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Residual T cell activation and skewed CD8+ T cell memory differentiation despite antiretroviral therapy-induced HIV suppression

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Abstract

HIV infection results in excessive T cell activation and dysfunction which may persist even during effective antiretroviral therapy (ART). The dynamics of immune ‘deactivation’ and extent to which T cell memory subsets normalize after ART are unclear. We longitudinally assessed the influence of 1 year of ART on the phenotype of T cells in HIV-infected African women, relative to matched HIV-uninfected women, using activation (CD38, HLA-DR) and differentiation markers (CD27, CD45RO). ART induced a substantial reduction in T cell activation, but remained higher than HIV-uninfected controls. ART largely normalized the distribution of CD4+ T cell memory subsets, while the distribution of CD8+ T cell memory subsets remained significantly skewed compared to HIV-uninfected individuals. Thus, there was a considerable but only partial reversal of T cell defects upon ART. Understanding T cell impairment may provide important insights into mechanisms of HIV pathogenesis in the era of ART.

Keywords

Chronic HIV; antiretroviral therapy; T cell activation; T cell differentiation

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Disclosures

All authors declare that they have no conflicts of interest.

1. Introduction

A hallmark of HIV infection is chronic immune activation, which plays a fundamental role in HIV pathogenesis, by contributing to CD4+ T cell loss and driving disease progression [1]. Systemic immune activation results in increased cell turnover, depletion and exhaustion of T cells [1–4]. Specifically, increased immune activation causes alterations in T cell phenotype in both the CD4 and CD8 compartments, inducing a decrease in naive T cells and a concomitant accumulation of highly differentiated cells [4–6]. This skewed differentiation profile may result from thymic dysfunction, where the renewal of naive T cells is slowed, or antigen-driven differentiation, increasing the proportion of effector cells [7–9]. Overall, this loss of memory resources could account for sustained incidence of opportunistic infections or cancers.

With the introduction of antiretroviral therapy (ART), the majority of HIV-infected individuals fully suppress viral load, display a progressive increase in CD4 counts and reduced opportunistic infections, resulting in a substantial improvement in their health [10–13]. HIV-induced T cell phenotypic defects can be partially corrected upon ART. HIV-infected individuals on ART show an overall decline in CD4+ and CD8+ T cell activation (commonly measured by CD38 and HLA-DR expression) and proliferation (measured by Ki67 expression), but these appear to remain higher compared to HIV-uninfected individuals [14–19]. Additionally, while ART partially restores the distribution of T cell memory subsets [20,21], persistent defects in the memory differentiation profiles of T cells have also been observed in treated patients [22–24].

Thus, despite the unquestionable clinical and immunologic benefits of ART, HIV-infected individuals on suppressive treatment may still display immune defects. In fact, we still do not completely understand the extent to which specific T cell memory subsets are recovered upon ART, their relationship with ongoing immune activation, and whether the recovery profile is similar between the CD4 and CD8 compartments. African cohorts, in particular, are less well-studied than Caucasian cohorts. With this in mind, the aim of this study was to determine the effect of ART on the activation, proliferation and memory differentiation profiles of CD4+ and CD8+ T cells in chronically HIV-infected South African women, before and 12 months after the initiation of ART. These profiles were compared to age- and ethnicity-matched HIV-uninfected women from the same community.

2. Material and Methods

2.1 Characteristics of study participants

Twenty-eight women were recruited from the Centre of AIDS Programme of Research in South Africa (CAPRISA) 002 HIV acute infection cohort in KwaZulu-Natal, a predominantly subtype C epidemic, as described previously [25,26]. The median age of the study participants was 31 years [Interquartile range (IQR): 27–34 years] and we found no association between age and restoration of T cell phenotypes after ART (data not shown). These participants were infected for a median of 4.2 years [Interquartile range (IQR): 2.7–5.6 years]. To estimate the time of HIV infection, a prospective RNA positive/antibody negative result or the midpoint between the last antibody negative test and the first antibody

