The Distribution and Immune Profile of T Cell Subsets in HIV-Infected Children from Uganda

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Abstract

T cell activation is an important mechanism in HIV-associated immune depletion. We have previously demonstrated an association between the hyperactivation of $CD4^+$ and $CD8^+$ T cells and low CD4 status in HIV-infected Ugandan children. In this study, we explore differences in activation between naive and memory T cells in HIV-infected Ugandan children. A significant correlation between CD4- and CD8-mediated immune activation and CD4 status was observed only in the memory T cells. Antiretroviral (ART) untreated and treated HIV-positive and HIV-negative children displayed similar profiles of activation and distribution within the $CD4^+$ naive T cells. In contrast, significantly higher immune activation of the memory $CD4^+$ T cell subset was seen in ART-untreated children when compared to ART-treated or HIV-negative children. ART-mediated viral suppression led to the correction of $CD4^+$ immune activation to levels seen in uninfected children but did not increase the size of the memory $CD4^+$ T cell population. High levels of $CD8^+$ immune activation were also found in both naive and memory cell subsets. Antiretroviral treatment led to the normalization of $CD8^+$ T cell activation but did not correct the distribution of naive $CD8^+$ T cells. We also assessed PD-1 expression on $CD8^+$ T cells as a measure of immune dysfunction. Upregulation of PD-1 was highest in untreated children but persisted in ART-treated children compared to uninfected children. The mechanisms of immunopathogenesis in pediatric HIV infection likely involve distinct contributions from individual naive and memory T cells subsets.

Introduction

HUMAN IMMUNODEFICIENCY VIRUS (HIV) infection in children leads to progressive immunodeficiency and immune dysfunction, although our understanding of T cell dynamics during viral infection remains limited.[1,2] High levels of immune activation have been associated with HIV disease progression in children. The gradual decline of naive T cell pools in HIV-infected children has been attributed to continuous activation of naive T cells and to thymic dysfunction. In addition, depletion of memory T cells likely occurs through direct lysis of infected cells and apoptosis of uninfected bystander cells via activation-induced cell death.[3]

The mechanisms of immune dysfunction and exhaustion are not well understood. The receptor programmed cell death-1 (PD-1), a member of the CD28 family, is involved in signaling T cell death, and negatively regulates T cell function.[4,5] The level of PD-1 expression on HIV-specific T cells is associated with a decrease in functional capacity.[6–8] High levels of PD-1 expression on HIV-1-specific T cells reflect a state of cellular exhaustion and may prevent optimal antiviral function. In the presence of high viral load, increased expression of PD-1 can be attributed to T cell activation.[6,7]

The level to which T cells are depleted in HIV infection depends on the level of immune activation. Homeostatic levels of naive and memory T cell subsets may be disrupted in chronic infection. We previously showed that the $CD4^+$ T cell count, but not the viral load, was significantly associated with both $CD4^+$ and $CD8^+$ T cell activation in Ugandan children.[9] Given the variable level of T cell maturity in children, the contributions of different subsets to immune exhaustion are uncertain. To clarify this area, we next evaluated the pattern of immune activation in different T cell subsets in HIV-infected children in Uganda.

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Materials and Methods

Study subjects and samples

Samples were obtained from the study cohort "Children with HIV and Malaria Project" (CHAMP, $N=61$), a prospective observational study investigating interactions between HIV and malaria coinfection in children. CHAMP has enrolled 300 HIV-infected children (ages 1–10 years) from the Pediatric Infectious Disease Clinic at Mulago Hospital in Kampala.

