Adenosine deaminase regulates T reg expression in autologous T cell-dendritic cell cocultures from patients infected with HIV-1

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ABSTRACT
Regulatory T cells have an important role in immune suppression during HIV-1 infection. As regulatory T cells produce the immunomodulatory molecule adenosine, our aim here was to assess the potential of adenosine removal to revert the suppression of anti-HIV responses exerted by regulatory T cells. The experimental setup consisted of ex vivo cocultures of T and dendritic cells, to which adenosine deaminase, an enzyme that hydrolyzes adenosine, was added. In cells from healthy individuals, adenosine deaminase led to a significant decrease of HIV-1-induced CD4+CD25 hi forkhead box p3+ cells, and to a significant enhancement of the HIV-1-specific CD4+ responder T cells. An increase in the effector response was confirmed by the enhanced production of CD4+ and CD8+ CD25- CD45RO+ memory cell generation and secretion of Th1 cytokines, including IFN-γ and IL-15 and chemokines MIP-1α/CCL3, MIP-1β/CCL4, and RANTES/CCL5. These ex vivo results show, in a physiologically relevant model, that adenosine deaminase is able to enhance HIV-1 effector responses markedly. The possibility to revert regulatory T cell-mediated inhibition of immune responses by use of adenosine deaminase, an enzyme that hydrolyzes adenosine, merits attention for restoring T lymphocyte function in HIV-1 infection.


Introduction
HIV-1 infection is characterized by a chronic immune activation and CD4+ T cell depletion that leads to a progressive immune dysfunction where suppressed function of T lymphocytes occurs, and HIV-specific T cell responses are particularly impacted [1]. CD4+CD25+Foxp3+ T reg are essential for the maintenance of immunologic homeostasis and self-tolerance [2]. T reg isolated from HIV-1-infected individuals suppress the HIV-1-specific CD8+ T cell cytolytic and CD4+-proliferating responses [1, 3]. On one hand, increased CD39 expression in HIV-1 infection is related to T reg-mediated cell suppression [4] through inhibition of effector T cell IL-2 production [5]. On the other hand, a gene polymorphism associated with reduced cell-surface CD39 expression slows the progression to AIDS, implicating Ado production in disease progression [4]. Furthermore, the expression of CD39 in Foxp3+ T reg correlates with AIDS disease progression [4, 6]. It should be noted that CD39 is a nucleotidase that converts ATP to AMP, which may be then catabolized to Ado by CD73, a cell-surface 5′-nucleotidase. Ado, generated from catabolism of adenine nucleotides by CD39 and CD73 [7], leads to T reg-mediated suppression in human and murine lymphocytes [8–10].

ADA is key to control Ado concentration. ADA is essential for immune responses, and its congenital deficit leads to SCID as a result of toxic levels of intracellular Ado [11]. ADA can also be

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Abbreviations: ADA = adenosine deaminase, Ado = adenosine, BAL = bronchoalveolar lavage, DC = dendritic cell, DIOC6 = 3,3′-dihexyloxacarbocyanine iodide, EC = elite controller, Foxp3 = forkhead box p3, HAART = highly active antiretroviral therapy, NC = noncontroller, NECO = 6′-N-ethylcarboxamidoadenosine, PEG = polyethylene glycol, PVL = plasma viral load, RC = responder cell, T reg = regulatory T cell, VC = viremic controller

The online version of this paper, found at www.jleukbio.org, includes supplemental information.
released to the extracellular medium and bind to the surface of T cells [12–14], where it contributes to control extracellular Ado concentrations. ADA interacts directly with a variety of cell-surface proteins: CD26, a surface glycoprotein with dipeptidyl peptide IV activity [13–15], and Ado A1, A2A, and A2B 7-transmembrane-domain receptors coupled to G proteins [16–18]. In all cases, the protein remains enzymatically active and contributes to a decrease in the concentration of the immunosuppressive Ado at places where antigen presentation occurs. Thus, cell-surface ADA may degrade Ado and act as a costimulatory molecule in T cell activation [19]. Although HIV-1 gp120 envelope protein disrupts ADA–CD26 interaction [20], thus contributing to the HIV-1-promoted immunodeficiency [21], ex vivo assays have shown that ADA is still able to enhance autologous T cell proliferation against heat-inactivated HIV-1, presented by DCs in individuals receiving HAART [22]. All of these observations indicate that ADA may boost safe cellular responses against the virus [23]. In fact, PEG-ADA (marketed as Adagen) is approved for the treatment of ADA-related SCID [24].