positive enzyme-linked immunosorbent assay test were used. ART was offered according to the South African National HIV treatment guidelines (at a CD4 count of <200 cells/mm³ prior to October 2012; <350 cells/mm³ from October 2012 to March 2015). The majority of the participants (25/28) were taking standard first-line therapy, namely TDF/3TC/EFV (n=14) or TDF/FTC/EFV (n=4) or ddI-EC/3TC/EFV (n=3) or TDF/3TC/NVP (n=1) or AZT/3TC/NVP (n=1) or d4T/3TC/EFV (n=1) or d4T/3TC/NVP (n=1), while only one individual was on a second-line regimen, consisting of AZT/3TC/Lpvr/r (as indicated in Table S1). Two participants switched drug regimens during the study period, namely CAP200 (ddI-EC /3TC/ EFV to TDF /3TC/EFV at month 11) and CAP255 (d4T/3TC/EFV to AZT/3TC/EFV at month 10). Peripheral blood samples were taken at two time-points: pre-ART initiation (median: 1.9 months [IQR: 0.4–2.7]) and post-ART initiation (median: 12.2 months of ART [IQR: 11.3–12.9]). An additional 23 HIV-uninfected women who were matched for age and ethnicity were included from the CAPRISA 004 1% Tenofovir microbicide gel trial [27]. These women were either in the pre-intervention or in the placebo phase of the trial. No participants had tuberculosis (TB) over the study period or immune reconstitution inflammatory syndrome following ART. Ethical approval for the study was obtained from the Research Ethics Committees at the University of KwaZulu-Natal and University of Cape Town. All participants provided written, informed consent.

2.2 HIV plasma viral load and T cell counts

Plasma HIV viral load and CD4 count were assessed at each study visit. During the study period, 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 in June 2010 (LDL 40 RNA copies/ml), and then to Roche Taqman version 2.0 in January 2012 (LDL 20 RNA copies/ml). The FACSCalibur TruCOUNT method (BD Biosciences) was used to measure absolute blood CD4+ and CD8+ T cell counts.

2.3 Sample processing

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque (Amersham Pharmacia) density gradient centrifugation and cryopreserved in freezing media consisting of heat-inactivated foetal calf serum (FCS; Invitrogen) containing 10% dimethylsulfoxide (DMSO; Sigma-Aldrich). Cells were stored in liquid nitrogen until use. Cryopreserved PBMC were thawed and rested in R10 (RPMI 1640 plus 10% heat-inactivated FCS and 50 U/ml of penicillin-streptomycin) at 37°C with 5% CO₂ for 3 hours before staining.

2.4 Monoclonal antibodies and staining

To measure T cell activation and differentiation, the following antibodies were used: CD14 Pacific Blue (Tük4), CD19 Pacific Blue (SJ25-CI), CD4 PE-Cy5.5 (S3.5), CD8 Qdot-705 (3B5), Ki67 FITC (7B11; all Invitrogen), CD3 PE-Cy7 (SK7), HLA-DR APC-Cy7 (L243), CD38 APC (HIT2; all BD Biosciences), CD27 PE-Cy5 (1A4CD27), CD45RO ECD (UCHL1; both Beckman Coulter), and a violet viability dye (Vivid; Molecular Probes). Optimal titers were determined for each antibody. Staining was performed as follow: PBMC were stained with a viability dye (Vivid), then labeled with antibodies against surface markers, fixed, permeabilized and subsequently stained intracellularly for Ki67. Cells were then resuspended in 1X CellFix (BD Biosciences) and kept at 4°C until acquisition. Samples

were acquired within 24 hours on a BD Fortessa using FACSDiva software and analyzed using FlowJo (version 9.9.3; TreeStar). Figure S1 shows the gating strategy used to identify activated, proliferating and memory T cells.

2.5 Statistical analyses

Statistical analyses were performed using Prism (GraphPad version 5.0). The Mann-Whitney U test and the Wilcoxon Signed Rank test were used for unpaired and paired samples, respectively. Correlations were determined by the non-parametric Spearman Rank correlation test. A p-value of <0.05 was considered to be statistically significant.