For comparison, samples from a total of 25 children were selected from a local parallel cohort of 601 HIV-uninfected children.[10] Samples from HIV-1-positive adult volunteers ($N=27$) were obtained from an existing cross-sectional cohort study in Uganda.[11–15]

Demographic information, CD4$^+$ T cell status (percentage or absolute count), and plasma HIV RNA levels were also obtained at the time of enrollment and blood draw. Peripheral blood mononuclear cells (PBMCs) were separated and cryopreserved in liquid nitrogen until assay time as previously described.[13,14,16–18]

Approvals for these studies were obtained from the California Department of Public Health, the University of California, San Francisco, the Makerere University Research and Ethics Committee, the Ugandan National Council of Science and Technology, and the Joint Clinical Research Centre Institutional Board Review. All study participants gave written informed consent. Parents or legal guardians consented on behalf of the children.

Immune activation and functional immunophenotype

Activation staining was performed by incubating PBMCs with the following antibodies: HLADR FITC, CD38 PE, CD3 PerCp-Cy5.5, CD4 APC-CY7, CD27 APC, CD45RA-PECy7, and CD8 Pacific Blue, and analyzed by flow cytometry.[18] Dead cells were first gated out using a violet excited viability dye (LIVE/DEAD Fixable Dead Cell Stain; Invitrogen). Immune activation was defined as the percent of CD38$^+$ HLA DR$^+$ T cells within the naive and memory subsets. We defined naive T cells as CD3$^+$CD8$^+$ (or CD3$^+$CD4$^+$) CD27$^+$CD45RA$^+$ and memory T cells as CD3$^+$CD8$^+$ (or CD3$^+$CD4$^+$) CD27$^-$CD45RA$^-$. PD-1 level was defined as the percent of expression of PD-1-APC on CD3$^+$CD8$^+$ T cells. Gating was standardized and set using fluorescence controls for HLADR, CD38, and PD-1.

A minimum of 30,000 CD3$^+$ cells per sample was acquired using an eight-color flow cytometer (LSRII, BD Biosciences). Analysis was performed by FLOWJO software (TreeStar, San Carlos, CA).

Statistical analysis

Groups were compared using the Mann–Whitney U test and analysis was performed with PRISM software version 4.02 (GraphPad, San Diego, CA). Spearman's correlation coefficient was used to determine the correlation between two variables, and a linear least-squares regression model was used in multivariate analysis. Statistical significance was defined as $p < 0.05$.

Results

Characteristics of study participants

The clinical characteristics of the pediatric and adult study volunteers are shown in Table 1. HIV-infected children on antiretroviral (ART) treatment in this study have undetectable HIV plasma RNA. Overall, there was no statistical difference in age ($p \geq 0.05$) between the HIV-positive and HIV-negative children (median 8 years, range$=3$–11). The median age, CD4$^+$ T cell count, and plasma HIV-1 RNA levels of the adult study population were 35 years (range$=31$–42), 240 cells/mm^3 (range$=4$–687), and 101,122 copies/ml (range$=678$–844,279), respectively.

Profile of immune activation in CD4$^+$ T cell subsets

T cell activation is believed to play a critical role in T cell depletion and immune recovery in children chronically infected with HIV.[19] We assessed immune activation, as measured by coexpression of surface CD38 and HLA-DR, in different CD4$^+$ T cell subsets in Ugandan children. Naive T cell subsets were defined as CD4$^+$ T cells expressing both CD27 and CD45RA. Memory T cells were defined as CD45RA$^-$ with variable expressions of CD27 in order to include both memory effector and central memory subpopulations. Because there was no significant difference in the level of immune activation between different subsets of memory T cells, data from memory T cells were grouped together as CD4$^+$ and CD45RA$^-$CD27$^{+/-}$.

The level of activation and the distribution of the naive CD4$^+$ T cell subset did not differ between HIV-infected and uninfected children (Fig. 1, and data not shown, $p > 0.05$).

