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CD4⁺CD73⁺ T cells are associated with lower T-cell activation and C reactive protein levels and are depleted in HIV-1 infection regardless of viral suppression

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Abstract

Background—The role of the adenosine (ADO) suppression pathway, specifically CD39-expressing and CD73-expressing CD4⁺ T cells in HIV-1 infection is unclear.

Methods—We evaluated the frequency and numbers of CD4⁺CD39⁺ and CD4⁺CD73⁺ T cells, activated T cells, and plasma C reactive protein (CRP) levels in 36 HIV-1-positive individuals and 10 normal controls (NC). Low-level plasma viremia was evaluated using single copy assay. Mass spectrometry was used to measure hydrolysis of ATP by ectoenzyme-expressing CD4⁺ T cells, whereas cyclic adenosine monophosphate (cAMP) levels were measured using enzyme immunoassay. Suppression of T-cell function by exogenous ADO and CD4⁺CD73⁺ T cells was tested by flow cytometry.

Results—CD39 and CD73 are expressed in different CD4⁺ T-cell subsets. CD4⁺CD73⁺ T cells do not express CD25 and FOXP3, and their frequency and numbers were lower in HIV-1-positive individuals regardless of virologic suppression ($P = 0.005$ and $P < 0.001$, respectively). CD4⁺CD73⁺ numbers inversely correlated with CD4⁺CD38⁺DR⁺ ($P = 0.002$), CD8⁺CD38⁺DR⁺ T-cell frequency ($P = 0.05$), and plasma CRP levels ($P = 0.01$). Both subsets are required for hydrolysis of exogenous ATP to ADO and can increase CD4⁺ T-cell cAMP levels when incubated with exogenous ATP. Low-level viremia did not correlate with activated T-cell frequency. *In vitro*, ADO suppressed T-cell activation and cytokine expression. CD4⁺CD73⁺ T cells suppressed T-cell proliferation only in the presence of exogenous 5'-AMP.

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Conflicts of interest

No conflicts of interest are declared.

Conclusion—The ADO-producing CD4⁺CD73⁺ subset of T cells is depleted in HIV-1-positive individuals regardless of viral suppression and may play a key role in controlling HIV-1-associated immune activation.

Keywords

adenosine; CD73; HIV; immune activation; suppression

Introduction

Effective antiretroviral therapy (ART) has led to a significant increase in the life expectancy of individuals with HIV-1 infection [1]. Despite this improvement, mortality rates in HIV-1-infected individuals on ART are still higher than those in the general population [2,3]. In HIV-1-infected individuals, persistent immune activation has been associated with disease progression [4,5]. It is hypothesized that the increased risk for end-organ diseases [6–8] and death is also due to persistent immune activation [9]. Moreover, increased frequency of activated immune cells has been linked to early senescence and increased morbidity in HIV-1 infection [10–12].

Excessive immune activation in HIV-1 infection has been attributed to persistence of the virus in the circulation [13]. However, chronic immune activation is also seen in individuals with undetectable plasma viremia. Thus, immune activation could alternatively be due to the preferential depletion of CD4⁺ T cells in the gut mucosa by HIV-1 infection, leading to enhanced translocation of microbial products to the circulation [14–16]; reactivation of other viruses such as cytomegalovirus [17]; and dysregulation of the T-cell homeostasis [18].

The role of regulatory T cells (Treg) in HIV-1-associated immune activation and disease progression remains unclear. It has been shown that Treg can control HIV-1 virus replication in conventional T cells (T_{conv}) but also suppress functions of activated T_{conv} [19,20]. Moreover, HIV-1-infected individuals have an increased frequency of Treg, which correlates with progressive disease [19,21]. The Treg subset responsible for these effects consists of CD4⁺CD39⁺CD25⁺FOXP3⁺ T cells. CD39 is an ectoenzyme, which, in concert with CD73, hydrolyzes ATP to adenosine (ADO), an important regulatory factor in inflammation [22]. Although murine Treg express CD39 and CD73 [23,24], few human CD4⁺ T cells (< 0.1%) coexpress both enzymes on the cell surface [25]. Recently, the CD39/ADO axis has been linked to Treg-mediated suppression in cancer as well as inhibition of HIV-1 replication [20,26]. However, little is known about CD4⁺ T-cell expression of CD73 in HIV-1 infection.

We hypothesized that extracellular ADO-mediated suppression plays a role in HIV-1-associated immune activation and that CD4⁺CD73⁺ T cells are essential in this immunoregulatory mechanism. We, therefore, conducted a cross-sectional study within the Multicenter AIDS Cohort Study (MACS) to seek evidence that CD4⁺CD73⁺ T cells regulate activation of T cells in the blood of HIV-1-positive individuals. We show that CD4⁺CD73⁺ T-cell numbers were decreased in HIV-1-positive individuals regardless of viral suppression. Depletion of CD4⁺CD73⁺ T cells correlated with increased numbers of activated CD4⁺ and CD8⁺T_{conv}, suggesting that CD4⁺CD73⁺ T cells play a previously unrecognized role in regulating HIV-1-associated immune activation.

