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HIV-Infected Children Have Elevated Levels of PD-1$^+$ Memory CD4 T Cells With Low Proliferative Capacity and High Inflammatory Cytokine Effector Functions

Julia Foldi,[1] Lina Kozhaya,[5,a] Bret McCarty,[1,2] Mussa Mwamzuka,[4] Fatma Marshed,[4] Tiina Ilmet,[1,2] Max Kilberg,[1,2] Adam Kravietz,[1,3] Aabid Ahmed,[4] William Borkowsky,[1,2] Derya Unutmaz,[1,3,a] and Alka Khaitan[1,2,3]

[1]New York University School of Medicine, [2]Department of Pediatrics, Division of Infectious Diseases and Immunology, and [3]Department of Microbiology, New York University School of Medicine; [4]Bomu Hospital, Mombasa, Kenya; and [5]Jackson Laboratory for Genomic Medicine, Farmington, Connecticut

Background. During human immunodeficiency virus (HIV) disease, chronic immune activation leads to T-cell exhaustion. PD-1 identifies "exhausted" CD8 T cells with impaired HIV-specific effector functions, but its role on CD4 T cells and in HIV-infected children is poorly understood.

Methods. In a Kenyan cohort of vertically HIV-infected children, we measured PD-1$^+$ CD4 T-cell frequencies and phenotype by flow cytometry and their correlation with HIV disease progression and immune activation. Second, in vitro CD4 T-cell proliferative and cytokine responses to HIV-specific and -nonspecific stimuli were assessed with and without PD-1 blockade.

Results. HIV-infected children have increased frequencies of PD-1$^+$ memory CD4 T cells that fail to normalize with antiretroviral treatment. These cells are comprised of central and effector memory subsets and correlate with HIV disease progression, measured by viral load, CD4 percentage, CD4:CD8 T-cell ratio, and immune activation. Last, PD-1$^+$ CD4 T cells predict impaired proliferative potential yet preferentially secrete the Th1 and Th17 cytokines interferon-γ and interleukin 17A, and are unresponsive to in vitro PD-1 blockade.

Conclusions. This study highlights differences in PD-1$^+$ CD4 T-cell memory phenotype and response to blockade between HIV-infected children and adults, with implications for potential immune checkpoint therapies.

Keywords. HIV infection; children; PD-1$^+$ CD4 T cells; immune exhaustion; Th17.

During human immunodeficiency virus (HIV) infection, viral reservoirs persist despite antiretroviral therapy (ART) and trigger chronic T-cell activation, which leads to immune exhaustion. T-cell exhaustion is defined by progressive loss of effector functions and occurs during chronic viral infections in mice, rhesus macaques, and humans, resulting in defective pathogen clearance [1]. Exhausted T cells express several inhibitory co-receptors, of which PD-1 is the most well-studied [2]. PD-1 is a member of the B7:CD28 superfamily that, upon binding to its ligands PD-L1 or PD-L2, functions as a coinhibitory molecule in T cell receptor (TCR) signaling [3]. During acute infections, T cells upregulate PD-1 after activation as a negative feedback mechanism [3–5]. Failure to downregulate PD-1 in the setting of chronic viral infections characterizes exhausted T cells with weak antigen-specific proliferative and cytokine responses [2]. The therapeutic potential for PD-1 inhibitors was highlighted in the chronic lymphocytic choriomeningitis virus murine infection model in which blockade of the PD-1/PD-L1 pathway restored survival and functionality of CD8 T cells [6]. Similarly, inhibition of PD-1 improved simian immunodeficiency virus–specific CD8 T-cell cytotoxic, proliferative, and cytokine responses in nonhuman primates [7, 8]. HIV-infected adults also have increased levels of PD-1$^+$ CD8 T cells with impaired function that recover HIV-specific effector responses with blockade in vitro [9–11].

While the role of PD-1 in HIV infection has been more thoroughly studied in CD8 T cells, its role in CD4 T-cell exhaustion has also been demonstrated. In HIV-infected (HIV$^+$) adults, PD-1 is upregulated on HIV-specific CD4 T cells [12, 13] and correlates with markers of disease progression [9, 12, 14, 15]. Additionally, PD-1$^+$ CD4 T cells were recently reported to be a reservoir for latent HIV in ART-suppressed adults [16]. Most importantly, blockade of PD-1/PD-L1 increases HIV-specific CD4 T-cell proliferation [9, 12, 13, 15], suggesting multiple potential therapeutic roles for immune checkpoint inhibitors in HIV infection. An important unresolved question is whether the effect of blocking the PD-1/PD-L1 pathway is limited to HIV-specific CD4 T cells, which comprise a small portion of all PD-1–expressing cells [17], or if blockade of PD-1 also modulates function in nonspecific CD4 T cells.

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[a]Present affiliation: Jackson Laboratory for Genomic Medicine, Farmington, Connecticut.

Correspondence: A. Khaitan, MD, New York University School of Medicine, 430 E 29th St, Alexandria Center for Life Sciences West Bldg, Rm 324, New York, NY 10016 (alka.khaitan@nyumc.org).

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HIV$^+$ children have tolerogenic immune systems with weak immune responses, leading to uncontrolled viral replication, rapid disease progression, and high mortality rates [18–21]. PD-1 in these children may mediate both immune tolerance and exhaustion. HIV$^+$ children have increased PD-1 expression on CD8 T cells that correlates with immune activation [22–24], yet the frequency, phenotype, and function of PD-1$^+$ CD4 T cells in the pediatric population are poorly characterized. In this study, we examined CD4 T cells in a perinatally infected HIV$^+$ pediatric cohort from Mombasa, Kenya, for PD-1 expression and correlation with markers of HIV disease progression and immune activation. We also determined the functional capacity of PD-1$^+$ CD4 T cells and effects of PD-1 blockade on CD4$^+$ T cells. We show that frequencies of PD-1$^+$ memory CD4 T cells are significantly elevated in HIV$^+$ children and correlate with HIV disease progression, measured by CD4 percentage, CD4:CD8 ratio, HIV plasma viremia, and T-cell activation. We further demonstrate that PD-1$^+$ memory CD4 T cells are hypoproliferative yet preferentially secrete Th1 and Th17 cytokines compared to PD-1$^-$ CD4 T cells and that these functional features are not reversed by in vitro PD-1 blockade.

MATERIALS AND METHODS

Study Subjects and Specimens

Ethical approval for this study was obtained from New York University and Kenyatta National Hospital/University of Nairobi. Written informed consent and age-appropriate verbal assent were obtained from all participants and/or parents. We enrolled a total of 71 perinatally infected HIV$^+$ and 40 HIV-unexposed (HU) children aged 5–18 years from Bomu Hospital in Mombasa, Kenya, between 2011 and 2012. HIV$^+$ children included 38 ART-naive (ART$^-$) and 33 HIV$^+$ children on ART for at least 6 months (ART$^+$). Plasma and peripheral blood mononuclear cells (PBMCs) were isolated with Ficoll-Paque PLUS (GE Healthcare) density gradient centrifugation from each subject. HIV RNA was quantified on diluted plasma samples with Roche, COBAS AmpliPrep/COBAS TaqMan HIV-1 Test, version 2.0 (limit of detection 110 copies/mL). Table 1 outlines demographic and clinical characteristics of the participants.

