), and when

adjusted for length of treatment, the replenishment rate of CD4<sup>+</sup> T cells was 20 cells/mm<sup>3</sup>/month (IQR: 14–26, data not shown); this was accompanied by a concomitant doubling of the CD4/CD8 ratio (0.27 versus 0.66; *p* = 0.0007; Supplemental Fig. 2C). As previously reported (21–23), the degree of CD4 reconstitution was inversely related to absolute CD4 count at ART initiation (*p* < 0.0001; *r* = −0.85) and the duration of HIV infection (*p* = 0.016; *r* = −0.61, data not shown). We also measured immune activation before and after ART using the markers CD38 and HLA-DR. There was a significant reduction in CD4<sup>+</sup> and CD8<sup>+</sup> T cells coexpressing CD38<sup>+</sup> and HLA-DR<sup>+</sup> (*p* = 0.0002 and *p* = 0.0001; Supplemental Fig. 2D), as well as HLA-DR<sup>+</sup> alone (*p* = 0.0001 for both subsets). Residual levels of immune activation after 1 y of ART remained significantly greater than healthy, HIV-uninfected individuals for both markers (Supplemental Fig. 2D). We found no association between the degree of CD4 reconstitution and CD4<sup>+</sup> T cell activation before or after ART (data not shown).

To determine whether the dynamics of copathogen-specific CD4<sup>+</sup> T cells mirror the reconstitution of the total CD4<sup>+</sup> T cell population, we measured the magnitude of *M. tuberculosis*- and CMV-specific CD4<sup>+</sup> T cell responses, as well as SEB-reactive CD4<sup>+</sup> cells, before and after ART initiation. Fig. 1A shows representative flow cytometry plots of cytokine production (IFN- $\gamma$ , IL-2, and TNF- $\alpha$ ) by CD4<sup>+</sup> T cells in response to *M. tuberculosis*, CMV, and SEB stimulation in one HIV-infected patient (CAP267). At baseline (before ART initiation), 11 out of 15 individuals tested had a detectable *M. tuberculosis* response, with a median frequency of 0.08% of total CD4<sup>+</sup> T cells (range, 0–0.9%; Fig. 1B). CMV responses were detected in 10 out of 12 individuals tested, and the median frequency was 0.35% (range: 0–4.1%). SEB responses were detectable in 12 out of 12 participants with a median frequency of 16% (range: 8.6–32.5%). The frequency of *M. tuberculosis*-specific and SEB-responding CD4<sup>+</sup> T cells were comparable pre- and post-ART, whereas the frequency of CMV-specific CD4<sup>+</sup> T cells was significantly lower after treatment (median 0.35 versus 0.14%, *p* = 0.003; Fig. 1B). To take into account variation in absolute CD4 counts across the cohort, the absolute number of Ag-specific CD4<sup>+</sup> T cells was calculated. The number of *M. tuberculosis*-specific and SEB-responding CD4<sup>+</sup> T cells increased significantly after ART (*p* = 0.033 and *p* = 0.016, respectively), whereas the number of CMV-specific CD4<sup>+</sup> T cells remained unchanged upon treatment (Fig. 1C), consistent with the decreased frequency in the context of increases in absolute CD4 numbers. Overall, these data reveal that the dynamics of reconstitution of Ag-specific CD4<sup>+</sup> T cells vary according to their pathogen specificity.