Materials and methods

Study population and collection of peripheral blood mononuclear cells

This study was part of the MACS, an investigation of the natural history of HIV-1 infection in MSM, and was approved by the University of Pittsburgh Institutional Review board (IRB#9505118). Blood was obtained from HIV-1-positive individuals ($n = 36$) and age-matched HIV-1-seronegative controls (NC; $n = 10$). HIV-1-positive individuals included ART-naïve individuals (noART; $n = 9$) and ART-treated, virally suppressed individuals with CD4⁺ T-cell counts more than 500 cells/ μ l (ART500; $n = 15$) and those with CD4⁺ T-cell counts 350 cells/ μ l or less (ART350; $n = 12$). For additional functional studies, blood was obtained from ART-treated, virally suppressed MSM (CD4⁺ T-cell counts > 500) in the Pitt Treatment and Evaluation Unit (PTEU, $n = 6$) and buffy coats from healthy anonymous donors (Central Blood Bank, Pittsburgh, USA). All participants gave written informed consent. Heparinized blood (20 ml) was used for recovery of plasma and peripheral blood mononuclear cells (PBMCs) by Ficoll-Hypaque gradient separation (GE Healthcare Bioscience, Piscataway, New Jersey, USA). EDTA-anticoagulated blood was used for single copy assays (SCAs) and for determining the frequency and absolute numbers of CD3⁺CD4⁺T cells and CD3⁺CD8⁺T cells per μ l by flow cytometry and the TetraONE system, respectively (Beckman-Coulter, Brea, California, USA).

Flow cytometry reagents

The following antihuman monoclonal antibodies (mAbs) were used for staining: CD4-PC5 (13B8.2), CD8-PE/ PC5 (SFCI21Thy2D3), CD26-PE (4EL-1C7), HLA-DR-ECD (Immu357), CD45RO-ECD (UCHL1) (Beckman Coulter); CD39-FITC/PE-Cy7 (A1), CD38-FITC (HIT2), tumor necrosis alpha (TNF- α)-eFluor450 (MAb11), FOXP3-FITC (PCH101) (eBioscience, San Diego, California, USA), CD4-AF700 (RPA-T4), interleukin 2 (IL-2)-APC (5344.111), (Biolegend, San Diego, California, USA), CD73-PE (AD2) (BD Pharmingen, San Diego, California, USA); CD25-PE (4E3) (Miltenyi, Auburn, California, USA), including their respective isotypes as negative controls. All mAbs were pretitrated to determine the optimal staining dilution.

Surface and intracellular staining for flow cytometry

PBMCs from NC and HIV-1-positive individuals (MACS) were incubated with mAbs in 50 μ l phosphate-buffered saline for 30 min at room temperature. For intracellular staining of FOXP3 and cytokines, cells were fixed and permeabilized using a commercial staining kit (eBioscience) [27]. Flow cytometry was performed using an EPICS XL-MCL and Galliosflow cytometer equipped with Expo32 and Kaluza software (Beckman Coulter). The acquisition and analysis gates were restricted to the lymphocyte gate based on forward and side scatter. At least 5×10^5 events were acquired for analysis.

Measurement of residual plasma viremia by single copy assay

Low-level residual viremia of less than 50 copies/ml of plasma was quantified by SCA as previously described [28]. Briefly, HIV-1 virions were pelleted from plasma by ultracentrifugation and digested with proteinase K. Guanidinium isothiocyanate supplemented with glycogen was added, and RNA was pelleted with isopropanol, washed with ethanol, and resuspended in Tris-HCl. The RNA was reverse transcribed to cDNA, which was then quantified by real-time PCR using primers specific for a conserved region of *Gag*.

Measurement of plasma C reactive protein

Plasma of NC and HIV-1-positive individuals was assayed for C reactive protein (CRP; Human Singleplex Bead Kit and Human Extracellular Protein Buffer Reagent Kit; Invitrogen, Grand Island, New York, USA) using the Luminex (Austin, Texas, USA) fluorescent microbead platform and the manufacturer's protocol.

Mass spectrometric analysis

CD39⁺ and CD73⁺ T-cell subsets were separated by the ectonucleotidase-specific biotinylated mAbs and anti-biotin magnetic beads using AutoMACS (Miltenyi). For determination of ATP and 5'-AMP hydrolysis, CD4⁺ T cells (either CD39⁺ or CD73⁺) were incubated with 20 μ mol/l ATP for various time periods. Controls included cells alone or ATP alone. Supernatants were collected, centrifuged, boiled to inactivate ADO-degrading enzymes, and purines were measured using liquid chromatography-tandem mass spectrometer as previously described [29].

Evaluation of intracellular cyclic adenosine monophosphate levels

CD4⁺ T cells were separated from PBMCs of NC and HIV-1-positive individuals using CD4⁺ T-cell separation kit (Miltenyi). Frequency of CD4⁺CD39⁺ and CD4⁺CD73⁺ T cells were determined. Cells were cultured in complete Roswell Park Memorial Institute (RPMI) medium for 48 h with anti-CD3/CD28 microbeads in the presence of IL-2 (150 IU/ml), washed, placed in a 96-well plate at 10⁶ cells/well, and incubated with or without ATP (50 μ mol/l). Concentration of intracellular cyclic adenosine monophosphate (cAMP) was measured using Amersham cAMP Enzyme Immunoassay Biotrak System (GE Healthcare).

In-vitro suppression of T cells by adenosine

PBMCs (10⁶) from NC or HIV-1-positive individuals in the ART500 group were activated with Staphylococcal enterotoxin B (SEB, 1 μ g/ml; Sigma-Aldrich, St Louis, Missouri, USA) in the presence of ADO (0.1–1 mmol/l) in RPMI medium for 6–12 h at 37°C. T-cell immune activation and expression of TNF- α and IL-2 were measured using flow cytometry. Cells were stained as described above. Brefeldin A (10 μ g/ml, Sigma-Aldrich) was added for cytokine measurements. The ADO-receptor antagonist DPSPX (100 μ mol/l, Sigma-Aldrich) was added for blocking experiments.