3 Results

3.1 Viral suppression and T cell changes after ART

Twenty-eight HIV-infected women were studied prior to and one year after the initiation of ART. We first examined how the study participants responded to ART, by analyzing clinical parameters associated with immune recovery. Pre-ART samples were obtained at a median of 1.9 months (IQR: 0.4–2.7) before ART initiation (Table S1). The median plasma viral load was 38,513 HIV RNA copies/ml (IQR: 8,205–76,793), while the median CD4 and CD8 counts were 279 cells/mm³ (IQR: 228–330) and 1,018 cells/mm³ (IQR: 642–1,395), respectively. Prior to ART, viral load inversely correlated with CD4 counts, as expected ($r = -0.52$, $p = 0.005$; data not shown). After a median of 12.2 months of ART (IQR: 11.3–12.9), plasma viral load was significantly reduced ($p < 0.0001$; Figure 1A) in the majority of participants (21/28), and was below the detection limit of the assay used (i.e. <20 copies/ml, <40 copies/ml or <400 copies/ml), Table S1). The remaining 7 participants still had detectable but low levels of virus in their plasma (median: 64 copies/ml). The decrease in plasma viraemia was accompanied by a significant increase in absolute CD4 counts ($p < 0.0001$; Figure 1B), which occurred in 26/28 individuals. The median increase in the absolute number of peripheral CD4 cells post-ART was 249 cells/mm³. In contrast to the increase in absolute CD4 counts, no significant change was observed in the absolute CD8 counts pre- and post-ART ($p = 0.19$, Figure 1C). However, it is worth noting that in the majority of subjects (71%, 20/28), the absolute CD8 counts decreased by a median of 122 cells/mm³ following ART. These changes in CD4 and CD8 cells led to a significant increase in the CD4:CD8 ratio ($p < 0.0001$; Figure 1D), with a median fold change of 2.5 after 1 year of ART. Two participants, CAP248 and CAP262, did not reconstitute their CD4 cells. CAP248 was one of the participants with low but detectable viral load (121 RNA copies/ml; Table S1) after 1 year of ART, while CAP262 exhibited viral suppression, low CD8 counts and a CD4:CD8 ratio that doubled after treatment. However, both participants recovered their CD4 counts at later time points (~3 years) on ART (data not shown).

Overall, these results demonstrate the expected effects of 1 year of ART, namely suppression of HIV and a significant improvement in absolute CD4 counts in the majority of treated patients.

3.2 Substantial decreases in T cell activation and proliferation after ART

Previous studies have demonstrated that ART is highly effective at reducing T cell activation and proliferation [3,16], but normalization is often incomplete and is dependent on the timing and duration of ART. Thus, we evaluated the extent to which ART led to ‘deactivation’ of T cells in our cohort of African women who initiated ART during chronic infection. We first measured the expression of activation markers CD38 and HLA-DR pre- and post-ART and compared them to profiles from HIV-uninfected controls. Figure 2A shows representative flow cytometry plots of CD38 and HLA-DR expression on CD4+ and CD8+ T cells from one HIV-uninfected and one HIV-infected individual before and after ART. As expected, the frequency of activated CD4+ T cells was significantly higher in HIV-infected individuals prior to ART compared to HIV-uninfected controls (CD38+: medians 8.5% vs 1.6%; HLA-DR+: median 7.4% vs 1.5% and CD38+HLA-DR+: 1.7% vs 0.1%; $p<0.0001$; Figure 2B). After ART, the frequency of activated CD4+ T cells decreased significantly (CD38+: median 5.5%; HLA-DR+: median 3.3%; and CD38+HLA-DR+: median 0.3%), but still remained significantly higher than HIV-uninfected subjects ($p<0.0001$ for CD38+ and CD38+HLA-DR+, and $p=0.0006$ for HLA-DR+; Figure 2B left panel). Similar to CD4+ T cells, HIV also induced elevated expression of CD38 and HLA-DR on CD8+ T cells. The frequency of activation markers expressed on CD8+ T cells during HIV infection was higher than on CD4+ T cells (CD38+: medians 14.4% vs 8.5%; HLA-DR+: medians 23.7% vs 7.4% and CD38+HLA-DR+: medians 9.2% vs 1.7%). While ART also induced a significant reduction in CD8+ activation as for the CD4 compartment, the activation levels observed after 12 months of treatment remained significantly higher than HIV-uninfected individuals for all three subsets of activated cells (Figure 2B, right panel).

There was a positive association for T cell activation prior to and after ART for all activation markers measured on both CD4+ and CD8+ T cell subsets (CD4: $r=0.78$, 0.71 and 0.68 for CD38, HLA-DR and dual expression, respectively; CD8: $r=0.77$, 0.81 and 0.70 , respectively; $p<0.0001$ for all; data not shown). When we examined the relationship between T cell activation and clinical variables, viral load pre-ART positively associated with CD4+ T cell activation pre-ART (HLA-DR expression; $p=0.008$, $r=0.49$; data not shown) while it maintained a similar albeit weaker association with residual CD4+ T cell activation post-ART ($p=0.047$, $r=0.38$; data not shown). With regards to the CD8 compartment, no relationship was found between CD8+ T cell activation and clinical variables. There was no significant relationship between absolute CD4 count or CD4 recovery with T cell activation (data not shown).

Next, the effect of ART on T cell proliferation was determined in 18 participants for whom Ki67 staining was performed. Figure 2C shows representative flow cytometry plots of CD4+ and CD8+ T cell proliferation measured by Ki67 expression, from one HIV-uninfected and one HIV-infected subject before and after ART. The frequency of Ki67+ T cells was significantly higher in HIV-infected subjects compared to uninfected controls (CD4+: median 2.9% vs 0.42% and CD8+: 2.1% vs 0.38%; $p<0.0001$; Figure 2D). Similar to activation status, ART led to a significant reduction in the frequency of Ki67+ T cells (medians of 1% and 0.6% for CD4+ and CD8+ T cells, $p=0.0003$ and $p=0.0002$,

respectively), however the frequency of Ki67+ T cells remained significantly greater than the uninfected control group ($p<0.0001$ for CD4+ and $p<0.02$ for CD8+ T cells).