TABLE 1. CLINICAL CHARACTERISTICS OF STUDY PARTICIPANTS[a]

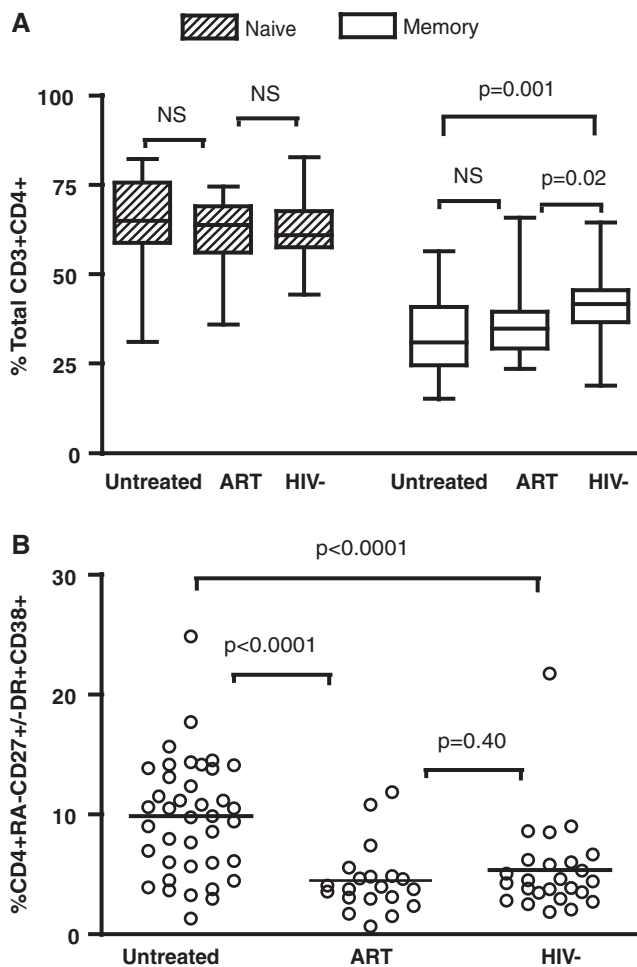| Study participants	Age (years, range)	CD4 (percent, range)	HIV plasma RNA (copies per milliliter, range)
Untreated HIV$^+$ children $N=41$	8 (2–11)	27 (12–43)	72,493 copies/ml (962–232,582)
ART-treated children $N=20$	6 (2–10)	25 (11–33)	<400 copies/ml —
HIV-negative children $N=25$	8 (3–11)	—	—
Untreated HIV$^+$ adults $N=27$	35 (31–42)	240 (4–687)	101,122 copies/ml (678–844,279)

[a]Median values and range.

FIG. 1. (**A**) Distribution of CD4$^+$ T cell subsets as defined by total naive (striped pattern) or memory (clear pattern) cells in a group of antiretroviral-naive children ($N = 40$), ART experienced with undetectable viral load ($N = 20$), or healthy HIV-negative children ($N = 25$). Horizontal lines represent medians and interquartile ranges (25th and 75th percentiles). (**B**) Immune activation in CD4$^+$ T cell memory subsets was defined as the percentage of CD4$^+$ T cells with the CD45RA$^-$CD27$^{+/-}$ phenotypes with surface coexpression HLA-DR and CD38. Immune activation was significantly higher in untreated children compared to ART-suppressed or HIV-negative children ($p < 0.001$).

FIG. 2. Correlation between the CD4% and memory T cell activation in children. The percentage of (**A**) CD4$^+$ memory T cell activation or (**B**) CD8$^+$ memory T cell activation is plotted against CD4%. A significant negative was observed in (**A**) ($r = -0.43$, $p = 0.007$) and in (**B**) ($r = -0.034$, $p = 0.04$).