The effect of ADA was studied here in ex vivo assays by use of cells from HIV-1-infected and noninfected individuals. Removal of extracellular Ado by ADA decreased the percentage of Tregs. Notably, the addition of ADA in cultures led to a decrease in Tregs and an increase in CD4+ responder T cells, in response to the inactivated HIV-1 Bal. antigen. Moreover, under the same conditions, ADA increased CD8+ T cell proliferation and CD4+ and CD8+ (CD25loCD45RO+) cell memory generation and increased the secretion of immunologically relevant chemokines and Th1 cytokines. The availability of a pharmaceutical formulation of ADA [24] makes it possible to consider the control of the nucleoside concentration as a therapeutic approach to improve T cell function and responses against HIV-1.

**MATERIALS AND METHODS**

**Sampling and study population**

Blood samples were obtained from the antecubital vein. EDTA-treated vacutainers (Becton Dickinson, San Diego, CA, USA) were used. All individuals gave informed consent. HIV-1-infected patients presenting CD4 cell numbers >200/µl and viral load ≤5 log/ml were classified as: ECs with PVLs <50 RNA copies/ml; VC with PVLs ≥50 and <5000 RNA copies/ml; and NCs with PVLs ≥5000 RNA copies/ml [25]. The characteristics of the HIV-infected patients from whom samples were taken are shown in Table 1.

**Antibodies and reagents**

FITC-conjugated mAb against CD4 and CD45RA; PE-conjugated mAb against CD8, CD25, and CD45RO; and PerCP-conjugated mAb against CD3, Alexa Fluor 700 mouse anti-human CD4 and CD69, allophycocyanin mouse anti-human CD25, V450 mouse anti-human CD127, and IgG1 isotype-matched controls were from BD Biosciences (Erembodegem-Aalst, Belgium). To quantify Treg, FoxP3 was detected by use of the commercially available human Treg staining kit (eBioscience, San Diego, CA, USA). NεCA was from Sigma-Aldrich (St. Louis, MO, USA).

**ADA preparation and activity determination**

ADA from calf intestine (Roche Diagnostics, Mannheim, Germany) was desalted by use of PD-10 columns (GE Healthcare, Cerdanyola, Spain), and enzyme activity was evaluated as reported previously [26].

**Cell death assay**

Cells from 4 days (d) cocultures performed in the absence or presence of ADA were treated as described elsewhere [27]. In brief, cells (2 × 10⁷) were washed with X-VIVO 15 media and then resuspended in 200 µl X-VIVO 15 media and incubated with 40 nM DIOC₁₆ (Molecular Probes, Invitrogen, Carlsbad, CA, USA) for 1 h at 37°C. For every sample, a minimum of 10,000 events was acquired (FACScanto; BD Biosciences) and analyzed with FlowJo software (Tree Star, Ashland, OR, USA). After gating on a lymphocyte population, apoptotic cells were identified by their low DIOC₁₆ fluorescence, whereas necrotic cells had propidium iodide staining and reduced DIOC₁₆ fluorescence.

**Isolation of CD4+CD25+ and CD4+CD25− T cells and cocultures**

CD4+CD25− and CD4+CD25+ T cells were freshly isolated from buffy coats by use of AutoMACS and the Regulatory T Cell Separation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). In brief, CD4+ T cells were first negatively selected from total PBMCs, followed by separation of CD25− and CD25+ by use of specific magnetic beads. Cocultures were performed by use of CD4+CD25− activated for 24 h with T-Activator CD5/CD28 Beads (Dynabeads; Life Technologies, Grand Island, NY, USA), CD3/CD28 plus 100 UI/ml IL-2, and CD4+CD25+ (responder cells, RCs) activated for 24 h with CD3/CD28 beads and stained with 5 µM CESE, following the instructions of the manufacturer (CellTrace CFSE; Molecular Probes, Invitrogen, Paisley, United Kingdom). Cocultures were performed in round-bottom, 96-well plates at 1:1 CD25−:CD25+ ratio for 5 d. Proliferation of RCs was quantified by measuring the dilution of the CFSE signal by flow cytometry.

