JLB

Article

Adenosine deaminase potentiates the generation of effector, memory, and regulatory CD4$^+$ T cells

José M. Martinez-Navio,[*,1] Víctor Casanova,*[*,1] Rodrigo Pacheco,[†,‡] Isaac Naval-Macabuhay,* Núria Climent,[§,||] Felipe Garcia,[||,¶] José M. Gatell,[||,¶] Josefa Mallol,* Teresa Gallart,[§,||] Carme Lluis,* and Rafael Franco*[,2]*

*Department of Biochemistry and Molecular Biology, Faculty of Biology, [§]Services of Immunology and Infectious Diseases & [||]AIDS Unit, Hospital Clínic, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), and [¶]HIVACAT, Hospital Clínic, Faculty of Medicine, University of Barcelona, Spain; [†]Fundación Ciencia para la Vida and Instituto Milenio de Biología Fundamental y Aplicada, Santiago, Chile; and [‡]Universidad San Sebastián, Chile

RECEIVED OCTOBER 27, 2009; REVISED SEPTEMBER 10, 2010; ACCEPTED SEPTEMBER 20, 2010. DOI: 10.1189/jlb.1009696

ABSTRACT

By interacting with CD26 on the CD4$^+$ T cell surface and with the AdoR A_{2B} on the DC surface, ADA triggers a costimulatory signal for human T cells. The aim of this study was to know whether ADA-mediated costimulation plays a role in the differentiation of T cells. The results show that irrespective of its enzymatic activity and dependent on TNF-α, IFN-γ, and IL-6 action, ADA enhanced the differentiation of CD4$^+$CD45RA$^+$CD45RO$^-$ naïve T cells toward CD4$^+$CD25$^+$CD45RO$^+$ Teffs and CD4$^+$CD45RA$^-$CD45RO$^+$ memory T cells. Furthermore, ADA potentiated generation of CD4$^+$CD25^{high}Foxp3$^+$ Tregs by a mechanism that seems to be mainly dependent on the enzymatic activity of ADA. Interestingly, an ADA-mediated increase on Teff, memory T cell, and Treg generation occurred, not only in cocultures from healthy individuals but also from HIV-infected patients. These data suggest that ADA is a relevant modulator of CD4$^+$ T cell differentiation, even in cells from immunologically compromised individuals. *J. Leukoc. Biol.* 89: 127–136; 2011.

Introduction

DCs are relevant APCs for activation of CD4$^+$CD45RA$^+$ naïve T cells and for their differentiation toward CD4$^+$CD25$^+$CD45RO$^+$ Teffs or CD4$^+$CD25^{high}Foxp3$^+$ Tregs. Whereas Teffs promote immunity against their cognate Ags, Tregs, by inhibiting function of Teffs, play an important physiological role limiting the effector phase of T cell responses, maintaining self-tolerance, and preventing autoimmunity [1, 2]. After a CD4$^+$ T cell response, reactivated Ag-specific T cells, which are a part of the pool of CD4$^+$CD45RA$^-$CD45RO$^+$ memory T cells [3, 4], coordinate a faster, stronger, and longer immune response [5, 6].

ADA, an enzyme involved in purine metabolism, catalyzes the hydrolytic deamination of Ado to inosine and ammonia. A congenital defect of ADA causes SCID, which is characterized by the absence of functional lymphocytes in affected individuals [7]. For many years, ADA was considered to be cytosolic, but it has been found on the cell surface of many cells. To date, it has been described that three different surface-anchoring proteins might mediate the binding of ecto-ADA to the cell surface, including the multifunctional protein CD26 and A_1R and A_{2B}R [8, 9]. A number of studies have evidenced that ADA, expressed on the cell surface, not only plays enzymatic activity-dependent functions, but also, it can trigger regulatory signals through its extraenzymatic properties [8, 9].

As a result of its enzymatic activity, ecto-ADA regulates the concentration of Ado available to bind to AdoRs [10]. Surface expression of AdoRs has been described in T cells [11–13] and in DCs [14–19]. In human T cells, the major AdoR expressed is the A_{2A}R [11–13], which is coupled with cAMP production. Elevated cAMP levels induce a marked impairment in T cell activation and IL-2 production [20], which promote anergy and tolerance in Th1 clones [21]. Accordingly, enzymatic activity of ecto-ADA decreases Ado levels available for stimulation of AdoRs expressed on the T cell surface, and this mechanism contributes to immune regulation [10, 22, 23].