Taken together, these data demonstrate that both CD4+ and CD8+ T cells are highly activated and have an increased turnover during HIV infection. ART significantly and substantially reduces activation and proliferation of both CD4+ and CD8+ T cells, but this reduction does not lead to complete normalization to levels found in HIV-uninfected individuals.

3.3 Association between CD4+ and CD8+ T cells activation before and after ART

We next investigated the relationship between CD4+ and CD8+ T cell activation pre- and post-ART initiation in our study participants ($n=28$). A significant positive correlation was observed between the frequency of CD4+ and CD8+ T cells expressing CD38 ($p<0.0001$, $r=0.89$), HLA-DR ($p=0.0002$, $r=0.65$) or co-expressing CD38 and HLA-DR ($p<0.0001$, $r=0.79$;) pre-ART (Figures 3A–C). These significant positive correlations were also observed after 1 year of ART (CD38: $p<0.0001$, $r=0.91$; HLA-DR: $p<0.0001$, $r=0.85$; and CD38+HLA-DR+: $p<0.0001$, $r=0.82$; Figure 3F–E). Moreover, there was a positive correlation between the frequencies of proliferating CD4+ and CD8+ T cells pre- and post-ART ($p=0.0005$, $r=0.74$ and $p=0.02$, $r=0.56$, respectively; data not shown).

Overall, these findings reveal that the activation and proliferation levels in CD4+ T cells reflect those in CD8+ T cells both prior to and after ART initiation. These data indicate that the ‘de-activation’ of CD4+ and CD8+ T cells after ART is related, and that the factors maintaining activation of T cells post-ART continue to affect both T cell subsets, as they did prior to ART.

3.4 CD4+ and CD8+ T cell differentiation before and after ART

HIV infection results in the skewing of T cell subsets from naive to more differentiated (effector and memory) phenotypes [28,29]. However, the extent to which the recovery of the different memory subsets occurs after ART has not been fully characterized. Therefore, we investigated the impact of ART on T cell differentiation in our HIV-treated participants ($n=28$) compared to HIV-uninfected controls ($n=23$). Figure 4A shows representative flow cytometric plots of CD27 and CD45RO expression on CD4+ T cells from one HIV-uninfected and one HIV-infected individual (pre- and post-ART). Based on these two markers, four separate CD4+ T cell subsets were identified, namely naive (CD27+CD45RO[−]), early differentiated (ED: CD27+CD45RO⁺, comprising central and transitional memory subsets), late differentiated (LD: CD27[−]CD45RO⁺, corresponding to effector memory cells) and terminally differentiated effector cells (TD: CD27[−]CD45RO[−]). Compared to HIV-uninfected controls, HIV-infected participants displayed significantly lower frequencies of naive and TD CD4+ T cells (Naive: medians 48% vs 38%, $p=0.02$ and TD: medians 4% vs 1%, $p<0.0001$), and higher frequencies of ED and LD CD4+ T cells (ED: medians 33% vs 43%, $p=0.002$ and LD: medians 10% vs 16%, $p=0.05$), although the latter was not significantly different (Figure 4B). ART led to an increased frequency of naive CD4+ T cells and a concomitant decrease in LD frequencies (Naive: medians 38% vs 44%, $p=0.006$ and LD: medians 16% vs 11%, $p=0.0003$). The median frequencies of ED and TD CD4+ T cell

subsets were comparable pre- and post-ART. While ART partly normalized the frequency of naive and LD subsets compared to HIV-uninfected controls, the ED and TD CD4+ subsets did not return to normal levels ($p=0.004$ and $p<0.0001$; Figure 4B).

To account for variation in absolute CD4 numbers pre- and post-ART, the changes in CD4+ T cell memory subsets were assessed in absolute number. We found a significant increase in the number of naive, ED and LD CD4+ T cell subsets (Naive: $p=0.0009$, ED: $p<0.0001$; LD: $p=0.02$; Figure 4C) after ART, with no significant change in the TD subset ($p=0.06$; Figure 4D). To compare the dynamics of CD4+ T cell memory subset reconstitution upon treatment, we examined the fold change in absolute number of each subset pre- and post-ART. Overall, all four subsets expanded following 1 year of ART, with naive CD4+ T cells exhibiting the largest expansion (median: 2.5), followed by ED CD4+ T cells (median: 1.9), which was higher than the increase in LD and TD CD4+ subsets (medians: 1.4 and 1.7, respectively; Figure 4D).