**Viral production and inactivation and DC pulsing**

HIV-1 Bal. strain was propagated on preactivated human PBMCs from healthy, HIV-1-negative donors. Preactivation was carried out with 5 µg/ml PHA (Sigma-Aldrich) for 48–72 h in RPMI-1640 medium (BioWhittaker, Cambrex Bio-Science, Verviers, Belgium), supplemented with 20% FBS (BioWhittaker, Cambrex BioScience) at 37°C in a humid atmosphere of 5% CO₂. Activated cells were washed with PBS, 3% before infection. Infected cells were then cultured for 21 d in RPMI 1640, supplemented with 20% FBS plus 10 U/ml IL-2 (Roche Diagnostics). The productive infection was determined by enzyme immunoassay (INNOTEST HIV-1 p24 Antigen mAb kit; Immunogenetics, Barcelona, Spain), and cell-free viral supernatant was collected and frozen at ~80°C until inactivation was performed. Viral supernatant (1.06 µg/ml p24) was fixed with 1.80 formalin (1:2000 formaldehyde) for 1 h at 37°C, followed by 10 min × 3 at 62°C. Inactivated preparations were ultra-filtered (Amicon Ultra-1 30k PLH, 100 kDa membrane; Millipore, Madrid, Spain), washed with PBS, and pelleted (15,000 g during 1 h at 4°C). To generate negative controls, the same process was performed, but infection was excluded. Control supernatants resulted as p24 negative and were diluted in the same proportion as p24-containing samples. Proliferation or cytokine production values obtained in negative controls were subtracted to the corresponding values obtained with HIV-1-pulsed DCs. When pulsing immature DCs, 1 µg/ml recombinant soluble p24 (Protein Sciences, Meriden, CT, USA) or ~5 × 10⁹ particles/ml (10 µg/ml p24) were used.

**DC isolation**

Monocyte-derived DC isolation and maturation were done as already reported [28]. Although in vitro cultures of monocyte-derived DCs from HIV-1-infected patients remain uninfected and functionally intact [29], we performed the procedure in the presence of zincodine.

**T cell isolation, T cell-DC cocultures, and proliferation assays**

As a source of the T cell-enriched population for T cell-DC cocultures [30], nonadherent PBMCs were collected and washed 4 times with X-VIVO 10 media. Unless otherwise stated, cells were cultured in X-VIVO 10. To measure T cell proliferation, 10⁷ cells/ml were stained with 5 µM CFSE by use of the
CellTrace CFSE proliferation kit (Molecular Probes, Invitrogen, Paisley, United Kingdom), as indicated by the manufacturer’s protocol. Autologous cocultures were performed in 96-well, round-bottom plates, containing control or HIV-1-pulsed DCs (20,000 cells/well) and fresh CFSE-stained T cells (2 × 10^5 cells) in a final volume of 200 μl X-VIVO 10. T cell proliferation was assessed by flow cytometry after 7 d of coculture in a humidified atmosphere with 5% CO₂ at 37°C.

Flow cytometry

Cells washed with PBS were incubated for 30 min at 4°C with PBS containing 10% rabbit serum and 0.1% NaN₃ before 30 min incubation (4°C) with primary labeled antibodies. Cells were washed with PBS, and 10,000–50,000 events were acquired in a FACSCalibur or FACSCount flow cytometer (BD Biosciences). Data were analyzed with FlowJo software (Tree Star). Lymphocyte populations were selected by forward- and side-scatter parameters.

Cytokine and chemokine determination

T cells from HIV-1 patients were cocultured with autologous, HIV-1-pulsed DCs, as described above, and the secretion of cytokines and chemokines was measured in supernatants at d 7 of culture. Multiplex Luminex assays (Cytokine Human 25-Plex Panel; Invitrogen, Carlsbad, CA, USA) were performed as already reported [31]. The following 25 mediators were tested: eotaxin, GM-CSF, IL-1β, IL-1R antagonist, IL-2, IL-4, IL-5, IL-6, IL-7, CXCL8 (IL-8), IL-10, IL-12p40/p70, IL-13, IL-15, IL-17, IFN-α, IFN-γ, CXCL10 (IFN-γ-induced protein 10), CCL2 (MCP-1), CCL9 (monokine induced by IFN-γ), CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), and TNF-α.

Statistical analysis

GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA, USA) was used for data handling and plotting graphs. Quantitative variables were analyzed by use of the median and interquartile ranges.

Table 1. Clinical information of HIV-1-infected patients

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*VL, Viral load; expressed as log viral copies/ml. HCV, hepatitis C virus; DL, detection limit.