During Ag presentation by DCs, T cell activation is subjected to regulation by several intercellular interactions mediated by surface and soluble molecules that stimulate their receptors expressed on T cells. These regulatory interactions might modulate the activation and differentiation of T cells. In this re-

Abbreviations: 3D=three-dimensional, ADA=adenosine deaminase, ADA-Hg=adenosine deaminase inhibited by $HgCl_2$, Ado=adenosine, A*n*R=adenosine receptor *n*, FL1–3=fluorescence 1–3, Foxp3=forkhead box p3, iDC=immature DC, IQR=interquartile ranges, SEA=staphylococcal enterotoxin A, Teff=effector T cell, Treg=regulatory T cell

The online version of this paper, found at www.jleukbio.org, includes supplemental information.

1. These authors contributed equally to this work.
2. Correspondence at current address: CIMA, University of Navarra, Avda Pio XII, 55, 31008 Pamplona, Spain. E-mail: rfranco@unav.es

gard, ADA, in a deaminase activity-independent way and by interacting with CD26 on the $CD4^+$ T cell surface and with the $A_{2B}R$ on the DC surface, triggers a strong costimulatory signal for T cell activation [14]. This ADA-mediated costimulation not only potentiates T cell proliferation but also the secretion of Th1 (IFN-γ) and proinflammatory (IL-6 and TNF-α) cytokines [8]. Importantly, we have recently described that this ADA-mediated costimulation is attenuated in $CD4^+$ T cells obtained from HIV-infected individuals, which contributes to the immunodeficiency [24]. However, when exogenous ADA is added on a coculture of HIV-pulsed DCs and autologous T cells, it is possible to generate a HIV-specific T cell response. This finding supports the possibility of using exogenous ADA to counteract the HIV-induced attenuation of ADA-mediated costimulation [25].

To know whether ADA may modulate naïve T cell differentiation, cocultures of naïve $CD4^+CD45RA^+CD45RO^-$ T cells and autologous superantigen-pulsed DCs were used as a model. ADA not only enhanced Teff generation but also increased memory T cell generation. Interestingly, ADA enhanced the generation of Tregs. The ADA-mediated effects were observed not only in healthy donors but also in immunologically compromised individuals, such as HIV-infected patients. In addition, we analyzed the contribution of IL-6, TNF-α, IFN-γ, and the deaminase activity to the ADA-mediated effects.

MATERIALS AND METHODS

Sampling and study population

Blood samples were obtained by venipuncture from the antecubital vein. EDTA-treated vacutainers (Becton Dickinson, San Jose, CA, USA) were used as collecting tubes, and every sample was processed immediately after extraction. Fifteen HIV-1-infected patients and 30 healthy control volunteers were included in the study. All individuals gave informed consent. The characteristics of HIV-infected patients are shown in **Table 1**.