A similar analysis was performed for CD8+ T cells. An additional CD8+ subset, namely intermediate cells (inter: CD27^{dim}CD45RO⁻) was characterized, as shown in the representative flow cytometric plots from one HIV-uninfected and one HIV-infected individual (pre- and post-ART; Figure 5A). As described previously, this subset is distinct from effector cells and is characterized by CD57 and CD127 expression, and appears to be a differentiation stage between central memory and effector memory cells [30]. Interestingly, as for CD4+ T cells, HIV infection led to a significantly lower proportion of naive CD8+ T cells (Figure 5B), and there was a concomitant increase in ED and LD CD8+ T cell subsets when compared to HIV-uninfected controls (Naive: medians 18% vs 48%, $p<0.0001$; ED: 24% vs 6%, $p<0.0001$; and LD: 8% vs 3%, $p=0.002$, respectively). In contrast to CD4+ T cells, although there was a trend towards a greater proportion of TD CD8+ T cells, their frequencies did not differ significantly between HIV-uninfected and HIV-infected individuals (medians: 24% vs 33%, respectively; $p=0.13$). There was also no significant difference in the frequency of Inter CD8+ T cells between the HIV-infected and the HIV-uninfected groups. Following ART, there was a significant increase in naive CD8+ T cell frequency, with a simultaneous decrease in ED and Inter CD8+ T cell frequencies (Naive: medians 31% vs 18%, $p<0.0001$; ED: 15% vs 24%, $p<0.0001$ and Inter: 5% vs 7%, $p=0.0005$; Figure 5B). No substantial differences in the proportions of LD and TD CD8+ T cell subsets were found between pre- and post-ART time points (LD: medians 8% vs 8%, $p=0.19$ and TD: 33% vs 29%, $p=0.89$). However, ART-induced restoration of the distribution profile of CD8+ T cell subsets was partial, as only naive cells significantly increased but still remained lower than HIV-uninfected subjects ($p=0.01$). These were compensated for by decreases in ED, Inter and LD subsets post-ART (Figure 5B).

While the median absolute CD8 count did not differ pre- and post-treatment, substantial variation in CD8 cell count was observed amongst participants. Thus, changes in the absolute number of each CD8+ memory subsets were assessed, and we observed a trend towards an increase in the number of naive CD8+ T cells ($p=0.08$) after treatment, with an increase occurring in 23/28 individuals (Figure 5C). The absolute number of ED CD8+ T cells and the Inter subset, which constitutes the smallest proportion of the CD8+ T cell population, decreased significantly post-ART (ED: $p<0.0001$ and Inter: $p=0.02$), whilst the

absolute number of LD and TD remained comparable pre- and post-ART. By calculating the fold change in absolute number of CD8+ T cell subsets pre- and post-ART (Figure 5D), overall only naive CD8+ T cells expanded (median fold change of 1.4), while the other four subsets all contracted.

Overall, our results reveal that HIV skews memory T cell profiles, promoting excessive T cell memory differentiation, with a decreased frequency of peripheral naive T cells, while augmenting the memory subsets (ED and LD cells). One year of ART was insufficient for complete normalization of CD4+ and CD8+ T cell subsets, despite suppression of viral load in most cases.