RESULTS

Effect of ADA treatment in expansion and regulatory activity of T cells

MACS was used to isolate CD4⁺CD25⁺ (Treg) cells from healthy donor buffy coats. Cells were activated with CD3/CD28 beads and 100 U/ml IL-2. ADA addition in the 24 h of treatment led to a dose-dependent reduction in the percentage and number of CD4⁺CD25⁺ Treg and to an increase, also dose dependent, in the percentage of CD4⁺CD25⁻ T cells (Fig. 1B and C). To address whether the addition of ADA influences cell viability, the presence of ADA at 2 different concentrations did not significantly decrease cell viability, which was higher than 90% (Supplemental Fig. 1). Foxp3 levels in the CD4⁺CD25⁺ and CD4⁺CD25⁻ subsets were determined by flow cytometry in samples incubated for 5 d with ADA (2 or 4 μM) or medium. CD4⁺CD25⁺ cells were Foxp3⁺, whereas <50% CD4⁺CD25⁻ cells expressed Foxp3 and with 1 order of magnitude lower mean fluorescence intensity (Fig. 1A).

Next, experiments were performed to understand whether ADA influences the phenotype of RCs cocultured with Treg.
CD4+CD25+ cells were treated with CD3/CD28 beads and 100 UI/ml IL-2 to generate CD4+CD25hiFoxp3+ cells (Tregs). CD4+CD25- responder T cells, previously stained with CFSE, were cocultured (1:1 ratio) for 3 d in the presence or absence of different doses of ADA. The percentage of CD4+CD25hi (B) and the percentage of CD4+CD25lo (C) T cells were determined by flow cytometry after 4 d of cell culture in the absence (dashed lines, value = 1) or in the presence (circles) of 2 or 4 μM (squares) ADA (n = 7). Each symbol represents a different donor, and values represent the ratio (in-fold) of the percentages of CD4+CD25hi or CD4+CD25lo cells obtained in the presence of ADA vs. those in the absence of ADA. *P < 0.05, Wilcoxon signed-rank test comparing median values of each group (thick lines) with 1 (control condition in the absence of ADA).

Figure 1. Expression of Foxp3 in CD4+CD25hi and CD4+CD25lo T cells. CD4+CD25+ T cells were isolated from buffy coat from healthy donors and activated for 48 h with CD3/CD28 beads at a (bead:cell) 1:1 ratio. (A) A representative dot plot with gates to select CD4+CD25hi and CD4+CD25lo populations (left) and an overlaid histogram showing Foxp3 expression in CD4+CD25hi and CD4+CD25lo cells (right). The percentage of CD4+CD25hi (B) and the percentage of CD4+CD25lo (C) T cells were determined by flow cytometry after 4 d of cell culture in the absence (dashed lines, value = 1) or in the presence (circles) of 2 or 4 μM (squares) ADA (n = 7). Each symbol represents a different donor, and values represent the ratio (in-fold) of the percentages of CD4+CD25hi or CD4+CD25lo cells obtained in the presence of ADA vs. those in the absence of ADA. *P < 0.05, Wilcoxon signed-rank test comparing median values of each group (thick lines) with 1 (control condition in the absence of ADA).

Expression of Ado-producing enzymes in T cells from HIV-infected donors and healthy subjects

The expression of CD39 and CD73, ectoenzymes responsible for extracellular Ado production, was analyzed in lymphocytes from healthy subjects and also from HIV-1-infected donors. PBMCs stained with CD4, Foxp3, CD25, CD39, and CD26, which anchors ADA to the cell surface, were analyzed by flow cytometry. The percentage of CD4+Foxp3hi cells was not significantly different between healthy and the different HIV-1-infected individuals (Fig. 3A). These cells were highly positive for CD25 and CD39, whereas they expressed very low levels of CD73 on their surface (Fig. 3B). CD4+Foxp3lo cells expressed much lower amounts of CD26 than their CD4+Foxp3hi counterparts (Fig. 3B). Taken together, these data suggest that ADA downwardly modulates CD25 expression. Ado, which has a marked suppressive potential, may be significantly produced by Tregs [9]. Thus, we determined whether signaling through activated AdoRs had functional consequences in our experimental model. The effect of an Ado analog (nonselective AdoR agonist, NECA), which is neither an ADA substrate nor inhibitor, was tested in cocultures. The addition of 4 μM ADA guaranteed that no endogenous extracellular Ado was available to AdoRs. In these conditions, NECA significantly reduced the percentage of CD25lo cells (Fig. 2G) and led to a trend toward increasing CD25hi cells (Fig. 2H). NECA actions clearly oppose those previously observed in the presence of ADA, suggesting that Ado contributes to the balance of the different T cell populations.