CD4⁺CD73⁺ suppression assays

For suppression of T-cell immune activation and TNF- α and IL-2 secretion, PBMCs were obtained from PTEU participants. CD4⁺CD73^{neg} and CD4⁺CD73⁺ cells were separated by magnetic beads as described above. CD4⁺CD73^{neg} cells were stimulated with SEB (1 μ g/ml) with or without CD4⁺CD73⁺ suppressor cells for 12 h. Levels of T-cell activation and TNF- α and IL-2 expression were measured as described above. For suppression of T-cell proliferation by CD4⁺CD73⁺ cells, we obtained PBMCs from NC. CD4⁺CD73^{neg} and CD4⁺CD73⁺ T cells were also separated by magnetic beads as described above. Carboxyfluorescein succinimidyl ester (CFSE)-labeled CD4⁺CD73^{neg} responder cells ($n = 10^5$) were stimulated with SEB in 96-well plates for 5 days. Proliferation of responder cells was measured by single-color flow cytometry.

Statistics

Data are expressed as medians. For samples with nonparametric distribution of values, Kruskal–Wallis and two-tailed exact Wilcoxon–Mann–Whitney tests were applied using SPSS software (IBM, Version 19). Correlations were calculated by the Spearman test. For the demographic data, groups were compared by Kruskal–Wallis with Dunn's Multiple

Comparison Test using GraphPad Prism v5.04. *P* values of 0.05 or less and *R*² values at least 0.4 were considered to be significant.

Results

Study populations

The majority of MACS participants on ART were infected with HIV-1 more than 10 years (16/29) as compared to the nine untreated participants who were infected less than 10 years (Table 1). Of the 29 participants on ART, the majority were on a nonnucleoside reverse transcriptase inhibitor or protease inhibitor-based regimen. CD4⁺ T-cell counts in the three groups of HIV-1-infected individuals were lower than those in the NC. CD4⁺ T-cell counts in the noART group were similar to those in the ART500 group.

Ectonucleotidase expression in CD4⁺ T cells

Whereas the absolute numbers of CD4⁺CD39⁺ and CD4⁺CD73⁺ T-cell subsets were decreased in HIV-1-positive individuals compared to NC, only the frequency of CD4⁺CD73⁺ T cells was decreased in HIV-1-positive individuals as compared to NC (8.8 vs. 5.2%; *P* = 0.005; Fig. 1a,b). In HIV-1-positive individuals and NC, CD39 and CD73 were expressed in different CD4⁺ T-cell subsets (Fig. 1c). Both subsets belonged to the memory compartment (CD45RO⁺). Commonly used Treg markers CD25 and FOXP3 were not expressed in CD4⁺CD73⁺ cells, unlike CD4⁺CD39⁺ cells (Figure S1), as has been previously reported [30]. In contrast to CD4⁺CD39⁺ Treg, nearly all CD4⁺CD73⁺ T cells were CD26⁺. For all HIV-1-positive individuals, the frequency and absolute numbers of CD4⁺CD73⁺ T cells positively correlated with CD4⁺ T-cell counts (*r* = 0.4, *P* = 0.01 and *r* = 0.8, *P* < 0.001, respectively; data not shown), whereas mean percentage of CD4⁺CD39⁺ T cells within the shrinking CD4⁺ compartment remained comparable to that in NC (Fig. 1a). These results suggest that CD4⁺CD73⁺ T cells are preferentially depleted in HIV-1-positive individuals.

CD4⁺CD73⁺ T-cell frequency and immune activation

Compared to NC, the absolute number and frequency of the CD4⁺CD73⁺ T cells were lower in all HIV-1-positive groups (Fig. 2a,b) regardless of viral suppression. The lowest absolute numbers of CD4⁺CD73⁺ T cells were seen in the ART350 group (15 vs. 89/μl in NC, *P* < 0.001; Fig. 2a). In contrast, the frequency of CD4⁺CD39⁺ T cells was similar in all four groups, whereas their absolute number was decreased in the noART and ART350 groups (Fig. 2c,d). As a group, HIV-1-positive individuals had a higher frequency of activated CD4⁺ (3.1 vs. 1.8%; *P* = 0.001) and CD8⁺ T cells (6.2 vs. 3.6%; *P* = 0.01) than NC (data not shown). All four HIV-1-positive groups had a higher frequency of CD4⁺ and CD8⁺ T cells coexpressing both activation markers than NC (Fig. 2e,f). The increased numbers and percentages of activated CD4⁺ and CD8⁺ T cells of HIV-1-positive individuals corresponded to the decreased numbers and percentages of CD4⁺CD73⁺ T cells (Fig. 2a,b,e,f).

Negative correlation of CD4⁺CD73⁺ T-cell numbers with activated T cells

In HIV-1-positive individuals, the absolute number of CD4⁺CD73⁺ T cells showed an inverse correlation with the frequency of activated CD4⁺ (*r* = -0.5, *P* < 0.002) and CD8⁺ T cells (*r* = -0.4, *P* = 0.05) as well as with plasma CRP levels (*r* = -0.5, *P* = 0.01; Fig. 3a). The frequency of CD4⁺CD73⁺ T cells was also inversely correlated to that of activated CD4⁺ T cells (*r* = -0.4, *P* = 0.03; data not shown). In contrast, absolute numbers of circulating CD4⁺CD39⁺ T cells in HIV-1-positive individuals did not correlate with the frequency of activated CD4⁺ or CD8⁺ T cells or with the plasma CRP levels (*r* = -0.3, *P* =

0.1; Fig. 3b). Majority of the individuals in ART350 and ART500 had no detectable viremia by SCA (limit of detection = 0.61 copies/ml). For those with detectable viremia, there was no correlation with T-cell immune activation (Fig. 3c). These data suggest that the depletion of CD4⁺CD73⁺ T cells in HIV-1-positive individuals plays a role in persistent immune activation and inflammation.