Antibodies and reagents

The following primary antibodies were used: FITC-conjugated mAb against HLA-DR, CCR5, CD4, CD14, CD19, CD25, CD45RA, and IgG-γ1 isotype-matched control; PE-conjugated mAb against HLA-DR, CXCR4, CD1a, CD11c, CD14, CD19, CD25, CD40, CD45, CD45RO, CD56, and IgG-γ1 isotype-matched control, and PerCP-conjugated mAb against CD3, CD4, and IgG-γ1 isotype-matched control from BD Biosciences (Erembodegem-Aalst, Belgium); nonconjugated mouse mAb against CD26 (Ba5), nonconjugated mouse IgG2a control isotype, and PE-conjugated mAb against CD80, CD83, and CD86 (Coulter-Immunotech Diagnostic, Marseille, France); PE-conjugated mAb against CD209 (eBioscience, San Diego, CA, USA); activity-neutralizing mouse mAb against human IL-6, TNF-α, and IFN-γ (Bender MedSystems Inc., Burlingame, CA, USA); affinity-purified, nonconjugated goat pAb against $A_{2B}R$ (N-19; Santa Cruz Biotechnology, Santa Cruz, CA, USA); and nonconjugated rabbit pAb against purified calf ADA [26]. The following secondary pAb were used: FITC-conjugated goat anti-rabbit IgG (Gene Tex, Inc., Irvine, CA, USA), rabbit anti-mouse Igs (Dako, Glostrup Denmark), and rabbit anti-goat IgG (Sigma-Aldrich, St. Louis, MO, USA). All antibodies were used at the concentration range recommended by the supplier. TNF-α, IL-6, SEA and irrelevant mouse IgGs were purchased from Sigma-Aldrich; IFN-γ (Actimmune) was from InterMune, Inc. (Brisbane, CA, USA). ADA from calf intestine (Roche Diagnostic Inc., Mannheim, Germany) was desalted by passage through a PD-10 column (Amersham Biosciences, Cerdanyola, Spain), and its enzymatic activity was evaluated for consumption of Ado, measured as decreases in absorbance at 265 nm [27]. To obtain ADA without enzymatic activity, desalted ADA was treated with $HgCl_2$, as described previously [28], and passed again through a PD-10 column to eliminate remaining $HgCl_2$. The enzymatic activity of Hg^{2+}-treated ADA was completely and irreversibly abolished.

Cocultures of monocyte-derived DCs and $CD4^+CD45RA^+CD45RO^-$ lymphocytes

Human PBMCs were obtained immediately after blood extraction using the standard Ficoll gradient method [29]. Monocytes were obtained as de-

TABLE 1. Clinical Information of Patients with HIV-1

					T CD4		T CD4 Nadir		T CD8	
Patient	HAART	Sex	Age	VL	Cells/μl	%	Cells/μl	%	Cells/μl	%
1	yes	M	42	<DL	858	31.4	364	19.0	858	31.2
2	no	M	30	4.3	802	37.4	565	29.0	672	31.1
3	yes	M	37	<DL	930	29.5	504	19.2	1395	45.0
4	no	F	41	2.2	891	49.2	646	41.8	655	35.7
5	no	F	43	3.6	548	26.3	513	26.3	907	43.1
6	no	M	36	4.0	1007	38.3	714	28.0	1166	43.7
7	yes	M	39	<DL	497	22.5	264	21.6	1401	62.5
8	no	M	32	2.5	1122	34.5	1073	32.4	693	21.0
9	yes	M	45	<DL	914	34.0	450	23.8	618	23.2
10	yes	M	27	2.3	517	31.0	331	17.9	768	46.2
11	yes	M	44	<DL	854	34.8	220	16.0	951	38.8
12	yes	M	39	<DL	665	38.1	437	30.9	577	32.6
13	yes	M	53	<DL	740	25.2	375	14.6	1302	44.0
14	no	M	36	3.14	576	32.0	351	26.0	1098	61.0
15	no	F	53	1.8	540	20.0	451	19.0	1944	72.0
Mean			40	3.0	764.1	32	484	24	1000	42
SD			7	0.9	198	7	211	7	384	14

HAART, Highly active antiretroviral therapy; VL, viral load; DL, detection limit.