Immune Phenotyping

PBMCs were stained with fixable viability dye (eBioscience) in phosphate-buffered saline (PBS) for 30 minutes, then stained with fluorescent-conjugated antibodies against CD3, CD4, CD8, CD45RO, CCR7, CD38, HLA-DR, and PD-1 (Supplementary Table 1) at 4°C for 30 minutes in PBS buffer containing 2% fetal calf serum and 0.1% sodium azide. For intracellular cytokine staining, cells were activated with phorbol 12-myristate 13-acetate (PMA; 40 ng/mL; Sigma), ionomycin (I; 500 ng/mL; Sigma), and GolgiStop (BD Biosciences) for 5 hours. Cells were stained with viability dye and surface markers CD3, CD4, CD8, CD45RO, and CCR6, then fixed, permeabilized (eBioscience kit), and stained with antibodies against interleukin (IL) 2, interferon gamma (IFN-γ), and IL-17A, then analyzed using an LSRII flow cytometer (BD Bioscience) and FlowJo software (Tree Star).

T-Cell Purification and Activation for PD-1 Sort Experiments

PBMCs were isolated from anonymous leukopaks obtained from the New York Blood Center under an institutional review board–approved protocol. CD4$^+$ T cells were isolated from PBMCs using Dynal CD4 Positive Isolation kit (Invitrogen) and were >99% pure. CD4$^+$ T cells were sorted into PD-1$^-$ and PD-1$^+$ memory cells (memory gate included CD45RO$^+$ and CR45RO-CD27$^-$ cells) with a FACSAria cell sorter (BD Bioscience). Monocytes were isolated using Dynal CD14 Positive Isolation kit (Invitrogen) or CD14 MicroBeads (Miltenyi Biotec), then cultured with granulocyte macrophage colony-stimulating factor (50 ng/mL) and IL-4 (50 ng/mL) for 3 days to generate dendritic cells. For proliferation experiments, cells were labeled with CFSE dye (Invitrogen), activated with soluble anti-CD3 (50 ng/mL clone OKT3, ATCC) and monocyte-derived dendritic cells (1:5 ratio), cultured for 4 days, then analyzed for proliferation by flow cytometry.

Proliferation and PD-1 Blockade Experiments in HIV-Infected Children

PBMCs were labeled with CellTrace Violet Cell Proliferation kit (Invitrogen), then stimulated with soluble anti-CD3 or HIV-specific pooled Gag peptide (2 μg/mL) and CD28/CD49 (1 μg/mL). HIV Gag peptides were obtained from the National Institutes of Health AIDS Reagents program (number 12437) as a peptide set containing 15 aa length peptides that are potential T-cell determinants [25]. On day 6–7, cells were stained with viability dye and surface markers CD3 and CD4, then analyzed by flow cytometry. For blockade experiments, cells were incubated with anti-PD-1 (clone EH12.2H7, 10 μg/mL) or isotype control antibodies (mouse IgG1, κ clone MG1-45, 10 μg/mL) for 30 minutes before stimulation.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism software. For comparison of multiple groups of subjects, the

Table 1. Subject Characteristics

Characteristic	HIV-Negative	ART-Naive	On ART	*P* Value
No.	40	38	33	
Age[a]	11 (9–15)	11 (8–14)	12 (8–13)	NS[b]
Female sex, No. (%)	17 (42)	22 (58)	20 (60)	NS[c]
CD4 percentage[a]	38 (33–42)	25 (16–28)	32 (26–39)	*P* < .0001[b]
Log HIV copies/mL[a]		4.9 (4.3–5.3)	2 (2–2) Undetectable: n = 27 (81%)	*P* < .0001[d]

Abbreviations: ART, antiretroviral therapy; HIV, human immunodeficiency virus; NS, not significant.

[a]Median values with interquartile range.

[b]Kruskal-Wallis test.

[c]χ^2 test.

[d]Two-sided Mann–Whitney test.

Kruskal–Wallis test followed by Dunn multiple comparisons test was performed. Multiple time points were evaluated with Wilcoxon matched-pairs signed-rank test. Correlations were assessed with the Spearman rank test. For linear regression analysis, the percent of proliferating cells was the dependent variable, and PD-1$^+$ memory CD4 T-cell percentage was the independent variable. Comparisons between PD-1$^-$ and PD-1$^+$ populations were performed with the ratio paired *t* test. Threshold of significance for all tests was .05.

RESULTS

HIV-Infected Children Have Elevated PD-1$^+$ Memory CD4 T Cells That Decrease With ART

Because PD-1 is mostly expressed on memory rather than naive CD4 T cells (Supplementary Figure 1*A*), we determined PD-1 expression on memory CD4 T cells (CD4 T_M), defined as the sum of central (T_{CM}, CD45RO$^+$CCR7$^+$), effector (T_{EM}, CD45RO$^+$CCR7$^-$), and RA$^+$ effector memory (EMRA) (T_{EMRA}, CD45RO$^-$CCR7$^-$) populations (Figure 1A). Both ART$^-$ and ART$^+$ subjects had significant increases in the percentage of PD-1$^+$ CD4 T_M compared with HU children ($P < .0001$ and $P = .02$, respectively; Figure 1A). Similarly, PD-1 mean fluorescence intensity in CD4 T_M was higher in HIV$^+$ than in HU children (Figure 1B). There was no correlation between age and PD-1$^+$ CD4 T_M in HU or HIV$^+$ children (Figure 1C). PD-1$^+$ frequencies in memory CD8 T cells were high in ART$^-$ but not ART$^+$ children compared with HU children (Supplementary Figure 1*B*). In ART$^-$ children, treatment initiation significantly lowered the frequency of PD-1$^+$ CD4 T_M, within 1 year ($P = .001$; Figure 1D). However, PD-1 levels remained higher than HU at 6 and 12 months post-ART ($P < .0001$; Supplementary Figure 1*C*). Longer durations of treatment in the ART$^+$ cohort were not associated with lower PD-1 expression in memory CD4 T cells (Supplementary Figure 1*D*).