CD4+CD25+ cells were treated with CD3/CD28 beads and 100 UI/ml IL-2 to generate CD4+CD25hiFoxp3+ cells (Tregs). CD4+CD25- responder T cells, previously stained with CFSE, were cocultured (1:1 ratio) for 3 d in the presence or absence of different doses of ADA. The percentage of proliferating RCs identified by low CFSE staining significantly increased with 4 μM ADA treatment (Fig. 2C), whereas the CFSE Treg population decreased with ADA treatment (Fig. 2B). A control was performed to confirm that cells with low CFSE staining did not include added Tregs (Supplemental Fig. 2). The effect of ADA was also analyzed in the gated population of CD25+ cells arising upon activation with CD3/CD8 beads [32]. The presence of ADA also dose dependently reduced the percentage of CD25hi cells (Fig. 2E) while increasing the CD25lo population (Fig. 2F). Taken together, these data suggest that ADA downwardly modulates Foxp3 and CD25 expression. Ado, which has a marked suppressive potential, may be significantly produced by Tregs [9]. Thus, we determined whether signaling through activated AdoRs had functional consequences in our experimental model. The effect of an Ado analog (nonselective AdoR agonist, NECA), which is neither an ADA substrate nor inhibitor, was tested in cocultures. The addition of 4 μM ADA guaranteed that no endogenous extracellular Ado was available to AdoRs. In these conditions, NECA significantly reduced the percentage of CD25lo cells (Fig. 2G) and led to a trend toward increasing CD25hi cells (Fig. 2H). NECA actions clearly oppose those previously observed in the presence of ADA, suggesting that Ado contributes to the balance of the different T cell populations.

Conclusion

Taken together, the data show that the Treg population of our study was CD4+ Foxp3+ CD25hi CD39+ CD73lo and CD26lo. Next, we compared CD4+Foxp3hi and CD4+Foxp3lo subsets in the clinically different, HIV-1-infected donor groups (Fig. 3C–F). Compared with data from healthy and ECs, CD39 expression in cells from infected individuals was slightly reduced in CD4+Foxp3hi cells from NCs and VCs (Fig. 3D). Interestingly, cells from the different groups of patients showed reduced CD73 expression compared with healthy donors (Fig. 3E). This observation was more pronounced in CD4+Foxp3lo cells from the NC group (Fig. 3E). Finally, and in accordance with Mandapathil et al. [9], a positive correlation between CD25 and either CD73 or CD39 expression and a trend toward a negative correlation (P = 0.07).
between CD26 and CD39 expression were observed in CD4⁺Foxp3⁺ cells from HIV-infected donors (Fig. 3G–I), thus confirming that CD39 is preferentially expressed in Tregs, whereas CD26 is preferentially expressed in RCs.

Effect of ADA on cytokine and chemokine production by HIV-1-specific T cell subsets
Several studies report higher amounts of Tregs in samples from HIV-1-infected patients compared with healthy individuals (see ref. [33] for review). As ADA treatment decreased the amount of Tregs in cell cultures from healthy individuals, we prepared autologous cocultures of lymphocytes with inactivated HIV-1 (BaL)- or rHIV-1 p24-pulsed DCs, isolated from fresh blood samples, collected from HIV-1-infected donors (Table 1). The expression of CD25 and Foxp3 was addressed by flow cytometry after 5 d of coculture in the presence or absence of 2 μM ADA (Fig. 4A). Remarkably, addition of ADA to cocultures with BaL-pulsed DCs led to a significant reduction (2-fold decrease) in the percentage of CD4⁺CD25⁺Foxp3⁺ cells coupled with a significant increase in the percentage of CD4⁺CD25⁺Foxp3⁻ cells (Fig. 4B and C). These effects were also noticeable in response to p24-pulsed DCs but without reaching statistical significance (Fig. 4B and C).

To investigate the effect of ADA addition on the proliferation of T cells in response to DCs presenting viral proteins, fresh blood samples were collected from HIV-1-infected donors, and monocyte-depleted lymphocytes were obtained. Lymphocytes were then stained with CFSE and cocultured with autologous DCs pulsed with BaL or p24. Cocultures were performed in the presence or absence of 2 μM ADA. T cell proliferation was measured by flow cytometry at d 7 of coculture (Fig. 5A). Upon Ado removal by ADA (Fig. 5B), a statistically significant increase in CD8⁺ T cell proliferation was observed in response to BaL- and p24-pulsed DCs. In addition, a trend was observed in CD4⁺ T cell proliferation in cocultures with BaL-pulsed DCs (Fig. 5B).
The ability to mount specific secondary memory T cell responses against HIV-1 antigens is gradually lost in the majority of HIV-1-infected subjects. However, some individuals show preserved memory responses for longer periods of time [34]. To determine the effect of ADA on potentiating HIV-1-T cell memory populations, lymphocytes from our cohort of HIV-1-infected patients were cocultured for 14 d with autologous DCs, pulsed with BaL or p24. CD4+ (Fig. 6A) or CD8+ (Fig. 6B) T cells with a memory phenotype (CD25+CD45RO+) were analyzed by flow cytometry. As shown in Fig. 6, ADA induced a statistically significant increase of memory CD4+ T cells in response to BaL (median range: 11–15%) and p24 (median range: 16–22%)-pulsed DCs. In response to BaL-pulsed DCs, the presence of ADA caused an increase in memory CD8+ T cells (Fig. 6E) from 13 to 18%. Therefore, ADA addition to HIV-1-pulsed DCs was able to increase the amount of CD4+ and CD8+ cells displaying a memory phenotype.