scribed previously [14] and were differentiated for 7 days to iDCs by adding 1000 U/ml IL-4 (Prospec-Tany Technogene Ltd., Rehovot, Israel) and 1000 U/ml GM-CSF (Prospec-Tany Technogene Ltd.) at Days 0, 3, and 5. DC immunophenotype ($CD3^-$, $CD4^-$, $CD8^-$, $CD14^-$, $CD19^-$, $CD56^-$, $HLA\text{-}DR^+$, $CD80^+$, $CD83^+$, $CD86^+$, $CD1a^+$, $CD11c^+$, $CD40^+$, $CD45RO^+$, $CD45RA^-$, $CD209^+$, $CXCR4^+$, $CCR5^-$) was confirmed by flow cytometry. A T cell-enriched fraction was obtained as described previously [14]. By using the human naïve $CD4^+$ T cell isolation kit (Miltenyi Biotec GmbH, Germany), a negative selection of these cells was performed, according to the manufacturer's instructions, to obtain $CD4^+CD45RA^+CD45RO^-CD25^-$ T cells. To confirm the purity and the naïve phenotype, the presence of CD4 and CD45RA and the absence of CD45RO and CD25 were tested by flow cytometry immediately after cell isolation. The purity of $CD4^+CD45RA^+CD45RO^-CD25^-$ T cells was between 94% and 98%. Autologous cocultures were performed in 96-well plates, containing naïve $CD4^+CD45RA^+CD45RO^-CD25^-$ T cells (2×10^5 cells/well) and iDCs (5×10^3 cells/well), pulsed or not with 100 pg/ml SEA for 3.5 h at 37°C in 200 μl/well XVIVO-10 medium containing or not 2 μM ADA, 20 ng/ml IL-6, 250 U/ml TNF-α, 1000 U/ml IFN-γ, or a combination of these cytokines. When indicated, cocultures were performed in the presence of 2 μM ADA and anti-IL-6 (150 ng/ml), anti-TNF-α (1.9 μg/ml), anti-IFN-γ (2 μg/ml), or irrelevant mouse IgG1 (2 μg/ml) as a negative control. Cocultures were incubated for the indicated times at 37°C in a humid atmosphere of 5% CO_2.

Immunostaining, quantification of Tregs, and flow cytometry

To analyze expression of cell surface markers, cocultured cells were collected, washed with PBS, resuspended at 2×10^6 cells/ml (50 μl/tube), and incubated with FITC-, PE-, and/or PerCP-conjugated antibodies (see above) for 30 min at 4°C. Cells were then washed with PBS, fixed with 1% formaldehyde in PBS, and analyzed by flow cytometry. FITC-, PE-, and PerCP-conjugated isotype-matched antibodies were used for negative control. When nonconjugated primary antibodies were used, cells washed with PBS were incubated for 30 min at 4°C with blocking buffer (PBS containing 5% BSA for ADA labeling or PBS containing 10% rabbit serum and 0.1% NaN_3 for CD26 or $A_{2B}R$ labeling) prior incubation with 10 μg/ml N-19, 20 μg/ml anti-ADA, 20 μg/ml irrelevant rabbit IgG (used as a negative control of anti-ADA), Ba5 (1:10), or mouse IgG2a (1:10) isotype control (used as a negative control of Ba5) for 30 min at 4°C. Cells were washed three times with blocking buffer and stained for flow cytometry analysis with the corresponding secondary antibodies: FITC-conjugated rabbit anti-goat IgG (1:400), FITC-conjugated rabbit anti-mouse IgG (1:20), and FITC-conjugated goat anti-rabbit IgG. Stained cells were analyzed on a FACSCan flow cytometer (Becton Dickinson). Cells were gated on the basis of forward- and side-scatter parameters, and this gating region was evaluated further for the expression of FL1, FL2, and FL3. To quantify Tregs, Foxp3 was detected using the commercially available human Treg staining kit (eBioscience), following the manufacturer's instructions. Cell samples were analyzed in a FACSCalibur flow cytometer (Becton Dickinson). In all cases, data were analyzed using CellQuest and FlowJo software.

Proliferation assays

T cell proliferation was determined by the CFSE method. Cells (10^7 cells/ml) were stained with 5 μM CFSE, as indicated by the manufacturer's protocol, using the CellTrace CFSE proliferation kit (Molecular Probes, Paisley, UK).

Statistical analysis

Data were analyzed using the GraphPad Prism 5.0 software. Quantitative variables were analyzed using medians and IQRs. The nonparametric Mann Whitney *U* test for unpaired data or the Wilcoxon signed rank test for paired data was used when comparing two groups. For multiple comparisons, the Kruskal-Wallis test, followed by Dunns post-test, was used. For all the tests used, a two-tailed *P* value <0.05 was considered statistically significant.

Online Supplemental material

Expression of cell surface ADA, CD26, and $A_{2B}R$ in T cells cocultured with SEA-pulsed DCs is shown in Supplemental Fig. 1. Kinetic analysis and the role of enzymatic and extraenzymatic activity of ADA in the generation of the $CD4^+CD25^{high}$ population are shown in Supplemental Fig. 2. Expression of $CD25^{high}$ on the $CD4^+$ T cell population from HIV-infected patients and healthy controls is also shown in Supplemental Fig. 2.