3.5 Activation levels within CD4+ and CD8+ T cell memory subsets before and after ART

Finally, in order to define whether HIV-induced hyperactivation affects all memory T cell subsets to the same extent, we determined the expression of activation markers on each T cell memory subset within CD4+ and CD8+ T cells (n=28; pre- and post-ART), and compared them to profiles in HIV-uninfected controls (n=23). During HIV infection, for both CD4+ and CD8+ T cells, all antigen-experienced subsets (ED, Inter, LD and TD) became activated (3–6 fold greater than the same subsets in HIV-uninfected subjects), as shown by a significant up-regulation of HLA-DR expression (CD4: $p<0.0001$ for ED, LD and TD; and CD8: ED, Inter, LD, TD: $p<0.0001$; Figures 6A and 6B). The most activated subsets were the ED, LD and TD cells for both CD4+ and CD8+ T cells, with activation being higher in the CD8 compartment compared to the CD4 compartment. With respect to naive cells, naive CD8+ T cells demonstrated ~11 fold higher levels of activation than the HIV-uninfected group, while naive CD4+ T cells showed little or no activation, apart from a single outlier. Following 1 year of ART, HLA-DR expression on all subsets was markedly reduced for both CD4+ (decrease of ~2 fold for ED and LD) and CD8+ T cells (decrease of ~3 fold for ED, ~2 fold for Inter, LD and TD). The degree of deactivation was comparable across all CD4+ and CD8+ memory subsets. Naive CD8+ T cells were also deactivated upon ART (decrease of ~4 fold). With regard to CD4+ T cells, none of the subsets fully normalized their activation levels post-ART to HIV-uninfected levels, whereas in three subsets of CD8+ T cells (ED, LD and TD), activation reached similar levels to those in HIV-uninfected individuals. The reduction in HLA-DR expression post-therapy is further demonstrated in Figures 6C and D, showing the fold change in activation for all CD4+ and CD8+ T cell subsets. Of note, similar profiles were observed when analyzing the activation marker CD38 (alone or in combination with HLA-DR), as well as using the proliferation marker Ki67 (Figure S2). Taken together, these data suggest that HIV activates all T cell subsets, irrespective of their differentiation stage, and partial normalization occurs after 1 year of suppressive therapy.

4. Discussion

HIV infection remains a considerable public health burden globally, and particularly in sub-Saharan Africa. With improved access to ART, there is now considerable improvement in the quality of life and life expectancy of HIV-infected individuals [12]. South Africa has the largest ART programme globally, yet there remains a gap in ART coverage, with only 3.4

million out of 6 million HIV-infected South Africans accessing therapy [31,32]. In addition to the challenges posed by the gap in access, the majority of those currently on treatment gained access at the chronic or late stage of infection, and the degree to which accumulated HIV-induced immune defects are reversed upon late treatment initiation is not fully understood. Thus, a better understanding of the impact of ART on T cell immune profiles is critical to improve therapeutic strategies. In this study, we investigated the impact of ART on the activation, proliferation and memory differentiation of CD4+ and CD8+ T cells in HIV-infected African women. Overall, our data showed that (i) whilst there was a decrease in T cell activation and proliferation, 1 year of ART was insufficient to completely normalize these immune defects compared to HIV-uninfected individuals; (ii) for T cell differentiation profiles, there was a partial normalization of the skewing of T cell subsets upon ART, with different dynamics for CD4+ and CD8+ T cells. These data reveal ongoing immunological perturbations during ART that warrant further study.

Here, we evaluated whether ART normalized T cell activation and proliferation in treated HIV-infected individuals compared to baseline levels in matched uninfected controls. We show that ART induces a substantial global decrease in the proportion of activated (CD38+ and/or HLA-DR+) and proliferating (Ki67+) CD4+ and CD8+ T cells. However, these parameters still remained significantly higher compared to HIV-uninfected individuals. These findings are consistent with previous reports, suggesting only a partial reduction in immune activation following treatment [3,16,33,34]. Several studies have highlighted some major consequences of ongoing immune activation on ART, including a high risk of non-AIDS related diseases and mortality [35]. The potential mechanisms contributing to this sustained immune activation have not been fully elucidated. Viral reservoirs established early in HIV infection may persist during ART either because the drugs are unable to reach these sites or due to the maintenance of long-lived latently infected cells that enable replication during natural homeostatic processes of cell turnover [36–39]. In addition, the breach in mucosal surfaces in the gastrointestinal tract (gut damage) as a result of HIV infection may be irreversible in many patients even with early initiation of ART, suggesting ongoing bacterial translocation may drive immune activation [40,41]. Although early ART initiation and long-term ART have been shown to significantly decrease immune activation, it remains elevated relative to HIV-uninfected individuals [42,43]. Other interventions to dampen or prevent immune activation may be necessary. ART intensification strategies show conflicting results [44,45], and administration of IL-21 in ART-treated SIV-infected rhesus macaques resulted in an increase in Th17 cells and decreased activation [46]. Thus, the effectiveness of these approaches at resolving immune activation in treated HIV infection are variable and may be limited, implying a need for the development of more effective methods to counteract the harmful effects of ongoing immune activation.

With respect to T cell differentiation, HIV-infection resulted in skewed CD4+ and CD8+ profiles, with a decrease in naive cell frequency and increased memory differentiation (ED and LD cells), which normalized only partially to levels observed in HIV-uninfected individuals. These data are in accordance with previous results [20,21,23]. Our data demonstrate that naive T cells also recover upon ART. It is known that during HIV infection, thymic dysfunction may impair the replenishment of naive T cells [7], and our observation could likely be explained by increased thymic output, as reported in other studies of HIV

patients (both children and adults) undergoing ART [47,48]. An important point to note is that despite a rebalancing of overall T cell memory profiles upon ART, certain memory T cells specific for particular pathogens may be preferentially depleted during HIV infection and not restored upon ART, raising the issue of not just quantity but also the 'quality' of immune recovery [49–51]. In fact, we showed that the replenishment of antigen-specific CD4⁺ T cells is associated with their memory phenotype pre-ART, where cells exhibiting an ED profile had a higher ability to replenish compared to cells with an LD profile [52]. This has important consequences for the health of those living in areas with a high burden of infectious diseases.