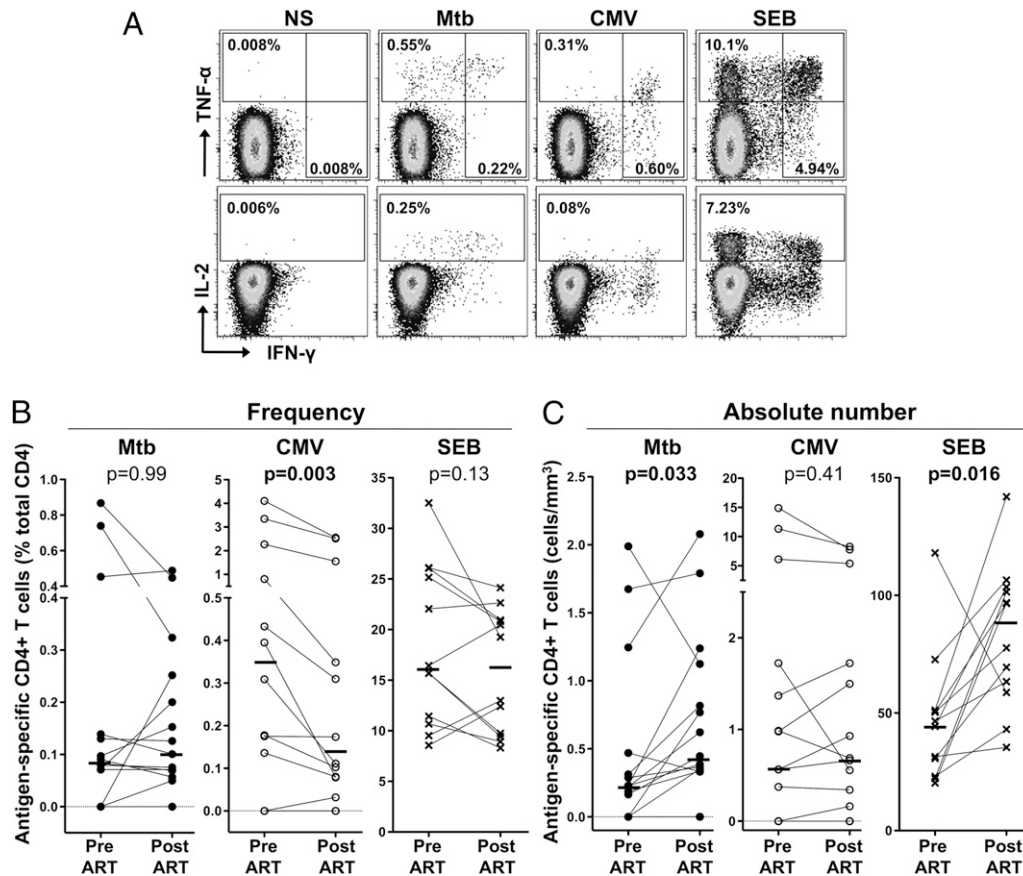
*Relationship between the dynamics of restoration of total and copathogen-specific CD4<sup>+</sup> T cells*

To further investigate the restoration dynamics of copathogen-specific CD4<sup>+</sup> T cells, we examined the relationship between the fold change in the absolute number of Ag-specific CD4<sup>+</sup> T cells pre- and post-ART (in individuals with detectable Ag responses at both time points, *n* = 11 for *M. tuberculosis*, *n* = 10 for CMV, and *n* = 12 for SEB), and the absolute total CD4 count variation. Fig. 2A shows that although the magnitude of cell replenishment of *M. tuberculosis*-specific and SEB-responding CD4<sup>+</sup> T cells was comparable to that of total CD4<sup>+</sup> T cells (an ~2-fold increase over the 12 mo of ART), the extent of restoration of CMV-specific CD4<sup>+</sup> T cells was significantly lower (*p* = 0.018, *p* = 0.013, and *p* = 0.0008, to *M. tuberculosis*, SEB, and total CD4<sup>+</sup> cells, respectively). Consistent with these observations, the degree of restoration of *M. tuberculosis*- and SEB-responding CD4<sup>+</sup> T cells corresponded closely with the overall CD4 recov-

Table I. Characteristics of study participants

Participant Identification	Pre-ART Duration of Infection (y)	Pre-ART				Post-ART			
		Viral Load (RNA Copies/ml)	CD4 Count (Cells/mm <sup>3</sup> )	CD4/CD8 Ratio	Months before ART Initiation	Viral Load (RNA Copies/ml)	CD4 Count (Cells/mm <sup>3</sup> )	CD4/CD8 Ratio	Months after ART Initiation
CAP200	3.8	423,000	135	0.24	2.5	<20	496	0.68	13.5
CAP206	5.2	198,277	289	0.15	0.2	<40	512	0.50	11.0
CAP222	6.1	3,049	319	0.61	3.2	<20	619	1.24	11.3
CAP237	5.2	4,743	308	0.57	0.0	<20	528	1.05	12.0
CAP244	7.3	21,955	318	0.32	0.0	273	647	0.53	12.3
CAP248	6.6	29,258	363	0.26	2.8	121	305	0.27	6.7
CAP255	3.7	8,830	226	0.18	3.8	<20	484	0.53	18.1
CAP257	4.8	52,800	170	0.19	2.0	<20	494	0.47	12.0
CAP262	5.8	1,130	338	1.69	1.7	<20	329	3.13	12.1
CAP267	5.4	41,338	269	0.27	1.3	<20	347	0.35	8.7
CAP268	4.2	4,537	186	0.18	0.9	<20	426	0.63	12.0
CAP276	1.4	22,000	232	0.37	2.3	<400	627	1.03	20.2
CAP277	4.9	7,997	322	0.22	1.0	29	535	0.66	10.0
CAP279	2.7	58,100	193	0.40	0.8	<20	400	1.48	12.0
CAP316	4.1	5,612	436	0.33	2.4	<20	708	0.67	11.8
Median	4.9	21,955	289	0.27	1.7	<20	496	0.66	12
IQR	3.8–5.8	4,743–52,800	193–322	0.95–0.40	0.8–2.5	<20–40	400–619	0.5–1.05	11–12.3