In contrast, the level of memory CD4$^+$ T cell population was lower in HIV-positive children, both untreated and ART treated, compared to that of HIV-negative children ($p = 0.0014$ and 0.02, respectively). CD4$^+$ memory T cell activation was comparable in the ART-treated and HIV-negative children ($p = 0.40$) and was significantly lower than in the untreated HIV-positive group ($p < 0.0001$). Furthermore, only memory, and not naive CD4$^+$ T cell immune activation correlated significantly with CD4 cell status (Fig. 2A, $r = -0.43$, $p = 0.007$ and $p > 0.05$, data not shown). CD4 percent, instead of absolute count, was used for correlation analyses of data from children because it is a more clinically relevant measure of CD4 status. Nevertheless, the statistical significance persisted when using absolute CD4 cell count in corre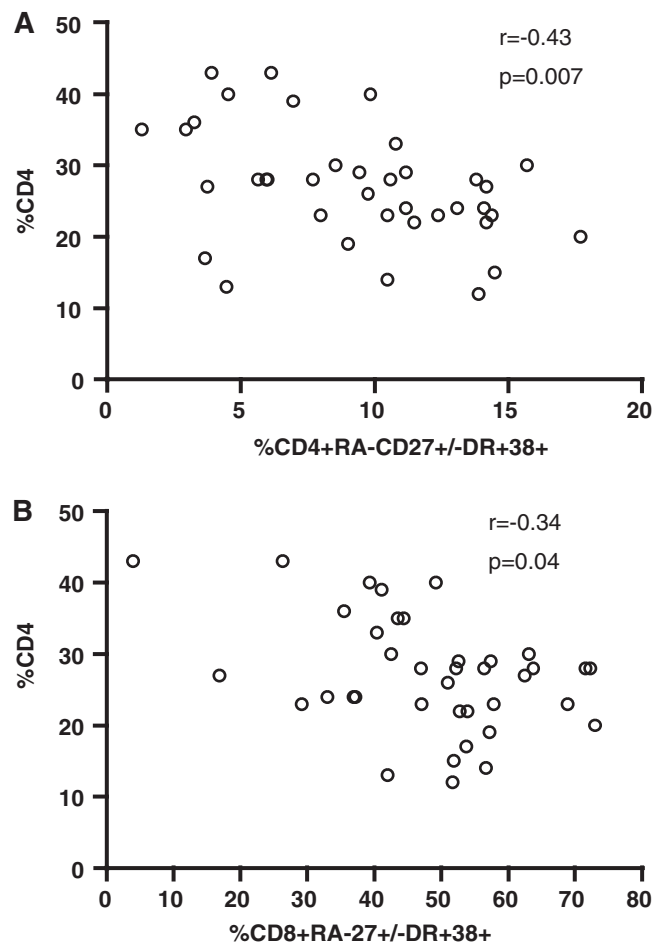lation analysis ($p < 0.05$, data not shown). Consistent with our previous findings,[9] we found no correlation between immune activation and viral load in any of the T cell subsets ($p > 0.05$, data not shown).

Profile of immune activation in CD8$^+$ T cell subsets

High CD8$^+$ T cell immune activation carries poor prognosis in HIV-infected children.[20,21] We first assessed the distribution of naive and memory T cells as defined above for CD4$^+$ T cells. HIV-infected children not treated with ART drugs had a distinct distribution of naive and memory CD8$^+$ T cells compared to ART-treated and HIV-negative children (Fig. 3A). Similarly, high levels of immune activation in the naive and memory T cell subsets were found in untreated children when compared to the ART-treated or uninfected groups (Fig. 3B and C). The level of activation of memory CD8$^+$ T cells negatively correlated with CD4 percent in the untreated population ($r = -0.34$, $p = 0.04$; Fig. 2C). This correlation was not observed for the naive CD8$^+$ T cell subset ($p > 0.05$, data not shown). In contrast to the CD4$^+$ immune profile, we found lower frequencies of the naive but not memory CD8$^+$ T cells in ART-treated children compared to the uninfected group,