To investigate whether modulation of Ado levels may induce cytokine and chemokine release in cells from HIV-1-infected patients, we analyzed the expression of 25 mediators of the immune response in the supernatant of a 7 d coculture of autologous lymphocytes and DCs pulsed with BaL or p24 in the

Figure 3. Phenotypic characterization of healthy and HIV CD4+ Foxp3+ Tregs. Cryopreserved PBMCs from healthy or HIV-1-infected donors were thawed, as indicated in Materials and Methods and stained for CD4, CD39, CD73, CD26, and Foxp3. (A) Dot plots showing the gating strategy to select CD4+Foxp3+ cells, with a fluorescence minus 1 (FMO) control (left) and a representative sample (right). (B) Histogram overlays indicating the expression of the different markers in CD4+Foxp3+ (red line) and in CD4+Foxp3− (blue) cells compared with an FMO control (gray). The percentage (upper) and the geometric mean (lower) of CD25 (C), CD39 (D), CD73 (E), and CD26 (F) expression in CD4+Foxp3+ (+) and in CD4+FOXp3− (−) cells from healthy and different groups of HIV-1-infected donors are shown. Each symbol represents a different donor, with box and whiskers indicating the median and the minimum to maximum values. Spearman test was used to calculate r and P in correlations between CD39 and CD25 (G) geo means, between CD39 and CD26 (H) geo means, or between CD73 and CD25 (I) geo mean. Correlations are within CD4+Foxp3+ cells (G and H) or within total cells (I). *P < 0.05 Kruskal-Wallis test, followed by Dunn’s multiple comparisons test, comparing CD73+ events (E, upper) with CD73 geo mean (E, lower) in a CD4+FOXp3− subset of healthy donors vs. a subset of HIV NCs.
presence or absence of 2 μM ADA. As depicted in Fig. 7A, the presence of ADA resulted in a consistent enhancement in the release of the Th1 cytokine IFN-γ, especially in cells pulsed with BaL (5- and 24-fold increase, respectively, for BaL- and p24-pulsed cocultures). The addition of the enzyme to the cultures also increased the secretion of the cytokines IL-1β (1.6- and 24-fold increase, respectively, in BaL- and p24-pulsed cocultures) and IL-6 (4- and 11-fold increase, respectively, in BaL- and p24-pulsed cocultures). The homeostatic cytokine IL-15 increased in the presence of ADA (2- and 5-fold increase, respectively, in BaL- and p24-pulsed cocultures). When chemokines were analyzed, a significant increase in RANTES/CCL5 was observed in BaL- and p24-pulsed cocultures (Fig. 7B). In addition, MIP-1α/CCL3 and MIP-1β/CCL4 levels obtained in the presence of ADA were increased but only reached statistical significance in, respectively, p24- and BaL-pulsed cocultures. Consistent with the observed enhancement in the release of Th1 cytokines, the Th2-related cytokines IL-13 and IL-5 were not increased in the presence of ADA, but rather, a tendency to decrease was observed. IL-10 levels were much lower, and a no statistically significant increase in response to p24 was induced by ADA (Supplemental Fig. 3).