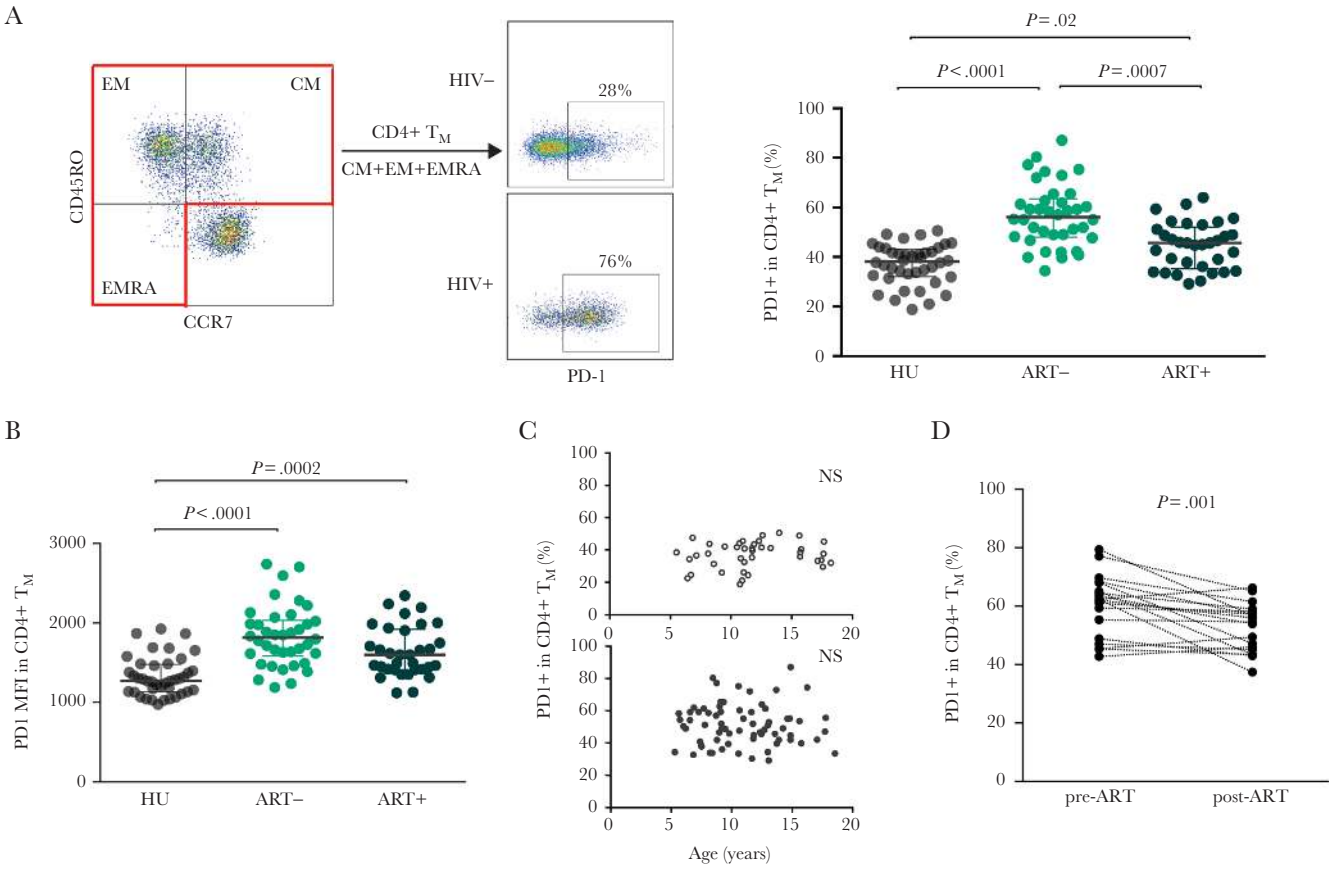

Figure 1. Increased frequency of PD-1$^+$ CD4 memory T cells in human immunodeficiency virus (HIV)–infected children declines with antiretroviral therapy (ART). *A*, Gating strategy to identify CD4 memory T cells based on CD45RO and CCR7 surface expression. Representative flow plots of cell surface PD-1 expression within CD4$^+$ memory T cells in an HIV-uninfected and an HIV-infected child are shown. The percentage of PD-1-expressing CD4$^+$ memory T cells from children aged 5–18 years in the following 3 categories are shown: HIV-unexposed (HU), HIV-infected ART-naive (ART$^-$), and HIV-infected on ART (ART$^+$). *B*, Mean fluorescence intensity of PD-1 in CD4$^+$ memory T cells in HU, ART$^-$, and ART$^+$ children. *C*, PD-1$^+$ memory CD4 T cells graphed vs age in HIV-uninfected (open circles) and HIV-infected (closed circles) children. *D*, PD-1$^+$ memory CD4 T-cell frequencies before (pre-ART) and 12 months after ART (post-ART). *P* values were calculated using Kruskal–Wallis test followed by the Dunn posttest for multiple comparisons and Wilcoxon matched-pairs signed-rank test for paired analysis. Bars on scatterplots represent median values with the interquartile range. Abbreviations: ART, antiretroviral therapy; CM, central memory; EM, effector memory; EMRA, RA+ effector memory; HIV, human immunodeficiency virus; HU, human immunodeficiency virus unexposed; MFI, mean fluorescence intensity; NS, not significant; T_M, memory T cell.

We next asked which memory subset accounted for high PD-1 levels in total memory CD4 T cells. PD-1 expression was increased in T_{CM} of ART$^-$ ($P < .0001$) and ART$^+$ ($P = .002$) children compared with HU children (Figure 2A). ART$^-$ children also had higher PD-1 expression in T_{EM} and T_{EMRA} compared with HU children (Figure 2A). HIV$^+$ children did not have similar increases in T_{CM}, T_{EM}, and T_{EMRA} compared to HU children (Supplementary Figure 2*A*). In each subject category, T_{CM} and T_{EM} comprised nearly equal proportions of the total PD-1$^+$ CD4 T_M population (Figure 2B). PD-1$^+$ CD4 T_M coexpressed minimal levels of other inhibitory molecules, CD160, 2B4, and TIM3, which were similar between HU and HIV$^+$ children (Supplementary Figure 2*B*).

PD-1 Expression on Memory CD4 T Cells Correlates With Disease Progression in HIV-Infected Children

We determined correlations between PD-1$^+$ CD4 T_M and clinical markers of disease progression. In HIV$^+$ subjects, PD-1$^+$ CD4 T_M frequency directly correlated with HIV viral load ($P = .02$; Figure 3A) in ART$^-$ children, and inversely correlated with CD4 percentage ($P < .0001$; Figure 3B) and CD4:CD8 ratio ($P < .0001$; Figure 3C) in HIV$^+$ children. These correlations were not present in HU (Supplementary Figure 3*A*). Immune activation markers CD38 and HLA-DR predict HIV disease progression [26]. The percentage of PD-1$^+$ CD4 T_M significantly correlated with CD38$^+$HLA-DR$^+$ CD8 ($P < .0001$) and CD4 T cells ($P < .0001$) and CD38$^+$CD45RO$^+$ CD4 T cells ($P = .003$; Figure 3D). There was no correlation between the PD-1$^+$ CD4 T_M and markers of immune activation in HU children (Supplementary Figure 3*B*).