RESULTS

ADA enhanced the switch from the naïve $CD45RA^+CD45RO^-CD25^-$ to the effector $CD45RA^-CD45RO^+CD25^+$ phenotype

To address whether ADA has an impact over the generation of Teffs, naïve $CD4^+CD45RA^+CD45RO^-$ T cells were freshly isolated and cocultured with autologous, SEA-pulsed iDCs. As markers of effector phenotype, expression of CD45RO and CD25 was determined by flow cytometry. In the absence of exogenous ADA, the percentage of $CD45RO^+$ and $CD25^+$ T cells increased with time, reaching a maximum at Day 7. ADA promoted an increase in the percentage of T cells expressing CD45RO or CD25 with similar kinetics (**Fig. 1A** and **B**). Control assays performed with nonpulsed iDCs did not lead to significant increases of CD45RO or CD25 expression in the absence or presence of ADA (Fig. 1A and B). These results indicate that ADA enhanced the TCR-mediated generation of Teffs, but it had no effect by itself. The ADA-mediated, costimulatory effect on $CD4^+$ T cell proliferation is impaired in HIV-infected individuals [24]. Thereby, the question of whether the generation of Teffs is attenuated in cocultures obtained from HIV-infected patients, compared with healthy donors, was investigated. ADA promoted a statistically significant increase of CD45RO and CD25 expression in every tested healthy or HIV-infected donor, showing individual-dependent variability (Fig. 1C and D). The ADA-mediated effect was not significantly different when comparing HIV-infected and healthy donors (Fig. 1C and D, insets), suggesting that ADA is also able to increase generation of the Teff phenotype in these immunologically compromised individuals.

ADA potentiates T cell proliferation in autologous cocultures with Ag-pulsed human iDC or mature DC. Costimulation is not a result of the enzymatic activity but of the interaction of ADA–CD26 complexes on T cells with $A_{2B}R$ expressed on DCs [14]. Accordingly, in cocultures of naïve $CD4^+CD45RA^+CD45RO^-$ T cells and autologous, SEA-pulsed iDCs, ADA induced an increase in the T cell proliferation that was independent of Ado deamination. Thus, active ADA and ADA-Hg enhanced T cell proliferation (**Fig. 2A**). In cocultures performed in the presence of ADA or ADA-Hg, it was observed that active and enzymatically inactive ADA promoted an increase in the percentage of T cells expressing CD45RO (Fig. 2B), demonstrating that the deaminase activity was not required to potentiate the generation of Teffs. As it has been described previously that ADA, independently of its deami-