Evaluation of adenosine production by CD4⁺CD73⁺ and CD4⁺CD39⁺ T-cell subsets

We tested the capability of CD4⁺CD39⁺ T cells to hydrolyze exogenous (e) ATP to 5'-AMP and of CD4⁺CD73⁺ T cells to hydrolyze e5'-AMP to ADO using mass spectrometry. The subsets were first separated using magnetic beads (purity > 85%; Figure S2). Exogenous ATP was hydrolyzed to 5'-AMP only in the presence of CD4⁺CD39⁺ T cells (44 ± 5 vs. 8 ± 2 ng/ml, $t = 60$ min, $P = 0.03$; Fig. 4a). 5'-AMP produced by CD4⁺CD39⁺ T cells was hydrolyzed to ADO only in the presence of CD4⁺CD73⁺ T cells (9.1 ± 2.9 vs. 2.7 ± 1.2 ng/ml, $t = 60$ min, $P = 0.03$; Fig. 4b). High levels of ADO were produced only when both T-cell subsets were present. Individually, these cell subsets were inefficient in converting eATP to ADO. Interestingly, the ability to hydrolyze e5'-AMP to ADO was greater in CD4⁺CD73⁺ T cells obtained from HIV-1-positive individuals ($P = 0.002$, Fig. 4c), despite no difference in expression of CD73 in CD4⁺ T cells of HIV-1-positive individuals and NC (data not shown).

Evaluation of cyclic adenosine monophosphate levels in CD4⁺ T cells

In lymphocytes, ADO mediates suppression by increasing cAMP levels in responder cells. We determined whether eATP-treated CD4⁺ T cells of HIV-1-positive individuals (PTEU) had similar cAMP levels as CD4⁺ T cells of NC. We found that the addition of eATP to total CD4⁺ T cells, which combine the CD4⁺CD39⁺ and CD4⁺CD73⁺ T-cell subsets, induced a greater increase of cAMP levels in NC than in HIV-1-positive individuals' cells (Fig. 4d). These results suggest that in HIV-1 infection, CD4⁺ T cells produce less ADO, increase cAMP to a lesser degree and, thus, induce less immunosuppression than CD4⁺ T cells of NC. This is likely due to the lower frequency of CD73⁺ T cells within the CD4⁺ population incubated with eATP in HIV-1-positive individuals than in NC (Fig. 4e). The results are consistent with the decreased numbers of CD4⁺CD73⁺ T cells in HIV-1 and with the persistent immune activation seen in HIV-1-positive individuals.

Suppressive activity of exogenous adenosine

We have shown above that low proportions of CD4⁺ T cells that express CD73, the rate-limiting enzyme in the ADO pathway, could account for low ADO levels and persistent immune activation in HIV-1-positive individuals. Next, we evaluated a potential role of ADO in suppression of cytokine expression in CD4⁺ T cells and CD4⁺ and CD8⁺ T-cell immune activation following in-vitro stimulation with SEB in NC and HIV-1-positive individuals (PTEU, $n = 5$, Fig. 5a,b). In the presence of eADO (250 μ mol/l, 6 h), the mean decrease in the percentage of cytokine-expressing CD4⁺ T cells was $8.1 \pm 3.6\%$ for TNF- α and $4.5 \pm 0.8\%$ for IL-2. However, the blockade of all ADO receptors by an antagonist, DPSPX, almost completely reversed ADO-mediated suppression of Th1 cytokine levels in CD4⁺ T cells. Similar results were obtained with CD8⁺ T cells (data not shown). ADO also suppressed T-cell activation. In the presence of ADO (500 μ mol/l, 6 h), the mean decrease in the frequency of activated T cells was $2.7 \pm 1.2\%$ for CD4⁺ and $2.5 \pm 1.6\%$ for CD8⁺ T cells (Fig. 5b). Thus, eADO had considerable inhibitory effects on functions of CD4⁺ and CD8⁺ T cells of NC and HIV-1-positive individuals.

Suppressive activity of CD4⁺CD73⁺ T cells

In preliminary ex-vivo experiments, we found that CD4⁺CD73⁺ T cells did not mediate suppression of TNF- α and IL-2 or CD38/DR expression in activated CD4⁺ T cells of HIV-1-positive individuals (PTEU) or NC (data not shown). The ability of CD4⁺CD73⁺ T cells to mediate suppression of responder cell proliferation was next evaluated. In CSFE suppression assays, we incubated CD4⁺CD25^{neg} responder cells alone or in the presence of isolated autologous CD4⁺CD73⁺ T cells and observed no suppression of responder cell proliferation by these cells (Fig. 5c). However, upon addition of e5'-AMP to the proliferation assay, increased suppression was observed. These results show that CD4⁺CD73⁺ T cells alone cannot mediate suppression. However, when supplemented with eATP in the presence of CD4⁺CD39⁺ cells or e5'-AMP, they acquire the ability to produce ADO and mediate suppression (Fig. 5a,b).

Discussion

In chronic HIV-1 infection, it is important to evaluate the integrity of immunoregulatory pathways, as persistent immune activation and increased levels of systemic inflammation go unchecked when immunoregulatory activities are compromised. In this study, we assessed the activity of the ADO suppression pathway, specifically the activity of ectoenzyme-expressing CD4⁺ T cells, in HIV-1 infection. Several groups have already reported on CD39⁺ Treg during chronic HIV-1. However, data regarding the role of CD4⁺CD73⁺ T cells in this disease are not available. As CD73 is a rate-limiting enzyme in the ADO pathway, its presence on a subset of CD4⁺ T cells potentially involved in immune regulation implicates ADO in HIV-1 pathogenesis.