A recent focus on curing HIV underlines the importance of studying ongoing immune activation during ART. This is due to the fact that residual immune activation and HIV replication during ART were found to be associated with the size of the latent viral reservoir, and HIV-infected individuals who initiated ART early were found to have lower T cell activation levels and a smaller HIV reservoir [42,53,54]. With regard to memory subset distribution, we made the interesting observation that the ED CD4⁺ T cell subset expanded by almost two-fold in the first year of ART. Whilst this increase may be partially attributed to the redistribution of CD4⁺ T cells from tissue sites to the blood upon viral suppression, expansion likely occurs mainly through homeostatic mechanisms [55–57]. The ED subset consists of central and transitional memory CD4⁺ T cells, previously identified as some of the main reservoirs of HIV in the blood of HIV-infected individuals [39]. Our data therefore suggest that at the onset of treatment, the HIV reservoir may expand substantially. Thus, further studies on CD4⁺ memory expansion and ongoing immune activation and their influence on the size and maintenance of the latent HIV reservoir are warranted. Ultimately, since current ART suppresses viral replication and is unable to eradicate integrated replication-competent provirus, there is an urgent need for novel therapeutic strategies to reduce and/or purge viral reservoirs to cure HIV infection.

Our study had several limitations. Firstly, we studied T cells in peripheral blood, and it is important to note that restoration of T cell memory phenotypes and deactivation in blood does not necessarily reflect T cell composition at other immunological sites of importance in HIV pathogenesis, such as the gastrointestinal tract or lymphoid tissue, where HIV causes significant damage and where low-level replication of HIV may persist under ART [58]. Furthermore, we focused on T cells, and abnormalities of other immune subsets such as B cells, dendritic cells and monocytes also occur during HIV infection [59–61] and may persist after ART [43,62]. We recently demonstrated that B cell activation and levels of soluble CD14 in plasma fail to normalize fully after 1 year of ART [63], likely as a result of persistent monocyte/macrophage activation [64–66]. Lastly, our cohort consisted of women only. There is evidence that women with untreated HIV infection have greater immune activation and more rapid HIV disease progression compared to men with the same viral load [67], and there may be differences that persist after ART. It is also worth noting that male sex was associated with lower CD4 gains after treatment independent of starting CD4 count [68], and thymic output has been described to be reduced in men compared to women [69,70]. Thus, testing whether our findings are applicable to men is warranted.

To the best of our knowledge, this is the first study to report T cell activation on different T cell memory subsets and examine, in addition to CD4 memory subsets, changes in CD8 memory subsets in African women with chronic HIV infection following ART. Our study demonstrated hyperactivation and skewing of T cell memory phenotypes in these women. Administration of ART during chronic HIV infection led to successful viral load suppression and CD4+ T cell increases. However, T cell activation and proliferation levels remained higher than HIV-uninfected subjects and the frequency of T cell memory subsets did not completely normalize, implying partial recovery following short-term (1 year) ART. In addition, whilst activation levels were nearly completely regularized within memory CD8+ T cells, they were only partially restored within memory CD4+ T cells, implying differential dynamics of deactivation between the two compartments. A follow-up study will be required to determine whether longer-term ART may enable full immune normalization of T cell phenotypes and deactivation in our cohort.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Clinical parameters before and after ART. (A) Plasma viral load, (B) absolute CD4 count, (C) absolute CD8 count, and (D) CD4/CD8 ratio before (pre-ART) and after 12 months of ART (post-ART) in 28 HIV-infected individuals. The horizontal dotted line indicates the detection limit of the assay. Horizontal solid lines indicate the median. Statistical significance was calculated using the Wilcoxon Signed Rank test.

Figure 2. Effect of ART on CD4+ and CD8+ T cell activation and proliferation.