PD-1$^+$ Memory CD4 T Cells Are Hypoproliferative and Predict Low Proliferative Capacity in HIV-Infected Children

We first compared proliferative potentials between PD-1$^-$ and PD-1$^+$ CD4 T cells isolated from anonymous leukopaks, due to limitations in blood volumes obtained from children. Sorted PD-1$^+$ and PD-1$^-$ memory CD4 T cells were activated through the TCR with anti-CD3, then assessed for proliferation. PD-1$^+$ CD4 T_M proliferated more than 2.5-fold less than PD-1$^-$ CD4 T_M from the same donor (Figure 4A and 4B). Next, we examined proliferative responses in relation to PD-1 expression on CD4 T cells in HIV$^+$ children (Supplementary Table 2). Total PBMCs were activated ex vivo with an antigen-independent stimulus using anti-CD3 antibody or an HIV-specific stimulus via pooled HIV Gag peptides (HIV Gag), then measured for proliferation of total CD4 T cells on day 6 (Figure 4C). Remarkably, higher baseline PD-1$^+$ CD4 T_M levels predicted decreased proliferative capacity within total CD4 T cells after anti-CD3 ($P = .02$; $R^2 = 0.54$) and HIV-specific stimulation ($P = .04$; $R^2 = 0.55$; Figure 4D). Notably, the frequency of T_{CM} or T_{EM} in memory CD4 T cells did not predict the proliferative potential of CD4 T cells after either stimulus (Supplementary Figure 4).

PD-1 Blockade Fails to Raise Proliferative Capacity of CD4 T Cells in HIV-Infected Children

We next evaluated whether blockade of PD-1 restored proliferative functions of CD4 T cells in HIV$^+$ children. The addition

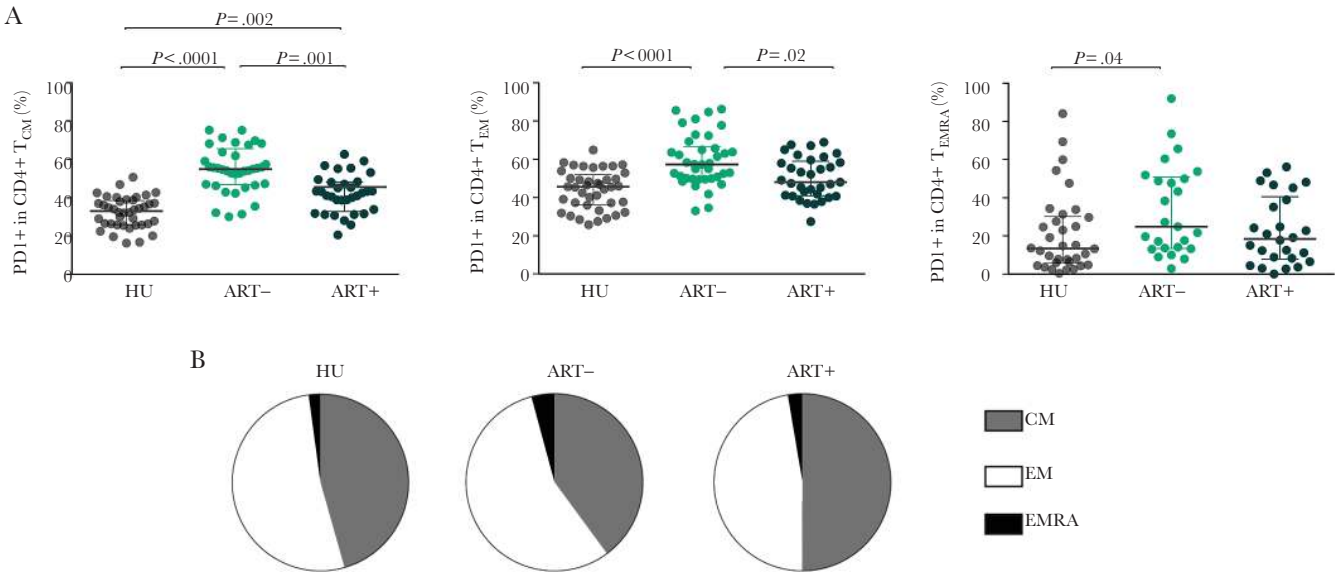

Figure 2. PD-1$^+$ CD4 T cells are central memory (CM) and effector memory (EM) CD4 T cells (T_{CM} and T_{EM}, respectively). *A*, The percentage of PD-1$^+$ cells in central (T_{CM}: CD45RO$^+$CCR7$^+$), effector (T_{EM}: CD45RO$^+$CCR7$^-$), and RA+ effector (EMRA) (T_{EMRA}: CD45RO$^-$CCR7$^-$) memory CD4 T cells in human immunodeficiency virus–unexposed (HU), antiretroviral therapy–naive (ART$^-$), and on-ART (ART$^+$) children. *B*, Pie charts demonstrating the mean proportion of T_{CM}, T_{EM}, and T_{EMRA} populations within PD-1$^+$ memory CD4 T cells in HU, ART$^-$, and ART$^+$ children. *P* values were calculated using Kruskal–Wallis test followed by the Dunn posttest for multiple comparisons. Bars on scatterplots represent median values with the interquartile range.