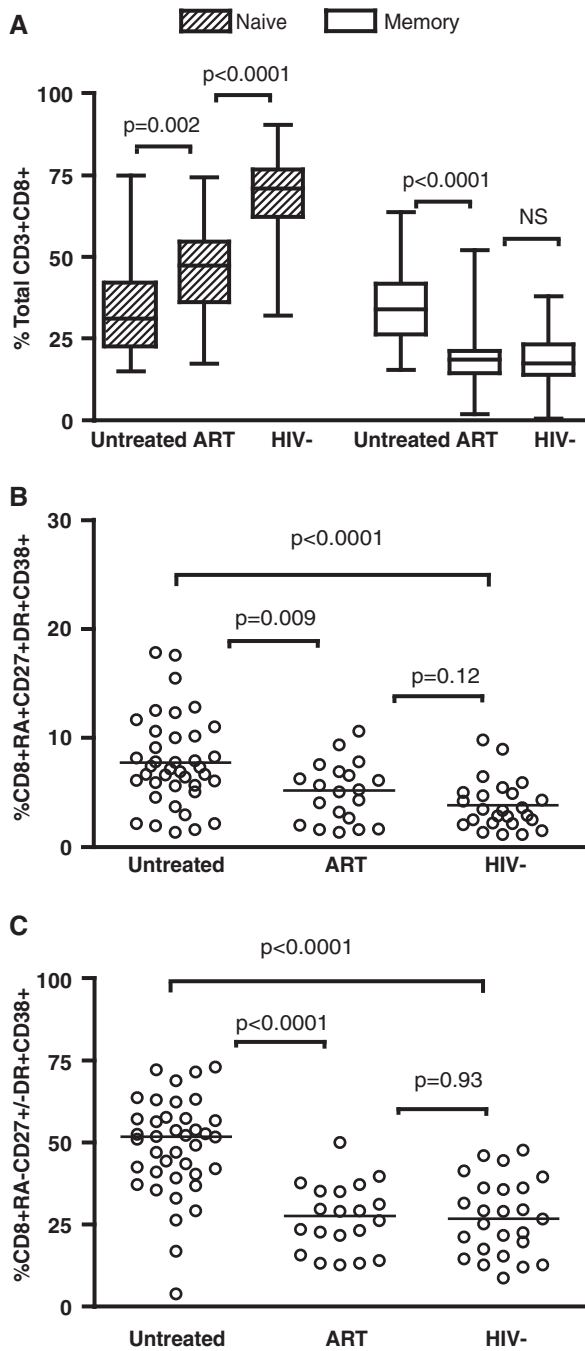**FIG. 3.** (**A**) Distribution of CD8^{+} T cell subsets as defined by total naive (striped pattern) or memory (clear pattern) cells in a group of antiretroviral-naive children ($N = 40$), ART experienced with undetectable viral load ($N = 20$), or healthy HIV-negative children ($N = 25$). Horizontal lines represent medians and interquartile ranges (25th and 75th percentiles). Immune activation in CD8 T cell subsets was defined as the percentage of total naive (**B**) or memory (**C**) CD8^{+} T cells expressing HLA-DR and CD38. Significant differences were observed in immune activation of the memory CD8^{+} T cell subpopulation between HIV-positive and HIV-negative children ($p < 0.001$).
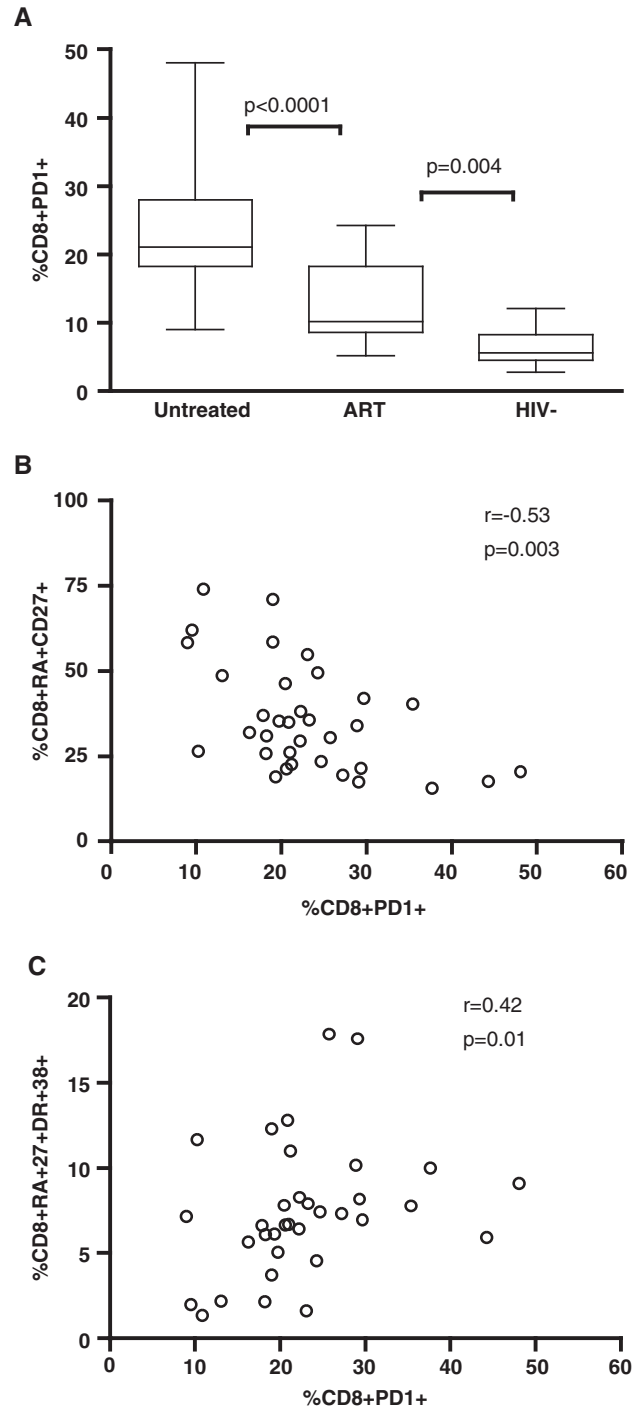