(A) Representative flow plots of CD38 and HLA-DR expression on T cells from one HIV-uninfected and one HIV-infected (pre- and post-ART) individual. (B) Frequencies of CD38+ (teal), HLA-DR+ (plum) and CD38+HLA-DR+ (blue) CD4+ (left panel) and CD8+ T cells (right panel) in 23 HIV-uninfected (open circles) and 28 HIV-infected (closed circles) individuals pre- and post-ART. (C) Representative flow plots of Ki67 expression in CD4+ and CD8+ T cells from one HIV-uninfected and one HIV-infected (pre- and post-ART) individual. Numbers represent the frequencies of proliferating (Ki67+) T cells. (D)

Frequencies of Ki67+ T cells in HIV-uninfected (n=23; open circles) and HIV-infected pre- and post-ART (n=18; closed circles) individuals. Horizontal lines represent the median. Statistical significance was calculated using a Mann-Whitney U test and Wilcoxon Signed Rank for unpaired and paired samples, respectively.

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Figure 3. Association between CD4+ and CD8+ T cell activation before and after ART. Correlation of the frequency of (A) CD38+, (B) HLA-DR+, and (C) CD38+HLA-DR+ CD4+ T cells and the frequency of CD38+, HLA-DR+ and CD38+HLA-DR+ CD8+ T cells (n=28), respectively, in HIV-infected individuals prior to ART initiation. Correlation of the frequency of (D) CD38+, (E) HLA-DR+, and (F) CD38+HLA-DR+ CD4+ T cells and the frequency of CD38+, HLA-DR+ and CD38+HLA-DR+ CD8+ T cells (n=28), respectively, post-ART. Statistical significance was calculated using a non-parametric Spearman Rank test. The dashed line represents a linear regression fit.

Figure 4. Memory differentiation profiles of CD4⁺ T cells before and after ART.

(A) Representative flow cytometry plots of the memory distribution of total CD4⁺ T cells in one HIV-uninfected and one HIV-infected individual pre- and post-ART. Naive: (CD27+CD45RO⁻, blue); Early Differentiated (ED: CD27+CD45RO⁺, green); Late Differentiated (LD: CD27⁻CD45RO⁺, red) and Terminally Differentiated (TD: CD27⁻CD45RO⁻, grey). The frequencies of each subset are indicated. Frequency (B) and absolute number (C) of CD4⁺ T cell subsets in HIV-uninfected (n=23; open circles) and HIV-infected individuals pre- and post-ART initiation (n=28; closed circles). Horizontal bars represent the

median. Statistical significance was calculated using a Mann-Whitney U test and Wilcoxon Signed Rank for unpaired and paired samples, respectively. **(D)** Fold change in the total, naive, ED, LD and TD absolute CD4⁺ T cell count over 12 months of ART. The horizontal dotted line indicates no change from the time point prior to ART. The solid lines at 0.8 and 1.2 represent 20% change above which a change was considered significant. Statistical comparisons were calculated using a one-way ANOVA test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 5. Memory differentiation profiles of CD8+ T cells before and after ART.

(A) Representative flow plots of total CD8 subset distribution in one HIV-uninfected and one HIV-infected individual pre- and post-ART. Naïve (blue), Early Differentiated (ED: green), Intermediate (Inter, brown), Late Differentiated (LD, red) and Terminally Differentiated (TD, grey). The frequencies of each subset are indicated. Frequency (B) and absolute number (C) of CD8+ T cell subsets in HIV-uninfected (n=23; open circles) and HIV-infected individuals pre-and post-ART initiation (n=28; closed circles). Horizontal bars represent the median. Statistical significance was calculated using a Mann-Whitney U test

and Wilcoxon Signed Rank for unpaired and paired samples, respectively. **(D)** Fold change in the total, naive, ED, Inter, LD and TD absolute CD8+ T cell count over 12 months of ART. The horizontal dotted line indicates no change from the time point prior to ART. The solid lines at 0.8 and 1.2 represent 20% change above which a change was considered significant. Statistical comparisons were calculated using a one-way ANOVA test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 6. Activation profiles of CD4+ and CD8+ T cell subsets before and after ART. Frequencies of (A) HLA-DR+CD4+ T cells and (B) HLA-DR+CD8+ T cells, in HIV-infected (pre- and post-ART; n=28; closed circles) individuals and HIV-uninfected (n=23; open circles). Blue, green, brown, red and grey represent the Naive, ED, Inter, LD and TD subsets. Data are shown as box and whisker (interquartile range) plots. Statistical significance was calculated using a Mann-Whitney U test and Wilcoxon Signed Rank test for unpaired and paired samples, respectively. Fold change in HLA-DR frequencies of (C) Naive, ED, LD and TD CD4+ T cells and (D) Naive, ED, Inter, LD and TD CD8+ T over 12 months of ART. The horizontal dotted line indicates no change from the time point prior to

ART. Statistical comparisons were calculated using a one-way ANOVA test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

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