DISCUSSION

According to current knowledge, high levels of Tregs dampen HIV-1-specific T cell responses and facilitate the establishment and maintenance of chronic infection [35]. The high expression of CD39 during AIDS progression [4] ultimately leads to an increase in extracellular levels of Ado. The ex vivo model used here proves advantages of ADA addition to boost anti-HIV-1 responses. Remarkably, ADA had a dual effect in cells from HIV-1-infected individuals, reducing the percentage of CD4+CD25hiFoxp3+ HIV-1-specific Tregs and increasing the HIV-1-specific CD4+CD25loFoxp3+ T cell population corresponding to CD4+ T effector cells. These results suggest that ADA could be beneficial in reducing the suppression of HIV-1-specific responses mediated by Tregs. Of note is that the experiments with the Ado analog, NECA, show that the effect is, at least in part, mediated by activation of AdoRs. NECA may activate the 2 AdoRs positively coupled to the adenylate cyclase, A2A and A2B, that are present in lymphocytes [36, 37]. Increases in cAMP levels seem responsible for the immunosuppressive action of Ado in sites of inflammation; cAMP signaling triggered by accumulation of the nucleoside protects tissues from excessive reaction by the immune system [37]. Recently, Almahariq et al. [38] have shown that Treg-mediated suppression is boosted by a protein that is activated by cAMP (exchange protein directly activated by cAMP), further underscoring the importance of cAMP for Treg function [5]. Depletion of Ado by ADA would revert these immunosuppressive actions. Costimulatory actions mediated by the interaction of ADA with ADA-binding proteins (CD26 and AdoRs) in lymphocytes and DCs at sites of antigen presentation have also been described [19]. It should be noted that CD26 is reported to be a negative selection marker for human Tregs [39, 40], and therefore, the ADA/CD26-mediated costimulation is unlikely in these cells. Tregs in our study were CD4+ Foxp3+ CD25hi CD39hi CD73+ and CD26–/low; accordingly, the Ado acting on Tregs may likely come from release at sites of antigen presentation or be generated by CD73 expressed on neighboring cells. In fact, the high local concentrations achieved by Ado in lymph nodes may come

Figure 4. ADA decreased the CD4+CD25hiFoxp3+ Tregs in cocultures, whereas it increased the HIV-1-specific CD4+ T effector cells. T cells, freshly isolated from patients infected with HIV-1, were cocultured for 5 d with autologous-inactivated HIV-1-BaL- or HIV-1-p24-pulsed DCs in the absence or in the presence of 2 μM ADA, and the expression of Foxp3 was determined. (A) Representative dot plots showing Foxp3 expression in the CD4+ T cell population in response to BaL-pulsed DCs in the absence (BaL) or in the presence (BaL+ADA) of 2 μM ADA compared with a FMO control (left). (Right) Histograms corresponding to CD25 expression within the CD4+Foxp3+ and CD4+Foxp3– subsets. Percentages of CD4+Foxp3–CD25hi (B; n = 7) or CD4+Foxp3+ CD25hi (C; n = 9) cells induced in response to BaL (circles) or p24 (triangles) in the absence (solid symbols) or presence (open symbols) of 2 μM ADA are given. Each pair of linked symbols represents data obtained by use of cells from a particular individual. Values are the ratio (in-fold) of the percentage of positive cells with respect to the negative controls obtained with nonpulsed DCs (reference value of 1, indicated by dotted lines). *P < 0.05, Wilcoxon signed-rank test comparing CD4+Foxp3–CD25hi (B) with CD4+Foxp3+CD25hi (C) in the absence vs. the presence of ADA.
from the action of cell-surface CD73 or from release by equilibrative nucleoside transporters [41].

In accordance with an enhanced CD4+ T effector/Th response, ADA increased CD8+ T cell proliferation in autologous cocultures with BaL- or p24-pulsed DCs. In cells from HIV-1-infected individuals and in these physiologic conditions, ADA enhanced the population of T cells involved in the control of HIV-1 infection. In fact, CD8+ T cells play a critical role in the host defense against viral infections [42]. The effect of ADA on CD8+ cells may be, in part, a result of the costimulatory action of ADA by interacting with cell-surface CD26 [43]. In this study, it was reported that CD26hi CD8+ T cells are among the subset of early effector memory T cells and that CD26-mediated costimulation is actually more robust than CD28-mediated costimulation [43]. As a result of sample limitation, we could not assess to which extent CD8+ cell-specific actions were a result of the enzymatic activity or of the costimulatory role of ADA by interacting with cell-surface CD26 [43]. In this study, it was reported that CD26hi CD8+ T cells are among the subset of early effector memory T cells and that CD26-mediated costimulation is actually more robust than CD28-mediated costimulation [43]. As a result of sample limitation, we could not assess to which extent CD8+ cell-specific actions were a result of the enzymatic activity or of the costimulatory role of ADA. When CD4+ T cell proliferation was assessed, a tendency toward an increase of CD4+ T cell proliferation was observed in response to BaL-pulsed DCs but not to p24-pulsed DCs. The structural diversity of antigens is key to generate specific immune responses [44], and therefore, it is likely that heat-inactivated BaL viruses have more immunogenic potential than soluble proteins, such as p24.