FIG. 4. PD-1 expression on CD8^{+} T cells is higher in HIV-positive children. Dead cells were first gated out using a violet excited viability dye (LIVE/DEAD Fixable Dead Cell Stain; Invitrogen). Samples were subsequently gated on the CD3^{+}/CD8^{+} lymphocyte population then the percent of PD-1-positive cells was determined. Gating was standardized and set using the fluorescence minus one (FMO) control for PD-1. (**A**) PD-1 expression on CD8^{+} T cells was highest in children not receiving ART ($p < 0.0001$). Viral suppression does not lead to normalization of PD-1 expression compared to samples from HIV-negative children ($p = 0.004$). (**B**) The presence of PD-1 expression negatively correlated with the frequency of naive CD8^{+} T cells ($r = -0.53$, $p = 0.003$) and (**C**) positively associated with naive CD8^{+} T cell activation ($r = 0.42$, $p = 0.01$).
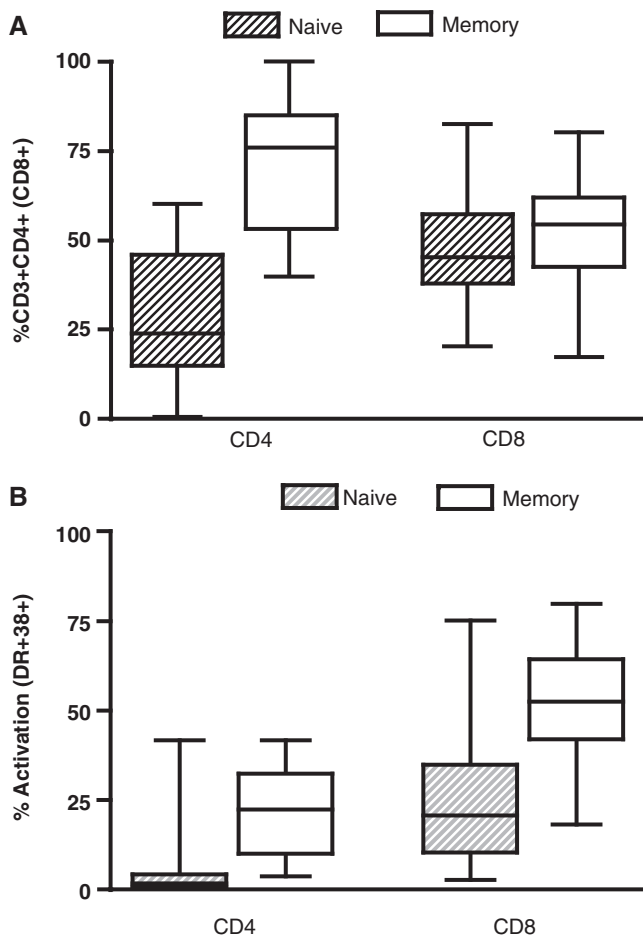
A