**FIGURE 1.** Effect of ART on *M. tuberculosis* (Mtb)-, CMV-, and SEB-specific CD4<sup>+</sup> T cell responses. **(A)** Representative flow cytometry plots of the expression of TNF- $\alpha$ , IFN- $\gamma$ , and IL-2 from CD4<sup>+</sup> T cells after stimulation with *M. tuberculosis* (Mtb) and CMV Ags and SEB, in one study participant. NS corresponds to unstimulated PBMC. The frequency of cytokine-producing cells expressed as a percentage of the total CD4<sup>+</sup> T cell population are indicated. Frequency **(B)** and absolute number **(C)** of *M. tuberculosis*-, CMV-, and SEB-specific CD4<sup>+</sup> T cell responses before and after ART. Horizontal bars represent the median. Statistical comparisons were performed using a Wilcoxon matched-pairs test.

ery, as measured in fold-change of the absolute CD4 count, with a slope close to 1 (Fig. 2B). In contrast, for CMV-specific CD4<sup>+</sup> T cell responses the slope was 0.37, indicating a reduced replenishment rate of these cells compared with the total CD4 compartment (Fig. 2B). Of note, although all study participants were CMV seropositive, none had detectable CMV viral replication at either visit, pre- or post-ART, indicating that a difference in CMV Ag load at the two visits was not a contributing factor to the reduced replenishment capacity of CMV-specific T cells.

#### Effect of ART on the functional capacity of copathogen-specific CD4<sup>+</sup> T cells

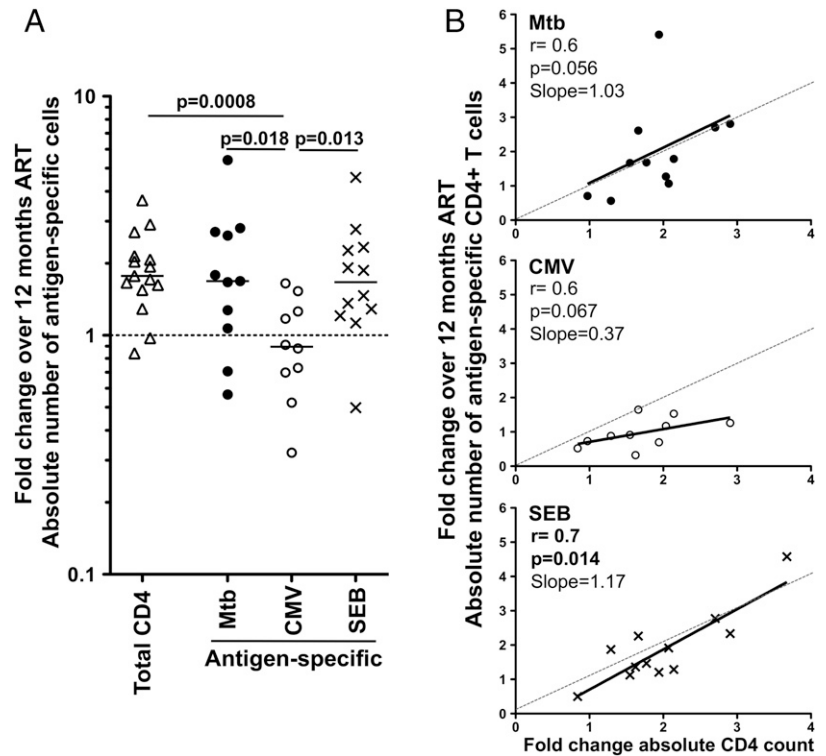
ART has been shown to gradually restore the polyfunctional capacity of HIV-specific T cells (28, 29), but the effect of ART on the functionality of CD4<sup>+</sup> T cells specific for other pathogens is unclear. Thus, we evaluated the cytokine secretion profiles of *M. tuberculosis*-, CMV-, and SEB-reactive CD4<sup>+</sup> T cells before and after ART initiation in detail (Fig. 3). We included nine HIV-uninfected individuals in this analysis for comparison. Our first finding was that the cytokine production profile of CD4<sup>+</sup> T cells varied significantly according to their specificity ( $p = 0.002$ ; Fig. 3A), with *M. tuberculosis*-specific responses consisting of a greater proportion of polyfunctional cells producing all three cytokines, in addition to a predominant TNF- $\alpha$  monofunctional subset (~40% of the response), whereas CMV-specific CD4<sup>+</sup> responses exhibited predominantly a IFN- $\gamma$ <sup>+</sup>TNF- $\alpha$ <sup>+</sup> phenotype (~60%; Fig. 3B). ART did not significantly alter the cytokine secretion profile of CD4<sup>+</sup> T cells from baseline, regardless of their