We found that CD73⁺CD4⁺ T cells represent a functionally and phenotypically distinct subset from CD4⁺CD39⁺FOXP3⁺Treg [20,31]. Unlike CD4⁺CD39⁺Treg, they do not express FOXP3 and CD25^{high}. We show that CD4⁺CD73⁺ T cells are depleted in HIV-1 infection regardless of virologic suppression and are not completely reconstituted with increasing CD4⁺ T-cell counts. Whereas a relative accumulation of Treg has been described during chronic HIV-1 infection [32], our findings suggest that CD4⁺CD73⁺ T cells are preferentially depleted. Possibly, depletion of these ADO-producing cells in HIV-1-positive individuals results in decreased ADO suppression, hence the inability to control persistent T-cell activation. Indeed, we noted inverse correlations between the number of CD4⁺CD73⁺ T cells and T-cell activation, as well as plasma CRP levels, which we did not observe with CD4⁺CD39⁺ T cells. Furthermore, CD4⁺CD73⁺ T cells have increased dipeptidyl peptidase-4 (CD26) expression, which can bind HIV-1 Tat [33,34] and lead to T-cell suppression. Further investigation should be done to evaluate whether Tat-binding leads to the inhibition of CD73 activity.

We also investigated the ability of ectoenzyme-expressing CD4⁺ T cells to produce ADO. Our ex-vivo results indicate that CD4⁺CD39⁺ T cells require cooperation from CD4⁺CD73⁺ T cells for optimal production of ADO, as they are predominantly expressed in different CD4⁺ T-cell subsets. Both subsets must work in tandem to convert eATP to ADO, with CD73 serving as a rate-limiting enzyme. ADO, in turn, suppresses functions of activated helper and effector T cells expressing A_{2A} receptors [35]. Our results suggest that the ability to produce ADO by the two ectoenzyme-expressing CD4⁺ T cells is not compromised in HIV-1-positive individuals. In fact, when eATP was added to CD4⁺ T cells containing both subsets, cAMP levels increased in CD4⁺ T cells obtained from NC and HIV-1-positive individuals. However, this increase in cAMP was greater in cells from NC, suggesting lower ADO engagement in HIV-1-positive individuals, which is likely due to lower numbers of CD4⁺CD73⁺ T cells. Together, these results suggest that fewer CD4⁺CD73⁺ T cells present in HIV-1 infection are responsible for lower ADO production and lesser cAMP upregulation.

leading to reduced suppression and persistent activation. To confirm that these mechanisms operate *in vivo*, it would be necessary to measure plasma ADO levels. However, ADO is highly unstable, and direct measurements of plasma levels are unlikely to be reliable and may be too low for measurements by mass spectrometry.

Unlike CD4⁺CD39⁺ Treg, which have been shown to mediate suppression by multiple mechanisms [36] in addition to ADO-mediated suppression [26], the CD4⁺CD73⁺ subset does not mediate suppression in CFSE suppression assays. However, by virtue of its ability to produce ADO, this subset can play an important role in immune regulation in HIV-1 infection. Indeed, our results confirm that ADO downregulates T-cell activation as well as TNF- α and IL-2 expression in T cells. Interestingly, Murray *et al.* [37] reported in 1984 that there was decreased 5' nucleotidase (5'-NT, CD73) activity in lymphocytes of patients with AIDS and asymptomatic MSM. Thus, early on in the AIDS epidemic, 5'-NT activity in T cells had already been considered an important factor in AIDS pathogenesis. CD73 activity can also prevent cardiovascular disease as ADO interferes with cellular adhesive properties in the vascular endothelium, thereby inhibiting atherogenesis [38]. Increased rates of cardiovascular disease and atherosclerosis have been reported in HIV-1-positive individuals despite effective ART [6,39]. The inverse correlation between CD4⁺CD73⁺ T-cell numbers and plasma CRP levels in HIV-1-positive individuals identifies a possible link between HIV-1 infection and increased rates of cardiovascular disease.