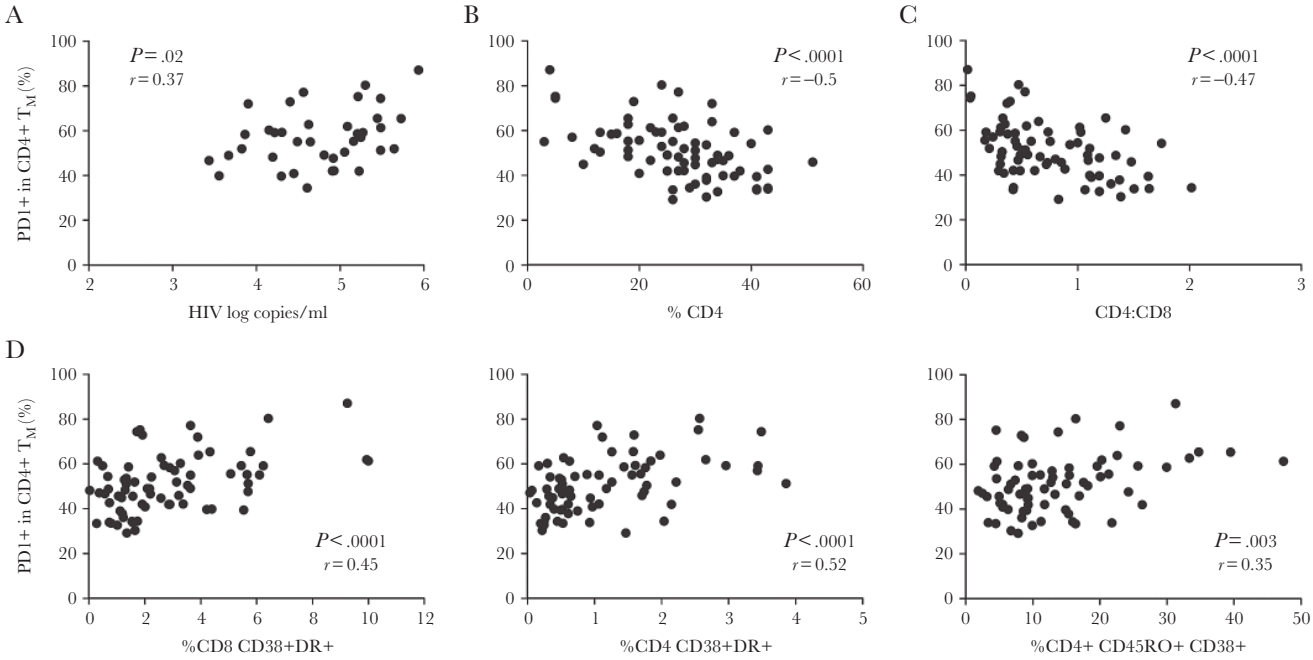

Figure 3. PD-1 expression on CD4 memory T cells (T_M) correlates with disease progression in human immunodeficiency virus (HIV)–infected children. The percentage of PD-1–expressing $CD4^+$ memory T cells directly correlates (*A*) with HIV RNA load (log copies/mL), and inversely correlates (*B*) with CD4 percentage and CD4:CD8 ratio (*C*). Peripheral blood mononuclear cells were stained with surface markers CD3, CD4, CD8, CD45RO, and activation markers CD38 and HLA-DR. The percentage of PD-1–expressing $CD4^+$ memory T cells directly correlates with the percentage of $CD38^+DR^+$ (*D*) CD8 and CD4 T cells as well as with $CD45RO^+CD38^+$ CD4 T cells. Statistical analysis was performed using Spearman correlation test.

of a monoclonal antibody to PD-1 to PBMC cultures with anti-CD3 maintained blockade of the PD-1 receptor for 8 days after stimulation (Supplementary Figure 5*A*). However, the percentage of proliferating CD4 T cells was similar between anti–PD-1 and isotype control antibodies after anti-CD3 (Figure 5A and 5B) and HIV Gag (Figure 5C and 5D) stimulation, despite high PD-1 expression. PD-1 blockade did not result in higher cell death, as the frequencies of live cells were similar between the isotype control and anti–PD-1 antibodies (Supplementary Figure 5*B*).

PD-1+ Memory CD4 T Cells Produce Inflammatory Th1 and Th17 Cytokines

In $PD\text{-}1^-$ and $PD\text{-}1^+$ sorted memory CD4 T cells from healthy adults that were stimulated with PMA/I, the $PD\text{-}1^+$ subset produced significantly less IL-2 and more of the inflammatory cytokine IFN-γ ($P = .007$ and $P = .0475$; Figure 6A) compared to the $PD\text{-}1^-$ memory CD4 T cells. Next we evaluated IL-17A production within $CCR6^+$ $PD\text{-}1^-$ and $PD\text{-}1^+$ cells, as all Th17 express CCR6. $PD\text{-}1^+$ $CD45RO^+CCR6^+CD4$ T cells had markedly higher IL-17A levels ($P = .007$) compared to their $PD\text{-}1^-$ counterpart (Figure 6A).

In HIV^+ children, we examined ex vivo cytokine production in $PD\text{-}1^+$ and $PD\text{-}1^-$ gated $CD45RO^+$ CD4 T cells by flow cytometry (Figure 6B). Activation with PMA/I did not significantly change PD-1 expression in HIV^+ children (Figure 6B), whereas it increased PD-1 levels in HU children (Supplementary Figure 6*A*). $PD\text{-}1^+$ $CD45RO^+CD4^+$ T cells from HIV^+ children also produced significantly less IL-2 ($P = .003$; Figure 6C) and significantly more IFN-γ ($P < .0001$; Figure 6C) and IL-17A ($P = .0003$; Supplementary Figure 6*B*) compared with $PD\text{-}1^-$ cells. CCR6 expression did not differ between $PD\text{-}1^-$ and $PD\text{-}1^+$ cells (Supplementary Figure 6*C*), and IL-17A was >2-fold higher in $PD\text{-}1^+$ $CD45RO^+CCR6^+$ cells compared with the $PD\text{-}1^-$ subset ($P = .0003$; Figure 6C). HU subjects also had higher IFN-γ and IL-17A production in $PD\text{-}1^+$ CD4 T cells (Supplementary Figure 6*D*). To confirm our findings in the entire HIV^+ cohort, we evaluated whether PD-1 expression on CD4 T_M correlated with cytokine production by total $CD45RO^+CD4^+$ T cells. Indeed, $PD\text{-}1^+$ CD4 T_M frequencies correlated inversely with IL-2 ($P = .008$; Figure 6D) and directly with IFN-γ and IL-17A levels in CD4 T cells ($P = .005$ and $P = .02$; Figure 6D). In HU, there were no significant correlations between $PD\text{-}1^+$ CD4 T_M frequencies and cytokines (Supplementary Figure 6*E*). PD-1 blockade with anti-PD-1 antibodies before stimulation with PMA/I or HIV Gag did not alter IFN-γ or IL-17A levels in $CD45RO^+$ CD4 T cells of HIV^+ children (Supplementary Figure 7).

DISCUSSION

In this study we demonstrate that perinatally-infected HIV^+ children aged 5–18 years have remarkable elevations in the frequency of $PD\text{-}1^+$ memory CD4 T cells that lower with ART, but fail to normalize. These cells are comprised of