specificity for *M. tuberculosis*, CMV, or SEB (Fig. 3A, 3B). Moreover, the polyfunctional characteristics of *M. tuberculosis*-specific and SEB-responsive CD4<sup>+</sup> T cell responses were comparable to those observed in HIV-uninfected individuals. In contrast, CMV responses were significantly skewed in HIV-infected individuals compared with HIV-uninfected subjects ( $p = 0.016$  and  $p = 0.045$  for pre- and post-ART; Fig. 3A), with a lower proportion of cells coproducing IFN- $\gamma$ , IL-2, and TNF- $\alpha$ , and a higher proportion of IFN- $\gamma$  monofunctional cells (Fig. 3B). These data demonstrate that, unlike for *M. tuberculosis*-specific CD4 responses, HIV infection impaired the functional potential of CMV-specific CD4 responses toward a less polyfunctional profile, and 1 y of ART did not restore the CMV functional profile.

#### Impact of the memory phenotype of pathogen-specific CD4<sup>+</sup> T cells on their replenishment potential upon ART

Depending on their memory differentiation profile, CD4<sup>+</sup> T cells are endowed with distinct survival and proliferation capacities (30, 31). Thus, to investigate whether the cell maturation phenotype could impact the replenishment capacity of copathogen-specific CD4<sup>+</sup> T cells, we first compared the differentiation profiles of *M. tuberculosis*- and CMV-specific CD4<sup>+</sup> T cells at baseline, prior to ART initiation. Fig. 4A shows a representative contour plot of distinct memory subsets in the CD4 compartment pre-ART in one HIV-infected individual. Based on the expression of CD45RO and CD27, we were able to discriminate four different memory subpopulations (32), namely: naive (CD45RO<sup>-</sup>CD27<sup>+</sup>), early differentiated (ED; CD45RO<sup>+</sup>CD27<sup>+</sup>), encompassing central and transitional

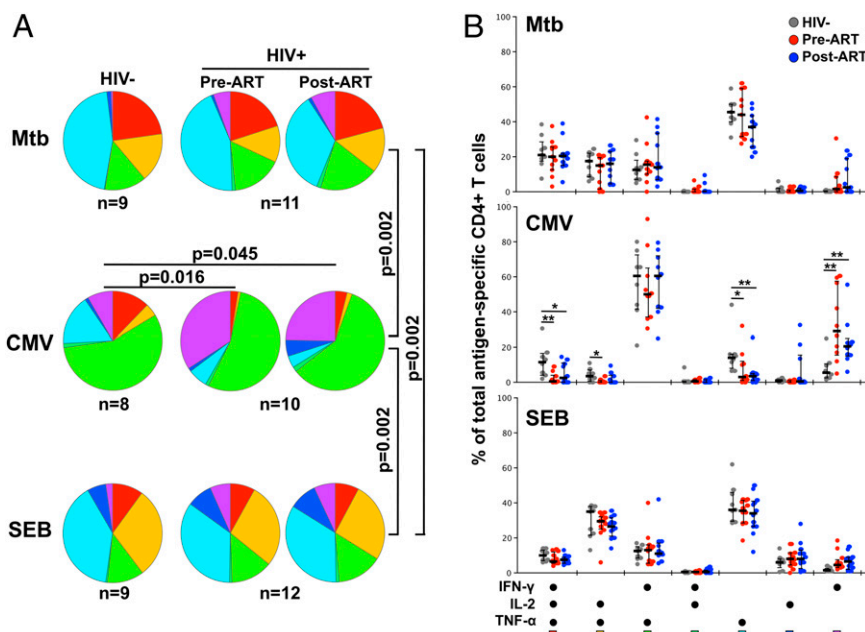
**FIGURE 2.** Restoration dynamics of *M. tuberculosis*- (Mtb), CMV-, and SEB-specific CD4<sup>+</sup> T cells after ART. **(A)** Fold change in the total, *M. tuberculosis*-, CMV-, and SEB-specific absolute CD4<sup>+</sup> T cell count over 12 mo of ART. The horizontal dotted line indicates no change from the baseline pre-ART time point. Statistical comparisons were performed using a non-parametric Mann–Whitney *U* test. **(B)** Association between the fold change of the absolute number of Ag-specific CD4<sup>+</sup> T cells and the fold change in the absolute CD4 count pre- and post-ART. Statistical associations were performed by a two-tailed nonparametric Spearman rank correlation. The solid line represents a linear regression fit, and the dashed line depicts the ideal slope of 1.