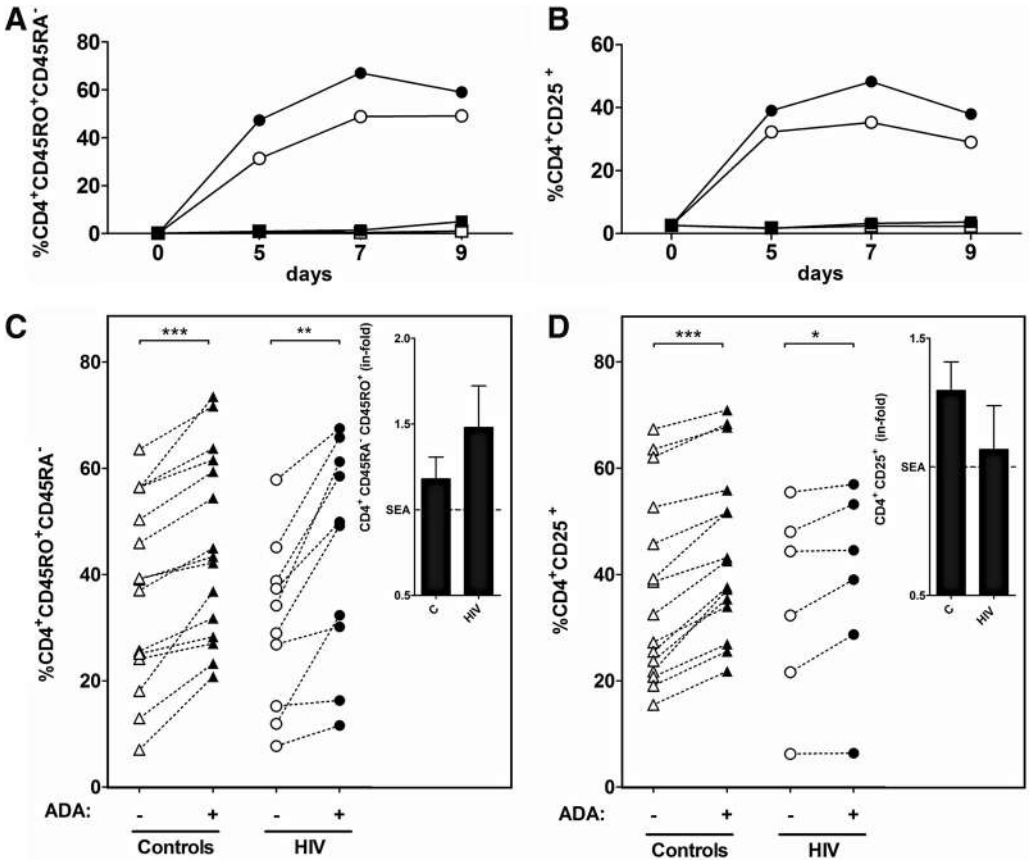

Figure 1. ADA effect on CD45RO and CD25 expression in naïve CD4$^+$ T-lymphocytes cocultured with autologous, SEA-pulsed iDCs. CD4$^+$CD45RA$^+$CD45RO$^-$ naïve T cells were freshly isolated and cocultured with autologous, SEA-pulsed iDCs in the absence or presence of 2 μM ADA, and subsequently, CD45RO and CD25 expression was determined by flow cytometry. (A and B) T cells were cocultured with nonpulsed (squares) or SEA-pulsed (circles) iDCs in the absence (open symbols) or presence (solid symbols) of ADA, and expression of CD45RO (A) and CD25 (B) was determined in the CD4$^+$-gated population. (C and D) The maximum level of expression of CD45RO (C) and CD25 (D) is depicted for cells from healthy donors (triangles) and HIV-infected individuals (circles) in the absence (open symbols) or presence (solid symbols) of ADA. Each pair of linked symbols represents results corresponding to cells from a particular individual. Values are represented as percentage of positive cells for the given surface marker. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. (C and D) Inset bar graphs represent the median ± IQR of the ratio (in-fold) of the percentage of positive cells obtained in the presence of ADA versus those obtained in its absence; the reference value of 1 is indicated by a dotted line. C, Control.

nase activity, leads to a marked increase in the production of the Th1 and proimmflamatory cytokines IFN-γ, TNF-α, and IL-6 [14], the role of these cytokines in the ADA-induced increase in CD45RO and CD25 expression was investigated. Accordingly, naïve CD4$^+$CD45RA$^+$CD45RO$^-$ T cells were cocultured with autologous, SEA-pulsed iDCs in the presence of ADA, IL-6, TNF-α, IFN-γ, or a combination of these factors, and CD45RO and CD25 expression on T cells was determined by flow cytometry. The concentration of cytokines used for these experiments was equivalent to the average of the maximum amount of cytokines produced in this experimental setup in response to ADA [14]. As shown in **Fig. 3A** and **C**, IL-6 and to a lesser extent, TNF-α and IFN-γ enhanced generation of Teffs. In magnitude, the IL-6-mediated effect was not significantly different from the ADA-mediated effect, and the maximum effect was obtained when using a cocktail of the three cytokines (Fig. 3A and C) or when using IL-6 plus TNF-α or IL-6 plus IFN-γ (data not shown). In cocultures performed in the presence of ADA and activity-neutralizing antibodies directed against IL-6, TNF-α, or IFN-γ, the ADA-induced effect was reduced significantly (Fig. 3B and D), something that was not encountered when an irrelevant mouse IgG1 was used (data not shown). Neither CD45RO nor CD25 expression was modified significantly in cocultures performed in the presence of IL-6 and the anti-IL-6 antibody, TNF-α and the anti-TNF-α antibody, or IFN-γ and the anti-IFN-γ antibody (data not shown), which confirmed the neutralizing action of these antibodies. Overall, these results indicate that IL-6, TNF-α, and IFN-γ may be involved in the ADA-induced pathway, leading to the potentiation of Teff generation.