Insights into the role of Treg in vaccines have been provided recently. Brezar et al. [45] have demonstrated that anti-HIV-1 immunization decreased HIV-1-specific Treg over time and inversely correlated with HIV-1-specific, IFN-γ-producing cells. This provides evidence that effective anti-HIV-1 immunization skews the T cell response from regulatory to effector. In such context, the results provided here show potential for the presence of ADA in anti-HIV-1 vaccination. Limited in time, more pronounced, Th1 vs. Th2 response is desired for successful control of HIV-1 infection [46, 47]. Our findings suggest that ADA could play a role in polarization toward a Th1 response, as suggested by the increased release of proinflammatory/Th-1 cytokines and chemokines observed in the presence of ADA. The effect of ADA inhibiting immunocompetent Tregs (from healthy individuals) was, as marked, as that achieved by PEG-ADA in ADA-deficient mice and in ADA-SCID patients [48]. It has also been described that IL-15 increases effector memory CD8+ T cells in rhesus monkeys immunized with a HIV-1 DNA vaccine, suggesting that cytokine addition may be an adjuvant for HIV-1 vaccines [49]. Alternatively, ADA presence may trigger the release of IL-15, which in turn, may contribute to the expansion and survival of antigen-specific CD8+ T cells [50].

In acute viral infections, naïve CD4+ and CD8+ T cells expand, differentiate, and acquire effector functions [51]. Several surface markers have been proposed to distinguish among naïve, memory, and RCs. One classification is based on the CD45RA/RO isoform expression [52]. Memory cells express

![Figure 5. ADA increased proliferation of CD8+ T cells to DCs pulsed with BaL or p24.](image-url)
the CD45RO isoform, whereas naïve or recently activated cells express the CD45RA isoform. ADA addition led to substantial generation of HIV-1-specific CD4+CD25−CD45RO+ memory T cells in autologous cocultures with BaL- or p24-pulsed DCs and enhanced the generation of CD8+CD25−CD45RO+ memory T cells in autologous cocultures with BaL-pulsed DCs but not with p24-pulsed DCs. Whereas DCs capture HIV-1 antigens and present them to MHC class II-restricted CD4+ T cells, MHC class I-restricted CD8+ T cells require crosspresentation [53]. On the one hand, the persistence of functional memory T cells represents the basis for a long-lasting protection after exposure to pathogens [54, 55]. On the other hand, a recent study suggested that a vaccine that elicits effector memory CD8+ T cells is able to induce early and durable control of viral replication in SIV-infected macaques [56]. Therefore, optimal therapeutic strategies would be those providing increases in CD4 and CD8 T cell HIV-1-specific memory pools. Our results indicate that an ADA-based therapy could be beneficial for potentiation of immunologic memory, perhaps effector memory, against HIV-1.

ADA addition to cocultures was able to increase the secretion of β-chemokines, such as MIP-1α/ CCL3, MIP-1β/ CCL4, and RANTES/CCL5. The global role of these chemokines is to recruit immune cells, such as neutrophils, monocytes, DCs, and activated T and B cells, to sites of antigen processing to mount the immune response further [57]. The ADA-mediated increase on these 3 β-chemokines is relevant to HIV-1 pathogenesis, as potent HIV-1 inhibitory activities of R5 HIV-1 strains are associated with these mediators [58, 59]. The findings suggest that the ADA/Ado axis might be a novel pathway to counteract the Treg-mediated suppression in HIV-1 infection. The commercially available form of ADA in pharmaceutical quality, Adagen, could be a suitable candidate for being tested in clinical trials in HIV-1-infected individuals. Not only chronic infections but also other diseases, such as cancer, where Tregs are mediating immune suppression, would potentially benefit with the use of Adagen.

**AUTHORSHIP**

Figure 7. ADA increased cytokines and chemokines secretion in cocultures of T cells and BaL- or p24-pulsed DCs. T cells freshly isolated from HIV-1 patients were cocultured with autologous BaL- or p24-pulsed DCs in the absence or presence of 2 μM ADA. Cytokine and chemokine release was determined in the supernatants at d 7 of coculture (n = 5). Cytokine (top row) or chemokine (bottom row) production in response to BaL (circles) or p24 (triangles) in the absence (solid symbols) or presence (open symbols) of 2 μM ADA is shown. Each pair of linked symbols represents an HIV-1-infected individual. *P < 0.05, **P < 0.01, ***P < 0.001, Student’s t test for paired samples in the absence (−) vs. in the presence (+) of ADA.
Adenosine deaminase regulates $T_{reg}$ expression in autologous T cell-dendritic cell cocultures from patients infected with HIV-1


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