memory subsets), late differentiated (LD; CD45RO<sup>+</sup>CD27<sup>-</sup>, encompassing effector memory cells), and terminally differentiated (CD45RO<sup>-</sup>CD27<sup>-</sup>, or effector cells). In HIV-infected subjects, although *M. tuberculosis*- and CMV-specific CD4<sup>+</sup> T cells were represented in both ED and LD subsets, they comprised significantly distinct profiles (Fig. 4B, left panel). *M. tuberculosis*-specific cells exhibited primarily an ED profile (median 51%, interquartile range [IQR] 42–62), whereas CMV-specific cells demonstrated a substantial enrichment of the LD phenotype (median 73%, IQR 48–86), as previously described (33). Of note, ART did not markedly alter the overall memory differentiation profile of *M. tuberculosis*-specific or CMV-specific CD4<sup>+</sup> T cells (Supplemental Fig. 3). In addition, HIV-uninfected individuals exhibited similarly distinctive memory CD4

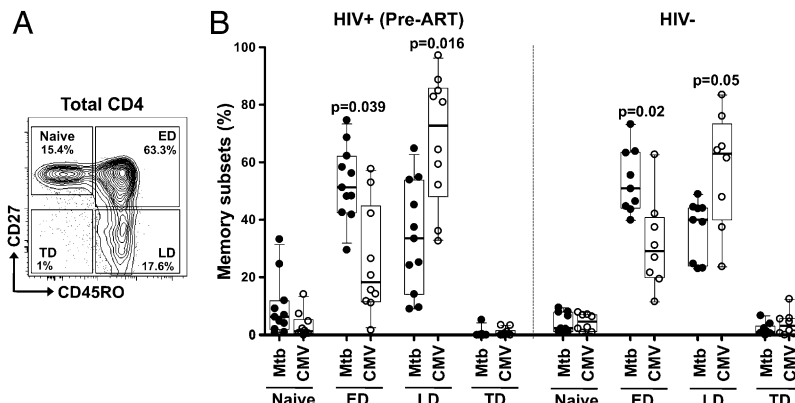
differentiation profiles for *M. tuberculosis* and CMV (Fig. 4B, right panel).

We next investigated the relationship between the memory phenotype of pathogen-specific CD4<sup>+</sup> T cells at the time of ART initiation and the capacity of these cells to be maintained and/or replenished upon treatment. Fig. 5A shows that the fold change in the absolute number of pathogen-specific CD4<sup>+</sup> T cells over 12 mo on ART inversely correlated with the proportion of cells exhibiting a late differentiated phenotype ( $p = 0.019$ ;  $r = -0.5$ ). Consistent with this, a positive correlation was observed between the fold change in the absolute number of pathogen-specific CD4<sup>+</sup> T cells and the proportion of these cells exhibiting an ED phenotype ( $p = 0.04$ ;  $r = 0.45$ ). Of note, the proportion of LD



**FIGURE 3.** Polyfunctional capacity of *M. tuberculosis*- (Mtb), CMV-, and SEB-specific CD4<sup>+</sup> T cells in HIV-uninfected and uninfected individuals pre- and post-ART. Pie charts **(A)** and graphs **(B)** representing the cytokine secretion ability of *M. tuberculosis*-, CMV-, and SEB-specific CD4<sup>+</sup> T cell responses in HIV-uninfected individuals ( $n = 9$ ) and HIV-infected individuals pre- and post-ART initiation. Each section of the pie chart represents a specific combination of cytokines, as indicated by the color at the bottom of the graph. Horizontal bars depict the median with interquartile range indicated. Statistical comparisons were performed using a Wilcoxon rank-sum test. \* $p < 0.05$ , \*\* $p < 0.01$ .

**FIGURE 4.** Memory profiles of *M. tuberculosis*- (Mtb) and CMV-specific CD4<sup>+</sup> T cells in HIV-infected and uninfected individuals. **(A)** Representative example of total CD4 memory subset distribution in one HIV-infected individual. Naive (CD45RO<sup>-</sup>CD27<sup>+</sup>), ED (CD45RO<sup>+</sup>CD27<sup>+</sup>), LD (CD45RO<sup>+</sup>CD27<sup>-</sup>), and TD (CD45RO<sup>-</sup>CD27<sup>-</sup>). The frequencies of each subset are indicated. **(B)** Memory profile of *M. tuberculosis*- and CMV-specific CD4<sup>+</sup> T cells in HIV-infected individuals pre-ART (left panel) and HIV-uninfected individuals (right panel). Results are shown as box and whisker (10–90 percentile) plots. Each dot depicts an individual, and the horizontal bar is the median. Statistical comparisons were performed using a one-way ANOVA nonparametric Kruskal–Wallis test.