B

FIG. 5. (**A**) Distribution of CD4+ and CD8+ T cell subsets as defined by total naive or memory cells with horizontal lines representing medians and interquartile ranges (25th and 75th percentiles) in a group of untreated HIV-positive Ugandan adults ($N = 27$). The median distributions of memory phenotypes on CD4+ and CD8+ T cells were 76% and 55%, respectively. (**B**) Immune activation in CD4+ and CD8+ T cell subsets. Naive and memory T cell activation was defined as the percentage of CD45RA+CD27+ or CD45RA−CD27+/− cells expressing HLA-DR and CD38, respectively. Median naive and memory CD4+ T cell activation was 22% and 2%, respectively. Median naive and memory CD8+ T cell activation was 53% and 21%, respectively.

despite no detectable differences in immune activation in these respective T cell subsets (Fig. 3).

Profile of T cell function in children

PD-1 is a member of the CD28 family and is expressed on T cells. It is primarily expressed on activated cells and its expression level is determined by T cell differentiation stage.[5,22,23] For CD8+ T cells, PD-1 was primarily expressed on CD45RA−CD27+ T cells (data not shown). The highest PD-1 expression on CD8+ T cells was found in untreated children, although viral suppression by ART treatment did not lower PD-1 expression to levels seen in HIV-negative children (Fig. 4A). High PD-1 expression was negatively correlated with the frequency of total naive CD8+ T cells (Fig. 4B, $r = -0.53$, $p = 0.003$), and positively associated with naive CD8+ T cell activation ($r = 0.42$, $p = 0.01$, Fig. 4C). Surprisingly, no asso-

ciation was observed between PD-1 levels and the distribution or the level of activation in the memory CD8+ T cell subsets ($p > 0.05$, data not shown), suggesting variable expression of PD-1 in the memory T cell population.

Profile of immune activation in adults

We previously reported higher immune activation in HIV-positive adults compared to children in Uganda.[9] We further studied the distribution of naive and memory CD4+ and CD8+ T cells and their respective level of activation in adults (Fig. 5). The median (and range) of memory CD4+ and CD8+ T cells in this adult population was 76% (41–99) and 55% (18–79), respectively. The median naive and memory CD4+ T cell activation was 2% (0.4–42) and 22% (4–42), respectively. The median naive and memory CD8+ T cell activation was 21% (18–78) and 53% (3–75), respectively. The frequencies of naive and memory CD4+ and CD8+ T cells in HIV-positive adults were significantly different compared to frequencies found in the untreated pediatric population ($p < 0.0001$, data not shown). Immune activation levels in the naive and memory CD4+ T cell subsets were higher in adults compared to children. Interestingly, despite similar viral loads (Table 1, $p = 0.56$), only the activation of CD8+ T cells within the naive subset, not in the memory subset, was higher in untreated adults when compared to children ($p > 0.0001$ and 0.34, respectively).