The cause of persistent immune activation and elevated levels of inflammation in chronic HIV-1 infection is unclear. Our study concentrated on why the immunoregulatory mechanisms, specifically the ADO pathway, are unable to control this increased inflammation. Our results support the hypothesis that loss of CD73-expressing CD4⁺ T cells and consequently, decreased ADO levels contribute to this lack of control. This loss is associated with increased proportions of activated T cells and systemic inflammation despite virologic suppression during ART. The CD73⁺ T-cell subset could, therefore, be a critical factor in the increased rates of HIV-1-associated chronic diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Effros RB, Fletcher CV, Gebo K, Halter JB, Hazzard WR, Horne FM, et al. Aging and infectious diseases: workshop on HIV infection and aging – what is known and future research directions. *Clin Infect Dis*. 2008; 47:542–553. [PubMed: 18627268]
2. van Sighem A, Danner S, Ghani AC, Gras L, Anderson RM, de Wolf F. Mortality in patients with successful initial response to highly active antiretroviral therapy is still higher than in non-HIV-infected individuals. *J Acquir Immune Defic Syndr*. 2005; 40:212–218. [PubMed: 16186740]
3. Lohse N, Hansen AB, Pedersen G, Kronborg G, Gerstoft J, Sorensen HT, et al. Survival of persons with and without HIV infection in Denmark, 1995–2005. *Ann Intern Med*. 2007; 146:87–95. [PubMed: 17227932]
4. Kuller LH, Tracy R, Belloso W, De Wit S, Drummond F, Lane HC, et al. Inflammatory and coagulation biomarkers and mortality in patients with HIV infection. *PLoS Med*. 2008; 5:e203. [PubMed: 18942885]
5. Hazenberg MD, Otto SA, van Benthem BH, Roos MT, Coutinho RA, Lange JM, et al. Persistent immune activation in HIV-1 infection is associated with progression to AIDS. *AIDS*. 2003; 17:1881–1888. [PubMed: 12960820]
6. Kaplan RC, Sinclair E, Landay AL, Lurain N, Sharrett AR, Gange SJ, et al. T cell activation and senescence predict subclinical carotid artery disease in HIV-infected women. *J Infect Dis*. 2011; 203:452–463. [PubMed: 21220772]
7. Smits HA, Boven LA, Pereira CF, Verhoef J, Nottet HS. Role of macrophage activation in the pathogenesis of Alzheimer's disease and human immunodeficiency virus type 1-associated dementia. *Eur J Clin Invest*. 2000; 30:526–535. [PubMed: 10849022]
8. Gibellini D, De Crignis E, Ponti C, Cimatti L, Borderi M, Tschon M, et al. HIV-1 triggers apoptosis in primary osteoblasts and HOBIT cells through TNFalpha activation. *J Med Virol*. 2008; 80:1507–1514. [PubMed: 18649336]
9. Grund B, Neuhaus J, Phillips A. Relative risk of death in the SMART study. *Lancet Infect Dis*. 2009; 9:724–725. [PubMed: 19926031]
10. Piconi S, Parisotto S, Rizzardini G, Passerini S, Terzi R, Argentero B, et al. Hydroxychloroquine drastically reduces immune activation in HIV-infected, antiretroviral therapy-treated immunologic nonresponders. *Blood*. 2011; 118:3263–3272. [PubMed: 21576701]
11. Giorgi JV, Hultin LE, McKeating JA, Johnson TD, Owens B, Jacobson LP, et al. Shorter survival in advanced human immunodeficiency virus type 1 infection is more closely associated with T lymphocyte activation than with plasma virus burden or virus chemokine coreceptor usage. *J Infect Dis*. 1999; 179:859–870. [PubMed: 10068581]
12. Liu Z, Cumberland WG, Hultin LE, Prince HE, Detels R, Giorgi JV. Elevated CD38 antigen expression on CD8+ T cells is a stronger marker for the risk of chronic HIV disease progression to AIDS and death in the Multicenter AIDS Cohort Study than CD4+ cell count, soluble immune activation markers, or combinations of HLA-DR and CD38 expression. *J Acquir Immune Defic Syndr Hum Retrovirol*. 1997; 16:83–92. [PubMed: 9358102]
13. Beignon AS, McKenna K, Skoberne M, Manches O, DaSilva I, Kavanagh DG, et al. Endocytosis of HIV-1 activates plasmacytoid dendritic cells via Toll-like receptor-viral RNA interactions. *J Clin Invest*. 2005; 115:3265–3275. [PubMed: 16224540]
14. Brenchley JM, Price DA, Schacker TW, Asher TE, Silvestri G, Rao S, et al. Microbial translocation is a cause of systemic immune activation in chronic HIV infection. *Nat Med*. 2006; 12:1365–1371. [PubMed: 17115046]
15. Douek D. HIV disease progression: immune activation, microbes, and a leaky gut. *Top HIV Med*. 2007; 15:114–117. [PubMed: 17720995]
16. Brenchley JM, Douek DC. The mucosal barrier and immune activation in HIV pathogenesis. *Curr Opin HIV AIDS*. 2008; 3:356–361. [PubMed: 19372990]
17. Papagno L, Spina CA, Marchant A, Salio M, Rufer N, Little S, et al. Immune activation and CD8+ T-cell differentiation towards senescence in HIV-1 infection. *PLoS Biol*. 2004; 2:E20. [PubMed: 14966528]