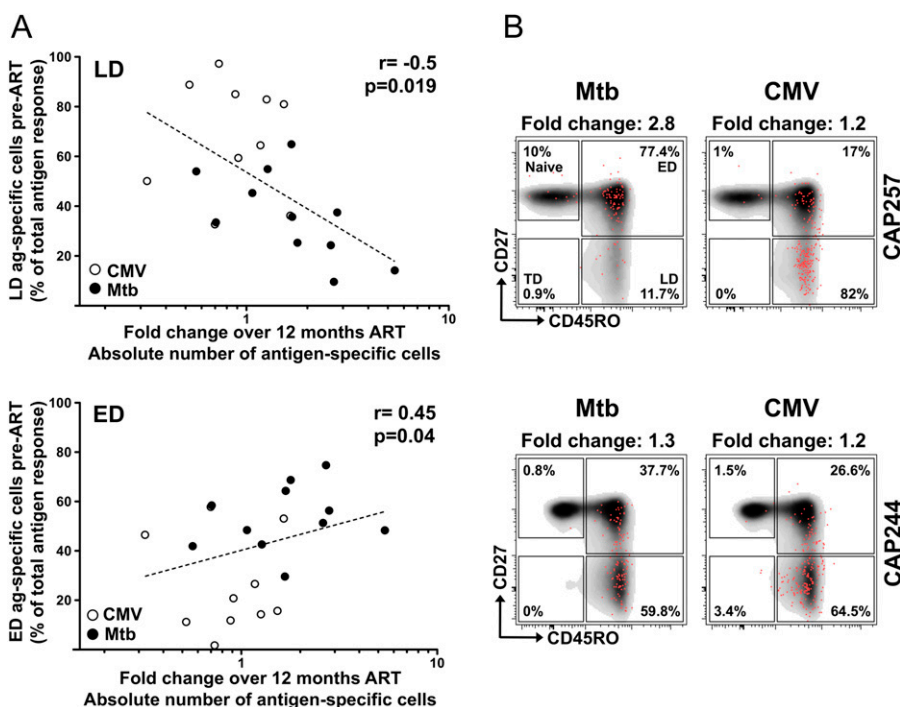
*M. tuberculosis*-specific CD4<sup>+</sup> T cell responses (in the absence of CMV responses) also correlated inversely with the fold change in the absolute number of *M. tuberculosis*-specific cells ( $r = -0.62$ ;  $p = 0.038$ ; data not shown), and so too when naive-like *M. tuberculosis*-specific cells were grouped together with ED cells, we observed a trend toward a positive association with the fold change in the absolute number of *M. tuberculosis*-specific cells ( $r = 0.54$ ;  $p = 0.05$ ; data not shown). This relationship between the memory profile and the ART-induced repletion potential of pathogen-specific CD4<sup>+</sup> T cells is further illustrated in Fig. 5B, showing specific examples from two participants. In CAP257, in which the pre-ART CMV-specific CD4 response showed an enrichment in cells with an LD phenotype (82%), their ability to expand over 12 mo on ART was considerably lower than *M. tuberculosis*-specific CD4 responses, characterized by an ED phenotype (77.4%) (fold change 1.2 for the CMV response versus 2.8 for the *M. tuberculosis* response). In CAP244, in which the

memory profile of both *M. tuberculosis* and CMV-specific responses were similar (~60% of LD cells), the fold change in the absolute number of CD4<sup>+</sup> T cells to both pathogens was comparable.

Overall, these data reveal that the ability of copathogen-specific memory CD4<sup>+</sup> T cells to expand upon ART appears to be related to their memory phenotype, in which ED cells have a higher replenishment capacity compared with LD cells.

## Discussion

The introduction of ART has changed the clinical pattern of HIV infection significantly, with considerable reductions in morbidity and mortality. Although it is clearly established that ART leads to a progressive replenishment of the CD4<sup>+</sup> T cell compartment in the majority of cases, the extent to which pre-existing copathogen-specific CD4<sup>+</sup> T cells are restored, and the causes of variable restoration, are poorly defined. In this study, we investigated the



**FIGURE 5.** Relationship between the memory profile of *M. tuberculosis*- (Mtb) and CMV-specific CD4<sup>+</sup> T cells and the dynamics of restoration of these cells. **(A)** Association between the proportion of Ag-specific CD4<sup>+</sup> T cells exhibiting an LD (top panel) or ED (bottom panel) profile with the fold change in Ag-specific CD4<sup>+</sup> T cell absolute count after ART. Statistical associations were performed by a two-tailed nonparametric Spearman rank correlation. **(B)** Representative examples of the memory profile of *M. tuberculosis*- or CMV-specific CD4<sup>+</sup> T cells pre-ART in two individuals. The frequencies of each subset are indicated. For each example, the fold change in Ag-specific absolute CD4<sup>+</sup> T cell count pre- and post-ART is indicated at the top of each plot.



dynamics of restoration of memory CD4<sup>+</sup> T cells specific for a bacterial copathogen (*M. tuberculosis*) and a viral pathogen (CMV) in 15 HIV-infected individuals in response to ART. Our main finding was that the extent of reconstitution of pathogen-specific cells was related to their memory differentiation profile at the time of ART initiation, in which cells exhibiting an ED memory profile (including central memory and transitional memory cells) had a higher replenishment potential compared with LD (effector memory) T cells.