Taking into account that ADA induces a costimulatory effect by interacting with CD26 on the T cell surface and with $A_{2B}R$ on the iDCs surface [14], control experiments were performed to test whether the T cell–iDC interaction is able to modulate the expression of ADA and ADA-binding proteins. Results show that ADA expression on the T cell surface was increased by coculturing them with SEA-pulsed iDCs; the effect was slightly potentiated by IL-6 and by a cocktail of IL-6, TNF-α, and IFN-γ (Supplemental Fig. 1A). It was also observed that the CD26 expression on the T cell surface was increased significantly when T cells were cocultured with SEA-pulsed iDCs, and this effect was also slightly potentiated by IL-6 and by a cocktail of IL-6, TNF-α, and IFN-γ but not by ADA (Supplemental Fig. 1B). All CD4$^+$ T cells expressed $A_{2B}R$ on the plasma membrane (Supplemental Fig. 1C), and levels of this receptor increased when naïve T cells were cocultured with SEA-pulsed iDCs, as measured by the geometric mean value of fluorescence intensity. Neither ADA nor the cytokines described above were able to further increase $A_{2B}R$ expression levels on CD4$^+$ T cells (Supplemental Fig. 1C and D).

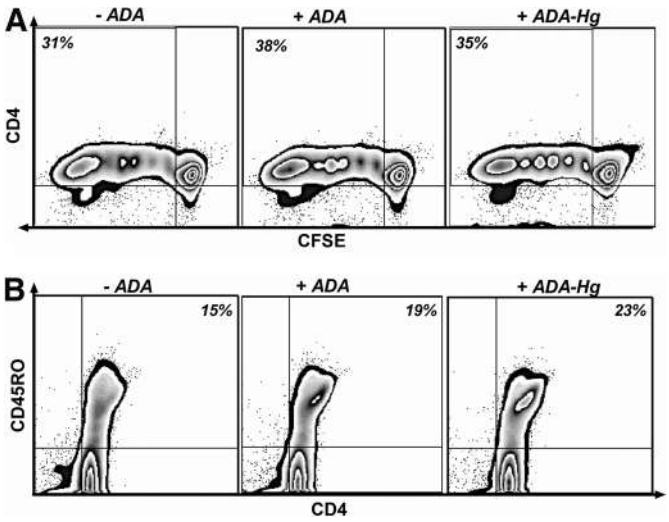

Figure 2. Role of ADA enzymatic activity on the proliferation and the expression of CD45RO in $CD4^+$ T cells cocultured with SEA-pulsed DCs. $CD4^+CD45RA^+CD45RO^-$ naïve T cells, freshly isolated from healthy donors, were labeled with CFSE and cocultured with autologous, SEA-pulsed iDCs in the absence or presence of 2 μM ADA or ADA-Hg. (A) $CD4^+$ T cell proliferation was analyzed by flow cytometry at Day 7 of coculture. (B) Contour plots showing CD45RO expression at Day 14 of coculture are shown. Representative data from one of three independent experiments are shown.

ADA enhanced the generation of $CD4^+CD45RO^+CD45RA^-CD25^-$ memory T cells

To determine the effect of ADA upon memory T cell generation after effector responses, T cells with the memory phenotype $CD4^+CD45RO^+CD25^-$ were identified in cocultures of naïve $CD4^+CD45RA^+CD45RO^-$ T cells and autologous, SEA-pulsed iDCs. The CD45RA expression in the $CD4^+CD25^-$ population decreased upon time (**Fig. 4A**, upper image), and this $CD45RA^-$ population was confirmed to be $CD45RO^+$ (Fig. 4A, lower image). Importantly, the presence of ADA induced a notable increase of memory T cells at Day 14 of coculture (Fig. 4A). The ADA-mediated effect was observed in cells from almost every healthy or HIV-infected donor (Fig. 4B) without significant differences when comparing HIV-infected and healthy individuals. Thus, the ADA induced enhancement of the Teff phenotype during the peak of T cell response results, after the contraction phase, in an increased generation of memory T cells.