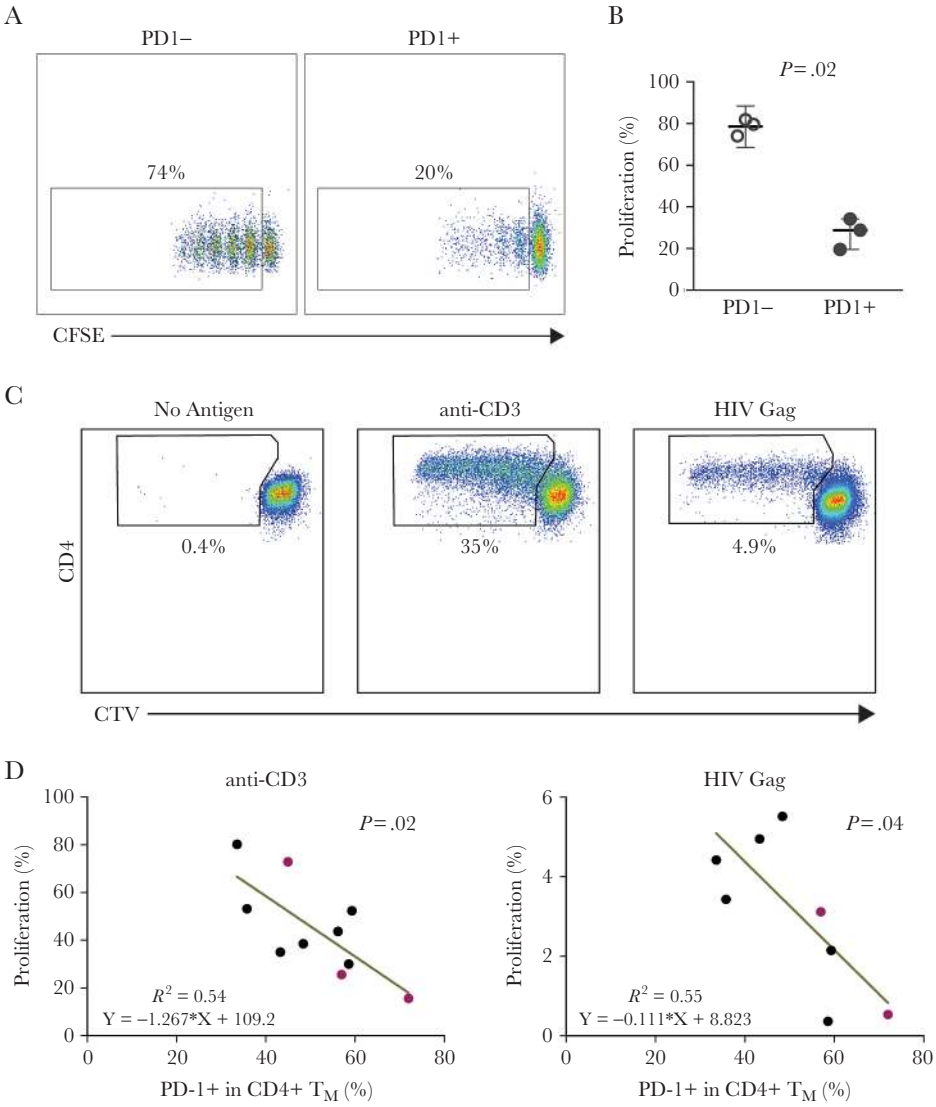

Figure 4. PD-1+ CD4 memory T cells (T_M) have decreased proliferative capacity in healthy adults and predict proliferative capacity in human immunodeficiency virus (HIV)–infected children. Peripheral blood mononuclear cells (PBMCs) from healthy adult donors were sorted into PD-1− and PD-1+ memory CD4 T-cell subsets by live cell sorting. *A* and *B*, Carboxyfluorescein succinimidyl ester (CFSE)-labeled PD-1− and PD-1+ CD4 memory T cells were stimulated with anti-CD3 (Clone OKT3) and monocyte-derived dendritic cells; cell proliferation was assessed on day 4. *A*, Representative flow plots show percent of proliferating cells measured by dilution of CFSE in PD-1− and PD-1+ CD4 memory T cells sorted from the same donor. *B*, Paired comparison of proliferation between PD-1− and PD-1+ sorted memory CD4 T cells from 3 adult donors. *P* value was calculated with a ratio paired *t* test. *C*, PBMCs from HIV-infected children were labeled with CellTrace Violet then either left unstimulated (no antigen), or stimulated with anti-CD3 or HIV Gag peptide pool. Representative flow plots of proliferation of CD4+ T cells on day 7 are shown from 1 donor. *D*, Linear regression analysis of baseline percentage of PD-1+ CD4 memory T cells vs the percentage of proliferating CD4 T cells after anti-CD3 and HIV Gag peptide stimulation. Triangles indicate viremic subjects.

central and effector memory subsets and correlate with HIV disease progression, measured by viral load, CD4 percentage, CD4:CD8 T-cell ratio, and immune activation. In HIV+ children, PD-1+ memory CD4 T cells predict both HIV-specific and -nonspecific proliferative capacity of total CD4 T cells. Despite weak proliferative potential, PD-1+ memory CD4 T cells preferentially produce Th1 and Th17 cytokines. Last, blockade of PD-1 with neutralizing antibodies failed to restore HIV-specific and -nonspecific proliferative responses or to dampen inflammatory cytokine production in HIV+ children.

Our findings of increased levels of PD-1+ CD4 T cells in HIV+ children that correlate with HIV disease progression are consistent with previous studies of HIV+ adult subjects [9, 12, 14, 15]. The significant correlations between PD-1+ CD4 T cells and immune activation markers raise the question whether PD-1+ CD4 T cells represent exhausted T cells or a recently activated T-cell population in HIV+ children. In 2 different cohorts of HIV+ children from the United Kingdom [22] and Uganda [23], PD-1 expression on CD8 T cells was positively associated with CD38+HLA−DR+ CD8 T cells; however, there are no similar pediatric reports of PD-1+ CD4 T cells. Interestingly,

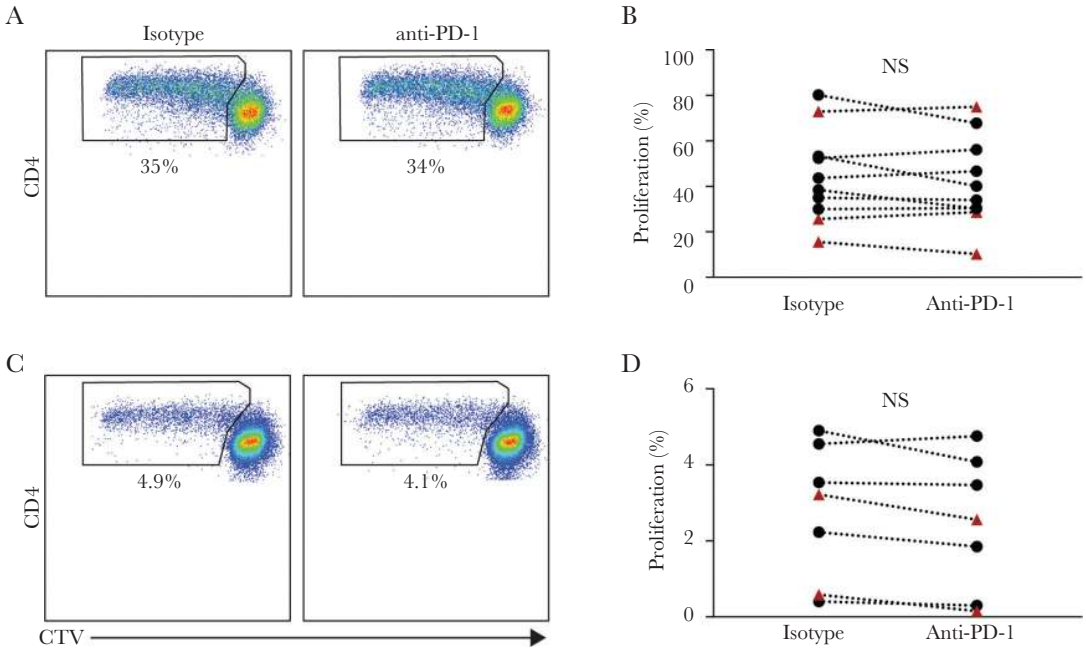

Figure 5. Proliferative capacity of CD4 T cells is not reversible by PD-1 blockade in human immunodeficiency virus (HIV)–infected children. CellTrace Violet (CTV)–labeled peripheral blood mononuclear cells from HIV-infected children were stimulated with anti-CD3 or HIV Gag peptide pool in the presence of PD-1 blocking antibody or isotype control antibody. Flow cytometry from a representative donor indicating the percentage of CTV^{dim} $CD4^+$ T cells after 7 days of anti-CD3(*A*) or HIV Gag (*C*) peptide stimulation is shown. Graphs depict summary of proliferative responses to anti-CD3 (*B*) and HIV Gag (*D*) peptide pool in the presence of PD-1 blocking antibody in HIV-infected children. Statistical analysis was performed using the ratio paired *t* test. Triangles indicate viremic subjects.