Multiple mechanisms contribute to the increase in CD4<sup>+</sup> T cells in blood in response to antiretroviral treatment for HIV (34). Over the first few months of ART, there is a redistribution of CD4<sup>+</sup> T cells from the lymph nodes to the blood (35, 36), leading to a rapid initial rise in CD4<sup>+</sup> counts. Moreover, homeostatic cell proliferation (37), decreased cell death (38) and increased thymic output (39, 40) also play a role in the replenishment of CD4<sup>+</sup> T cells. Although the CD4 absolute cell count at the time of treatment initiation is one of the main factors dictating the level to which CD4<sup>+</sup> T cells are restored (21, 23), other parameters such as the activation level of T cells at the time of treatment (41), age (42, 43), or active coinfections (44, 45) also influence the degree of reconstitution of the CD4 compartment. Thus, CD4<sup>+</sup> T cell recovery upon ART appears to be dependent on both the extent of immune damage at the time of treatment initiation and the regenerative capacities of these cells.

We demonstrate in this study that despite successful viral suppression and robust CD4 gains in the majority of participants, the dynamics of *M. tuberculosis*- and CMV-specific CD4<sup>+</sup> T cell recovery were distinct. This shows that not all T cell subpopulations have the same potential to be replenished. Several factors and/or cellular attributes could account for these differences. First, the regenerative capacities of pathogen-specific CD4<sup>+</sup> T cells could be determined by their intrinsic ability to survive or proliferate, and it has been clearly established that CD4<sup>+</sup> T cells exhibiting an ED memory profile have an enhanced survival and proliferative potential compared with cells with an LD phenotype (30, 31). Our data are in accordance with these observations, showing that the extent of replenishment of pathogen-specific CD4<sup>+</sup> T cells correlates with their memory status, with superior recovery observed for Ag-specific CD4<sup>+</sup> T cells exhibiting an early memory phenotype. Mechanistically, cell responsiveness to common  $\gamma$ -chain cytokines may account for the distinct renewal potential of these different cell subsets (46–48). In our study, the replenishment potential of *M. tuberculosis*- or CMV-specific CD4<sup>+</sup> T cells did not associate with their ability to produce IL-2 (data not shown); however, we have observed that responsiveness to IL-7 distinguishes ED and LD CD4<sup>+</sup> subsets, with ED memory T cells being more responsive to exogenous IL-7 and IL-2 (as measured by Stat5 phosphorylation), compared with LD memory CD4<sup>+</sup> T cells (C. Riou, unpublished observations). Further analysis comparing the level of expression of cytokine receptors and cytokine-induced proliferation potential between *M. tuberculosis*- and CMV-specific CD4<sup>+</sup> T cells would be needed to define the role of these signaling pathways in the restoration of immune memory cells upon ART.

Although *M. tuberculosis* responses displayed primarily an ED phenotype, there were substantial interindividual differences, ranging from 30–75% of the response. The CD4<sup>+</sup> memory profile is dictated by numerous factors, including continual Ag exposure in high burden settings (49), previous TB, TB treatment, and cure (50–52). A recent study carried out in a similar geographic setting as the current study noted dynamic changes in the T cell response to *M. tuberculosis* in the context of latent TB infection and HIV coinfection (53). It is worth noting that we also detected Ag-specific cells with a naive-like phenotype, previously described

for *M. tuberculosis* and other infections (51, 54, 55). Although making up only a minor proportion of the total *M. tuberculosis*- and CMV-specific response, these cells expanded post-ART in the majority of individuals compared with other memory subsets, that were characterized by more variable expansion and contraction. For CMV, this expansion led to a significantly greater frequency of this subset after ART. These cells may fall within the recently described memory stem cell subset, comprising memory cells endowed with a greater ability to proliferate and persist long-term compared with central memory (56, 57). Thus, the composition of the memory pool at the time of ART initiation may influence the restoration of Ag-specific CD4<sup>+</sup> T cells.