18. Catalfamo M, Wilhelm C, Tcheung L, Proschan M, Friesen T, Park JH, et al. CD4 and CD8 T cell immune activation during chronic HIV infection: roles of homeostasis, HIV, type I IFN, and IL-7. *J Immunol.* 2011; 186:2106–2116. [PubMed: 21257970]
19. Nikolova M, Carriere M, Jenabian MA, Limou S, Younas M, Kok A, et al. CD39/adenosine pathway is involved in AIDS progression. *PLoS Pathog.* 2011; 7:e1002110. [PubMed: 21750674]
20. Moreno-Fernandez ME, Rueda CM, Rusie LK, Chougnet CA. Regulatory T cells control HIV replication in activated T cells through a cAMP-dependent mechanism. *Blood.* 2011; 117:5372–5380. [PubMed: 21436067]
21. Schulze Zur Wiesch J, Thomssen A, Hartjen P, Toth I, Lehmann C, Meyer-Olson D, et al. Comprehensive analysis of frequency and phenotype of T regulatory cells in HIV infection: CD39 expression of FoxP3+ T regulatory cells correlates with progressive disease. *J Virol.* 2011; 85:1287–1297. [PubMed: 21047964]
22. Kumar V, Sharma A. Adenosine: an endogenous modulator of innate immune system with therapeutic potential. *Eur J Pharmacol.* 2009; 616:7–15. [PubMed: 19464286]
23. Borsellino G, Kleinewietfeld M, Di Mitri D, Sternjak A, Diamantini A, Giometto R, et al. Expression of ectonucleotidase CD39 by Foxp3+ Treg cells: hydrolysis of extracellular ATP and immune suppression. *Blood.* 2007; 110:1225–1232. [PubMed: 17449799]
24. Deaglio S, Dwyer KM, Gao W, Friedman D, Usheva A, Erat A, et al. Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *J Exp Med.* 2007; 204:1257–1265. [PubMed: 17502665]
25. Dwyer KM, Hanidziar D, Putheti P, Hill PA, Pommey S, McRae JL, et al. Expression of CD39 by human peripheral blood CD4+ CD25+ T cells denotes a regulatory memory phenotype. *Am J Transplant.* 2010; 10:2410–2420. [PubMed: 20977632]
26. Schuler PJ, Schilling B, Harasymczuk M, Hoffmann TK, Johnson J, Lang S, et al. Phenotypic and functional characteristics of CD4+ CD39+ FOXP3+ and CD4+ CD39+ FOXP3neg T-cell subsets in cancer patients. *Eur J Immunol.* 2012; 42:1876–1885. [PubMed: 22585562]
27. Law JP, Hirschhorn DF, Owen RE, Biswas HH, Norris PJ, Lanteri MC. The importance of Foxp3 antibody and fixation/permeabilization buffer combinations in identifying CD4+CD25+ Foxp3+ regulatory T cells. *Cytometry A.* 2009; 75:1040–1050. [PubMed: 19845018]
28. Palmer S, Wiegand AP, Maldarelli F, Bazmi H, Mican JM, Polis M, et al. New real-time reverse transcriptase-initiated PCR assay with single-copy sensitivity for human immunodeficiency virus type 1 RNA in plasma. *J Clin Microbiol.* 2003; 41:4531–4536. [PubMed: 14532178]
29. Jackson EK, Ren J, Mi Z. Extracellular 2',3'-cAMP is a source of adenosine. *J Biol Chem.* 2009; 284:33097–33106. [PubMed: 19801686]
30. Schuler PJ, Harasymczuk M, Schilling B, Lang S, Whiteside TL. Separation of human CD4+CD39+ T cells by magnetic beads reveals two phenotypically and functionally different subsets. *J Immunol Methods.* 2011; 369:59–68. [PubMed: 21513715]
31. Burton CT, Westrop SJ, Eccles-James I, Boasso A, Nelson MR, Bower M, et al. Altered phenotype of regulatory T cells associated with lack of human immunodeficiency virus (HIV)-1-specific suppressive function. *Clin Exp Immunol.* 2011; 166:191–200. [PubMed: 21985365]
32. Moreno-Fernandez ME, Presicce P, Chougnet CA. Homeostasis and function of regulatory T cells in HIV/SIV infection. *J Virol.* 2012; 86:10262–10269. [PubMed: 22811537]
33. Gutheil WG, Subramanyam M, Flentke GR, Sanford DG, Munoz E, Huber BT, et al. Human immunodeficiency virus 1 Tat binds to dipeptidyl aminopeptidase IV (CD26): a possible mechanism for Tat's immunosuppressive activity. *Proc Natl Acad Sci U S A.* 1994; 91:6594–6598. [PubMed: 7912830]
34. Weihofen WA, Liu J, Reutter W, Saenger W, Fan H. Crystal structures of HIV-1 Tat-derived nonapeptides Tat-(1–9) and Trp2-Tat-(1–9) bound to the active site of dipeptidyl-peptidase IV (CD26). *J Biol Chem.* 2005; 280:14911–14917. [PubMed: 15695814]
35. Deaglio S, Robson SC. Ectonucleotidases as regulators of purinergic signaling in thrombosis, inflammation, and immunity. *Adv Pharmacol.* 2011; 61:301–332. [PubMed: 21586363]
36. Schmidt A, Oberle N, Krammer PH. Molecular mechanisms of treg-mediated T cell suppression. *Front Immunol.* 2012; 3:51. [PubMed: 22566933]

37. Murray JL, Reuben JM, Munn CG, Mansell PW, Newell GR, Hersh EM. Decreased 5' nucleotidase activity in lymphocytes from asymptomatic sexually active homosexual men and patients with the acquired immune deficiency syndrome. *Blood*. 1984; 64:1016–1021. [PubMed: 6091811]
38. Buchheiser A, Ebner A, Burghoff S, Ding Z, Romio M, Viethen C, et al. Inactivation of CD73 promotes atherogenesis in apolipoprotein E-deficient mice. *Cardiovasc Res*. 2011; 92:338–347. [PubMed: 21955554]
39. Ho JE, Hsue PY. Cardiovascular manifestations of HIV infection. *Heart*. 2009; 95:1193–1202. [PubMed: 19564432]

Fig. 1. Expression of ectonucleotidases in human CD4⁺ T cells

(a) In HIV-1-positive individuals ($N = 36$), the absolute number of CD4⁺CD73⁺ and CD4⁺CD39⁺ T cells was decreased compared to the normal controls (NC; $N = 10$). Also, the frequency of CD4⁺CD73⁺ T cells was decreased in HIV-positive individuals, whereas the frequency of CD4⁺CD39⁺ Treg was comparable to NC. Box plots in this and other figures show the median, 25 and 75% quartiles, and the minimum and maximum values for NC and HIV-1-positive individuals. (b) Representative density plots showing decreased CD4⁺ T-cell compartment in an HIV-1-positive individual vs. NC (left column) and the percentages of CD39⁺ and CD73⁺ T cells in this compartment and expression of CD39 (right column). (c) Representative density plots show that CD39 and CD73 are expressed on different subsets of CD4⁺ T cells. CD4⁺CD73⁺ cells are CD45RO⁺memory T cells and do not express surface markers commonly associated with Treg, for example, CD25^{high} and FOXP3. Also, they are CD26⁺. All density plots are gated on CD4⁺ T cells and show results from a representative HIV-1-positive individual.