PD-1 expression after activation by PMA/I remained stable in HIV^+ children but increased in HU children, suggesting that a portion of $PD\text{-}1^+$ memory CD4 T cells in HIV disease is activation-induced. Additional markers are necessary to discern between recently activated and exhausted $PD\text{-}1^+$ CD4 T cells. Although subjects with recent viral illness or active coinfections were excluded from our study, the dynamics of PD-1 expression during and after acute infection remain poorly understood. Another potential explanation for the correlation between $PD\text{-}1^+$ CD4 T cells and immune activation derives from the recent report of a viral reservoir within $PD\text{-}1^+$ CD4 T cells [16]. Nearly half of $PD\text{-}1^+$ CD4 T cells from HIV^+ children are T_{CM}, which contains a viral reservoir in children [27, 28]. Although speculative, it is conceivable that a portion of this reservoir may intermittently replicate and trigger T-cell activation.

In our cohort of HIV^+ children, more than half of memory CD4 T cells were $PD\text{-}1^+$, yet HIV-specific cells account for only 1%–5% of the total CD4 T-cell population in chronic HIV infection [17]. Moreover, in healthy children, nearly 40% of memory CD4 T cells expressed PD-1. What functional role does PD-1 play on HIV-nonspecific T cells or in healthy hosts under physiologic conditions? Our experiments on $PD\text{-}1^+$ and $PD\text{-}1^-$ sorted populations from healthy adults demonstrated >2.5-fold lower proliferative potential in $PD\text{-}1^+$ CD4 T cells after stimulation with anti-CD3 antibodies. While previous reports describe hypoproliferative $PD\text{-}1^+$ CD4 T cells in the context of chronic viral infections [9, 15], to our knowledge this is the first report demonstrating a marked difference in proliferative capacity between $PD\text{-}1^+$ and $PD\text{-}1^-$ sorted CD4 T cells in response to TCR stimulation in healthy adults. Remarkably, $PD\text{-}1^+$ CD4 T_M frequencies in HIV^+ children similarly predicted both HIV-specific and -nonspecific CD4 T-cell proliferative responses. Whether this muted proliferative potential derives from an exhausted or terminally differentiated state is unclear. Alternatively, regulatory T cells expressing PD-1, which we previously reported [29], may exert suppressive functions leading to impaired proliferation.

In studies of adults, blockade of the PD-1 pathway increased HIV-specific CD4 T-cell proliferation [9, 12, 13, 15], yet in HIV^+ children, PD-1 blockade failed to raise proliferative responses to both anti-CD3 antibodies and HIV Gag peptides. Of note, most adult studies used blocking antibodies against PD-L1, whereas we used monoclonal antibodies to PD-1, as the majority of currently US Food and Drug Administration–approved PD-1-modulating antibodies target the receptor itself. However, PD-L1 blockade in a small subset of HIV^+ children replicated our results with PD-1 antibodies and had no effect on proliferative capacity (data not shown). While PD-1 blockade generally yielded larger effects in untreated viremic adult subjects [13, 15], PD-1 blockade failed to rescue proliferative capacity in both viremic and ART-suppressed children. Early studies of PD-L1 interactions with PD-1 on human CD4 T cells