To determine whether short-term ART affected not just quantitative but also qualitative aspects of copathogen immunity, we assessed the ability of cells to simultaneously secrete multiple cytokines, which for some infections, such as HIV and leishmaniasis, are a correlate of immune control or protection (58, 59). Although the significance of polyfunctional cells in TB is unclear (48), we demonstrated that >50% of *M. tuberculosis*-specific CD4<sup>+</sup> cells produced three or two cytokines simultaneously, a profile that did not significantly change after ART; moreover, this was similar to the profile from HIV-uninfected individuals. Our data are in apparent contrast to previous findings (18) showing that ART leads to an improved polyfunctional profile in *M. tuberculosis*-specific CD4<sup>+</sup> T cell after short-term treatment, but this may be due to cohort differences, as the participants in that study were characterized by more advanced immunosuppression and higher viral loads than in the current study. Because it has been reported that the impairment of the functional capacity of *M. tuberculosis*-specific CD4<sup>+</sup> T cells is related to HIV disease status (60), it is likely that differences in the severity of HIV pathology at the time of treatment initiation between the two studied groups could account for these discrepancies. The polyfunctional profile we observed for CMV-specific CD4<sup>+</sup> cells is consistent with that previously reported (33), with simultaneous IFN- $\gamma$  and TNF- $\alpha$ , and IFN- $\gamma$  alone dominating the response. The CMV profile also did not change after ART and in this case was less polyfunctional compared with HIV-infected individuals. Our study only examined paired samples from 12 mo after ART, and it is possible that prolonged ART may restore CMV functional profiles (29).

It is important to note that although we demonstrate an expansion of *M. tuberculosis*-specific CD4<sup>+</sup> T cells after ART, restoration of the magnitude and measures of functionality of the *M. tuberculosis*-specific CD4<sup>+</sup> Th1 response determined by the production of three cytokines is a coarse measure of functional immunity to TB and may not reflect protective immunity. Several additional CD4<sup>+</sup> T cell subsets and cytokines contribute to TB immunity (61–64), and we have found an even greater diversity of CD4<sup>+</sup> T cell subsets specific for *M. tuberculosis* (C. Riou, N. Strickland, A.P. Soares, B. Corleis, D.S. Kwon, E.J. Wherry, R.J. Wilkinson, and W.A. Burgers, submitted for publication). An additional issue is that we do not know the extent of pathogen-specific CD4<sup>+</sup> recovery in tissues upon ART, which appears defective for TB in the lungs even after prolonged viral suppression (20). These considerations may account for the persistently greater susceptibility of successfully treated HIV-infected individuals to TB (65).

Our study had several limitations. We were constrained by the availability of paired samples prior to and after ART, limiting us to studying 15 individuals in detail. Testing the generalizability of our findings in larger cohorts is warranted. Also, we focused our study on short-term restoration of pathogen-specific immunity (after 1 y of ART), and although study participants exhibited suppressed HIV replication, decreased immune activation, and improved CD4 counts, studying responses after longer-term therapy may provide further important insights and more definitive conclusions on the



restoration of pathogen-specific immunity. Furthermore, the participants we studied had nadir CD4 counts of  $>135$  cells/mm<sup>3</sup> (median 289 cells/mm<sup>3</sup>), and we did not have access to participants commencing treatment at lower CD4 counts (e.g.,  $<50$  cells/mm<sup>3</sup>). These highly immunocompromised patients would be the most relevant group to study with respect to susceptibility to CMV disease. In this context, although CMV-specific cells replenished more poorly than *M. tuberculosis*-specific cells after ART, they were present at a high frequency prior to ART initiation, which may well have been sufficient to provide protective immunity to CMV. Moreover, the extent of CD4 depletion at the time of ART initiation could impact the degree and dynamics of Ag-specific CD4<sup>+</sup> T cell recovery. Lastly, we delineated memory subsets based on two markers, CD27 and CD45RO. These markers cannot differentiate between central memory and transitional memory cells within the CD27<sup>+</sup>CD45RO<sup>+</sup> ED subset, and inclusion of a marker such as CCR7 would afford an additional degree of distinction that may reveal further differences in the ability of these memory subsets to expand after ART and should be included in future studies of reconstitution of pathogen-specific immunity. Further research addressing these important limitations is needed to confirm and extend our findings.

Understanding the host and cellular factors that contribute to successful immune restoration on effective antiretroviral treatment for HIV, including both the overall CD4<sup>+</sup> T cell compartment and copathogen-specific CD4 immunity, is of crucial importance, as only partial recovery of these cells could result in a persistently heightened risk of particular opportunistic infections. In this report, we showed that the renewal potential of pre-existing pathogen-specific CD4<sup>+</sup> T cells was related to their memory differentiation profile. It will be important to determine whether this principle holds true in a wider context for other copathogens. Incomplete restoration of functional immunity and the persistence of elevated susceptibility to particular coinfections have implications for targeted interventions in the treated HIV-infected population. Overall, our findings underscore the complexity of immune reconstitution on ART and the importance of preserving functional immunity with early ART (66).

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## Disclosures

The authors have no financial conflicts of interest.

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