Discussion

CD4 T cell loss is a surrogate marker of HIV pathogenesis and the extent of immune activation is associated with T cell apoptosis. We report here studies of activation of naive and memory T cells in a cohort of HIV-positive Ugandan children. Untreated, ART-experienced, and HIV-uninfected children displayed similar levels and a similar profile of activation of naive CD4+ cells. In contrast, the level of memory CD4+ T cells was lower in HIV-positive children, regardless of treatment status, and elevated activation was specifically observed in untreated, HIV-infected children. One explanation for these findings is the presence of an active thymic output that permits the maintenance and relative preservation of the CD4+ naive T cell population. Alternatively, repletion after ART may favor the naive cell subset, which appears to be less susceptible to HIV-induced immune activation. Activation of the CD4 memory population is expected to increase the division rate and relative cell count. However, activated CD4+ T cells are also at higher risk of bystander killing and direct HIV infection.[24–26] The fact that ART treatment corrects the abnormal immune activation without achieving full restoration of the memory CD4+ T cell pool indicates ongoing immune destruction. Our data indicate that viral suppression does not fully correct the disrupted CD4+ homeostasis in HIV infection, but may reset the balance between activation and destruction of T cells. One possible consequence is to delay the collapse of the immune system by strengthening its renewal capacity.[27,28] Nevertheless, immune homeostasis and restoration in HIV infection is a complex dynamic process and our study number and its cross-sectional design limit any definitive conclusions.

Elevated CD8+ T cell activation is an independent risk factor in HIV-mediated disease progression. The level of immune activation in both naive and memory CD8+ T cell

subsets in untreated children was significantly higher than in ART-treated or uninfected children. HIV infection also perturbed the balance between naive and memory CD8^{+} T cell distribution in children, and this imbalance failed to normalize fully with viral suppression. These findings suggest the presence of ongoing maturation and turnover of the CD8^{+} T cell population in the absence of detectable viremia and immune activation. Activation markers alone may thus undervalue the dynamics of CD8^{+} T cell turnover in untreated as well as treated children. We have previously described lower immune activation in children compared to HIV-infected Ugandan adults.[9] In this study, the level of CD8^{+} T cell activation in the memory T cell subset of untreated children was similar to levels seen in untreated HIV-positive adults. These findings underscore the need for a clear designation of cell subsets when measuring immune activation in children.

Upregulation of inhibitory molecules such as PD-1 receptor impairs T cell responses to HIV infection. PD-1 signaling inhibits the CD3/CD28^{−} activation pathway[29–31] and blocking by antagonistic PD-1 antibodies restores T cells functions *in vitro*.[7,32] We saw elevated levels of PD-1 on CD8^{+} T cells in HIV-infected children who were untreated or treated with ART. Interestingly, there was no significant association between PD-1 and the distribution or activation of the memory CD8^{+} T cell population, suggesting variable expression of PD-1 on this T cell subset. In contrast, PD-1 levels negatively correlated with the frequency and activation of the naive CD8^{+} T cells. Thus, PD-1 engagement may play a significant role in regulating T cell homeostasis in children in addition to its association with antigen-specific T cell dysfunction. The perturbation of the naive CD8^{+} T cell population may also be a sensitive measure of the altered homeostasis and immune dysfunction observed in pediatric HIV infection.

The mechanisms involved in HIV pathogenesis in children are not fully understood. Uniquely in children, quantitative and functional factors leading to full immune restoration are confounded by T cell dynamics involved in maturation of the immune system. We describe differential correlations of naive and memory T cells in HIV-infected children in Uganda. Many questions remain unanswered in the treatment of HIV-infected children. In particular, the optimal timing of ART initiation and the consequence of immune repopulation of different T cell subpopulations have not been fully established.[19,33–36] Our findings help to further characterize the complex immune derangements elicited by HIV infection in children.

Acknowledgments

Disclosure Statement

The authors have no commercial or other association that might pose a conflict of interest. I.S. and C.B. contributed equally to this work.

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