Fig. 2. Frequency and absolute number of CD4⁺CD73⁺ and CD4⁺CD39⁺ T cells in HIV-1-positive individuals

The absolute number (a) and frequency (b) of CD4⁺CD73⁺ T cells were decreased in all HIV-1-positive individuals regardless of viral suppression. For CD4⁺CD39⁺ Treg only the absolute number was decreased in HIV-1-positive individuals (c), whereas their frequency showed no significant difference as compared to normal controls (NC) (d). The frequency of activated CD4⁺CD38⁺DR⁺ T cells in the peripheral blood of the three groups of HIV-1-positive individuals was increased (e), whereas the frequency of CD8⁺CD38⁺DR⁺ T-cell activation was higher only in the noART and ART350 group comparable to NC (f). **P* < 0.05, ***P* < 0.01.

Fig. 3. Correlations between the percentage of activated T cells and absolute numbers of $CD4^+CD73^+$ T cells

(a) The absolute number of $CD4^+CD73^+$ T cells was inversely correlated with the frequency of immune-activated $CD4^+$ (left panel) and $CD8^+$ (center panel) T cells, defined by surface expression of CD38 and HLA-DR. The absolute number of $CD4^+CD73^+$ T cells was also inversely correlated to inflammation status measured by C reactive protein (CRP) plasma levels (right panel). (b) There was no correlation between the absolute number of $CD4^+CD39^+$ Treg and T-cell activation or CRP plasma levels. (c) The low-level plasma viremia of HIV-1-positive individuals from the ART350 ($n = 11$) and ART500 ($n = 11$) was measured by single copy assay (SCA) and correlated with T-cell immune activation. Majority of the participants had undetectable plasma viremia by SCA. No correlation was observed in those with low-level plasma viremia.

Fig. 4. Hydrolysis of ATP and 5' AMP by purified CD4⁺T-cell subsets

CD4⁺CD39⁺ and CD4⁺CD73⁺ T cells were separated from peripheral blood mononuclear cells (PBMCs) of normal controls (NC) by magnetic beads. (a) Purified cells were incubated in the presence of eATP individually or together. 5'-AMP production was measured by mass spectrometry. The highest levels of 5'-AMP were produced by eATP hydrolysis in the presence of CD4⁺CD39⁺ T cells. (b) When adenosine (ADO) production was measured by mass spectrometry, the highest levels ADO were produced in the presence of both CD39⁺ and CD73⁺ subsets. (c) CD4⁺CD73⁺ T cells were purified from PBMCs of HIV-1-positive individuals (ART500) and NC and incubated in the presence of e5'-AMP. ADO production levels were significantly higher with cells obtained from HIV-1-positive individuals than with those of NC. Bar graphs are mean values $\pm$ SD from five independent experiments. (d) Isolated CD4⁺ T cells from NC ($n = 3$) and HIV-1-positive individuals (PTEU, $n = 3$) after stimulation with anti-CD3/CD28 microbeads and IL-2. Levels of cyclic adenosine monophosphate (cAMP) (fM) were obtained after incubation $\pm$ eATP. Bar graphs show the mean fold-increase in cAMP levels with addition of eATP (left column). (e) Representative density plots show CD4⁺CD39⁺ and CD4⁺CD73⁺ T-cell frequency in NC vs. HIV-1-positive individuals (right column).

Fig. 5. Immune suppressive activity of exogenous adenosine

(a) Cytokine expression by CD4⁺ T cells following in-vitro stimulation with Staphylococcal enterotoxin B (SEB) was determined in normal controls (NC; $n = 3$) and HIV-1-positive individuals (Pitt Treatment and Evaluation Unit, PTEU, $n = 5$). In the presence of adenosine (ADO; 250 μ mol/l, 6 h), the mean decrease in frequency of cytokine expressing CD4⁺ T cells was $8.1 \pm 3.6\%$ for TNF- α and $4.5 \pm 0.8\%$ for IL-2. However, the blockade of all ADO receptors by DPSPX (100 μ mol/l) almost completely reversed ADO-mediated suppression of Th1 cytokine levels in CD4⁺ T cells. (b) Exogenous ADO also suppressed T-cell activation as measured by surface expression of CD38 and HLA-DR in a concentration-dependent manner. This was especially true for HIV-1-positive individuals with high immune activation. In the presence of ADO (500 μ mol/l, 6 h), the mean decrease in the frequency of activated T cells was $2.7 \pm 1.2\%$ for CD4⁺ and $2.5 \pm 1.6\%$ for CD8⁺ T cells. * $P < 0.05$ (c) CFSE-labeled CD4⁺CD73^{neg} responder cells were incubated with CD4⁺CD73⁺ suppressor cells. Proliferation of responder cells was suppressed only in the presence of 5'AMP. RC, responder cell; SC, suppressor cell.

Table 1

Study group demographics.

	NC	noART	ART350 ^a	ART500 ^a
Number of participants	10	9	12	15
Age	54 (38–74)	45 (31–53)	56 (47–71)	53 (35–71)
HIV-1 RNA (copies/ml)	–	1259 (50–24 344)	< 50	< 50
Years of viral suppression	–	–	4 (1–12)	5 (1–14)
CD4 ⁺ T cells (cells/μl)	934 (347–1752)	572* (333–966)	272** (150–394) ^b	677* (441–1019) ^c

Median (range) age, plasma HIV-1 RNA, number of years suppressed on an antiretroviral regimen, and CD4⁺ T-cell counts for the four groups of Multicenter AIDS Cohort Study (MACS) participants.

^aTen participants on a nonnucleoside reverse transcriptase inhibitor-based regimen, eight on a protease inhibitor-based regimen, three on an integrase inhibitor-based regimen, and six on various combinations.

^bOne participant with CD4⁺T-cell count of 394; all previous counts < 350.

^cTwo participants with CD4⁺T-cell counts < 500; all previous counts > 500.

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P < 0.05/0.005 compared to the control population, median (range).