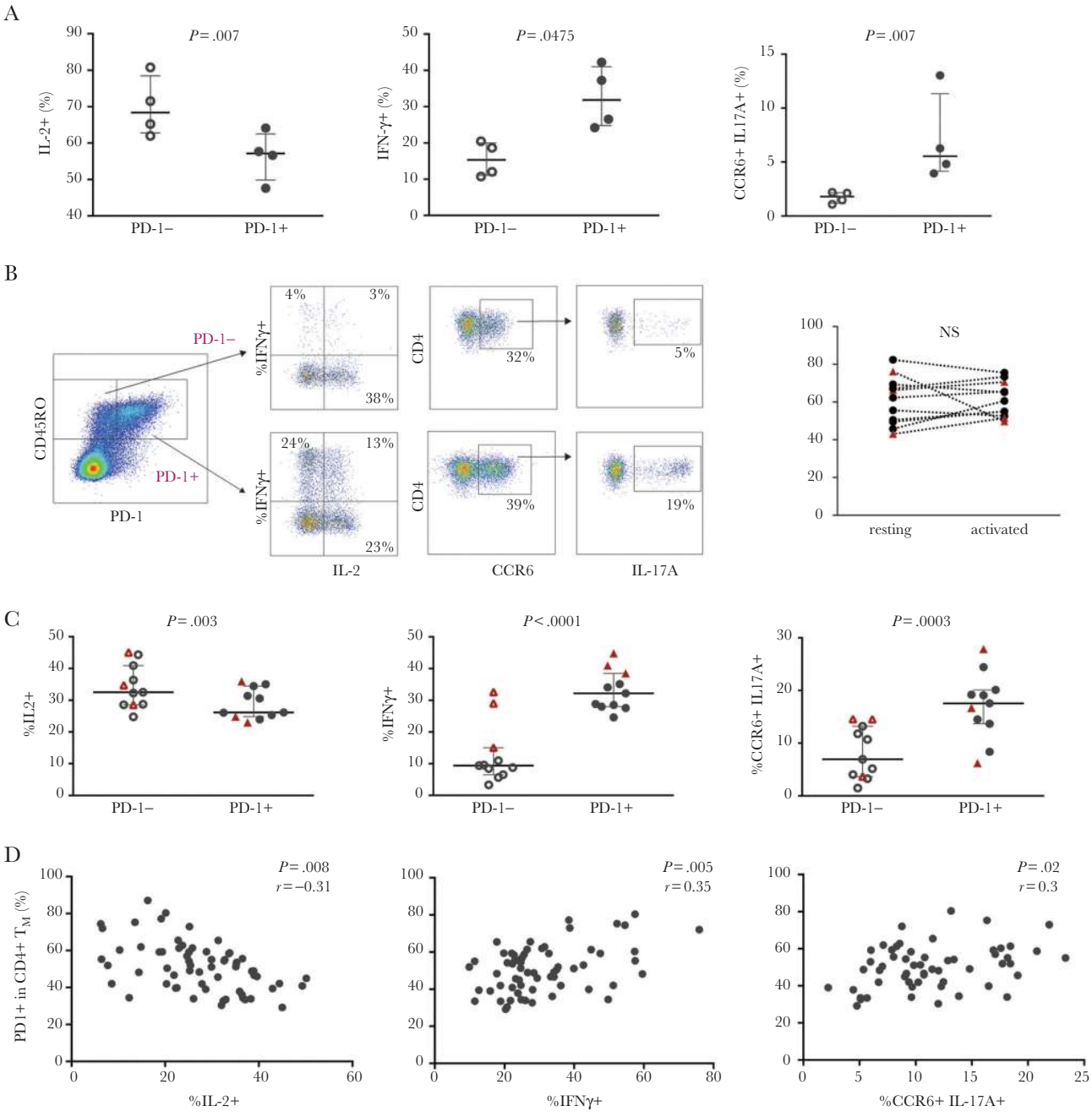

Figure 6. PD-1⁺ CD4 memory T cells preferentially produce Th1 and Th17 inflammatory cytokines. *A*, PD-1⁻ and PD-1⁺ sorted populations from healthy adults were stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin in the presence of GolgiStop for 5 hours, then assessed for intracellular cytokine production. Cumulative data comparing cytokine levels between PD-1⁻ and PD-1⁺ sorted memory CD4 T cells are shown for 4 donors. *B*, Total peripheral blood mononuclear cells from human immunodeficiency virus (HIV)–infected children were activated with PMA and ionomycin in the presence of GolgiStop for 5 hours, then evaluated for cytokine production by intracellular cytokine staining. Gating strategy to identify cytokines within PD-1⁺ and PD-1⁻ populations is shown. Interleukin (IL) 2 and interferon gamma (IFN-γ) were gated within PD-1⁻ and PD-1⁺ CD45RO⁺ CD4 T cells. IL-17A was gated within PD-1⁻ and PD-1⁺ CD45RO⁺CCR6⁺ CD4 T cells. Comparison of PD-1 expression before and after activation in HIV-infected children is shown on the right. *C*, Comparisons of cytokine levels between PD-1⁻ and PD-1⁺ memory CD4 T cells (T_M) in HIV-infected children are shown. Triangles indicate viremic subjects. *D*, Correlation graphs between resting PD-1⁺ memory CD4 T-cell frequency and total cytokine production by memory CD4 T cells. Statistical analysis was performed using the ratio paired *t* test (*A* and *C*), Wilcoxon signed-rank matched pairs test (*B*), and Spearman correlation test (*D*). Bars on scatterplots represent median values with the interquartile range.

demonstrated that PD-L1 failed to inhibit proliferation under saturating conditions of TCR activation with anti-CD3/CD28 [30]. Due to limited blood volumes obtained from children, our experiments were optimized on adult PBMCs. It is conceivable that our ex vivo stimuli may have been too strong to overcome with blockade because optimal antigen concentrations differ

between the developing pediatric and mature adult immune systems. Indeed, immune development in perinatally infected HIV$^+$ children amidst chronic antigenic stimulation may trigger irreversible T-cell exhaustion. Recently, Corneau et al characterized PD-1$^+$ CD4 T cells in healthy and HIV$^+$ adults by large multiplex mass cytometry, and demonstrated that PD-1$^+$ CD4 T cells were predominantly T_{EM} and T_{EMRA} with limited expression on T_{CM} [31]. In HIV$^+$ children, we found a nearly equal divide between T_{CM} and T_{EM} in PD-1$^+$ CD4 T cells; the functional effects of anti-PD-1 may differ between T_{CM} and T_{EM} subsets.

While producing significantly less IL-2 consistent with their proliferative dysfunction, PD-1$^+$ CD4 T cells preferentially produced inflammatory Th1 and Th17 cytokines in both healthy adults and HIV$^+$ children, suggesting these cells retain robust effector properties. Porichis et al also demonstrated the PD-1^{hi} sorted population was the main cytokine-secreting population in chronically HIV$^+$ adults [15]. A more recent report revealed that a subset of early differentiated CD27^{high}CD45RA^{low}CD127^{high} PD-1$^+$ cells are highly functional with elevated cytokine production [14]. While PD-1 blockade consistently restores HIV-specific T-cell proliferation in studies of adults, results vary on whether blockade also restores cytokine production. In HIV$^+$ children, anti–PD-1 antibodies did not alter cytokine production in CD4$^+$ T cells. Previous studies that report increased inflammatory cytokine secretion after PD-1 blockade [15, 32] measured cytokine levels in the supernatant and observed significant variability among subjects. D'Souza et al demonstrated no difference in cytokine production by HIV-specific CD4 T cells with intracellular cytokine staining assays after PD-1 blockade [12], similar to our findings.

Interestingly, in a comparison of the gene profiles of PD-1^{hi} CD8 T cells in healthy vs HIV$^+$ adults, Duraiswamy et al demonstrated that the gene signature of PD-1^{hi} CD8 T cells in healthy subjects did not match the exhausted gene profile of PD-1^{hi} CD8 T cells from HIV$^+$ adults, suggesting that PD-1^{hi} CD8 T cells in healthy humans represent effector memory cells rather than exhausted cells [33]. In PD-1$^+$ CD4 T cells, we found identical proliferative and cytokine functions in healthy adults and HIV$^+$ children, warranting further investigation of genetic and transcriptional signatures of PD-1$^+$ CD4 T cells in children. Most importantly, our data demonstrate that PD-1 expression alone does not identify exhausted CD4 T cells. Rather PD-1$^+$ CD4 T cells in children comprise a heterogeneous central and effector memory population and likely mark differentiated, exhausted, and recently activated CD4 T cells.

In summary, both treated and untreated HIV$^+$ children have significantly increased frequencies of PD-1$^+$ memory CD4 T cells that correlate with HIV disease progression and immune activation. In healthy adults, PD-1$^+$ CD4 T cells are hypoproliferative after TCR stimulation, and in HIV$^+$ children frequencies of these cells predict HIV-specific and -nonspecific proliferative capacity of CD4 T cells. Despite proliferative defects, PD-1$^+$ memory CD4 T cells preferentially produce Th1 and Th17 cytokines, IFN-γ and IL-17A, respectively. Last, in HIV$^+$ children, PD-1 blockade fails to restore proliferative potential or dampen cytokine production. Together, these findings demonstrate that PD-1$^+$ CD4 T cells are a heterogeneous subset of central and effector memory T cells with impaired proliferative capacity, yet with the potential to secrete proinflammatory cytokines. These cells are elevated in children with perinatally acquired HIV infection, correlate with disease progression, and are unresponsive to in vitro blockade. This study highlights a need for further investigation into the precise role of the PD-1 pathway in HIV$^+$ children in light of recent phase 1 clinical trials of PD-1 immune modulators in HIV$^+$ adults [34].

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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