To test whether the ADA enzymatic activity is involved in the ADA-induced increase of memory T cell generation, cocultures were performed in the presence of ADA or ADA-Hg. As shown in Fig. 4C, ADA and ADA-Hg promoted an increase in the percentage of CD45RO-expressing cells within the $CD4^+CD25^-$-gated population of T cells. These results indicate that ADA enzymatic activity was not required to enhance memory T cell generation and suggest that similarly to ADA-enhanced Teff generation, augmented memory T cell production is mediated by interaction of ADA with ADA-binding proteins. To investigate the role of IFN-γ, TNF-α, and IL-6 on the ADA-induced increase of memory T cell generation, cocultures of naïve $CD4^+CD45RA^+CD45RO^-$ T cells and autologous, SEA-pulsed iDCs were performed in the absence or in the presence of ADA, IFN-γ, TNF-α, or IL-6 and in the presence of a combination of these factors. As shown in **Fig. 5A** and **B**, IL-6 and to a lesser extent, IFN-γ and TNF-α were able to emulate the ADA effect on the $CD4^+CD25^-CD45RO^+$ T cell generation. When the three cytokines were added together, an additive effect was observed. Moreover, when IL-6 was combined with IFN-γ or TNF-α, but not when TNF-α was combined with IFN-γ, a significant increase in the generation of memory $CD4^+$ T cells was reached (data not shown). In cocultures performed in the presence of ADA and neutralizing antibodies directed against IL-6, TNF-α, or IFN-γ, the ADA-induced effect was reduced (Fig. 5C), and it did not change in the presence of irrelevant mouse IgG1 (data not shown). As a negative control, the generation of memory $CD4^+$ T cells was not significantly modified in cocultures performed in the presence of IL-6 and the anti-IL-6 antibody, TNF-α and the anti-TNF-α antibody, or IFN-γ and the anti-IFN-γ antibody (data not shown). Taken together, these results indicate that mainly IL-6, and to a minor extent, IFN-γ and TNF-α are involved in the ADA-promoted enhancement of the memory $CD4^+$ T cell generation.

ADA enhanced the generation of $CD4^+CD25^{high}Foxp3^+$ Tregs

According to the important role played by Tregs in the regulation of immune responses and as a result of the fact that ADA may modulate the generation of other T cell phenotypes (Figs. 1 and 4), the question of whether ADA is able to modulate the generation of Tregs was investigated. To determine the presence of a $CD4^+CD25^{high}Foxp3^+$ Treg subset in the $CD4^+CD25^+$ T cell population, cocultures of naïve $CD4^+CD45RA^+CD45RO^-$ T cells and autologous, SEA-pulsed iDCs were performed. According to the expression of $CD25^+$, three different $CD4^+$ T cell subpopulations were identified by flow cytometry: cells not expressing CD25, cells moderately expressing CD25, and cells with a high expression of CD25 ($CD25^{high}$; Supplemental Fig. 2). The $CD25^{high}$ subset of $CD4^+$ T cells appeared at Day 5 of coculture, and it became almost undetectable at later time periods (Supplemental Fig. 2A). In the presence of ADA, but not in the presence of ADA-Hg, the percentage of $CD25^{high}$ from HIV-infected and healthy donors was higher than in its absence (Supplemental Fig. 2B and C). To gain further evidence about the role of ADA on the Treg generation, the expression of the transcriptional factor Foxp3 within the $CD4^+CD25^{high}$ population was also assessed in coculture experiments at Day 5. ADA induced an increased generation of $CD4^+CD25^{high}Foxp3^+$ T cells in cocultures from healthy and HIV-infected donors (**Fig. 6A**). The effect was not significantly different on comparing HIV-infected patients and healthy controls. Controls performed using nonpulsed iDCs did not lead to the generation of the $CD4^+CD25^{high}Foxp3^+$ T cell phenotype, in the absence or in the presence of ADA (data not shown). Enzyme-inactive ADA (Fig. 6B) was unable to potentiate the generation of $CD4^+CD25^{high}Foxp3^+$ T cells in cells from healthy or HIV-infected donors. Taken together, these results suggest that deamination of endogenous Ado promoted an enhanced generation of Tregs during the effector phase of the T

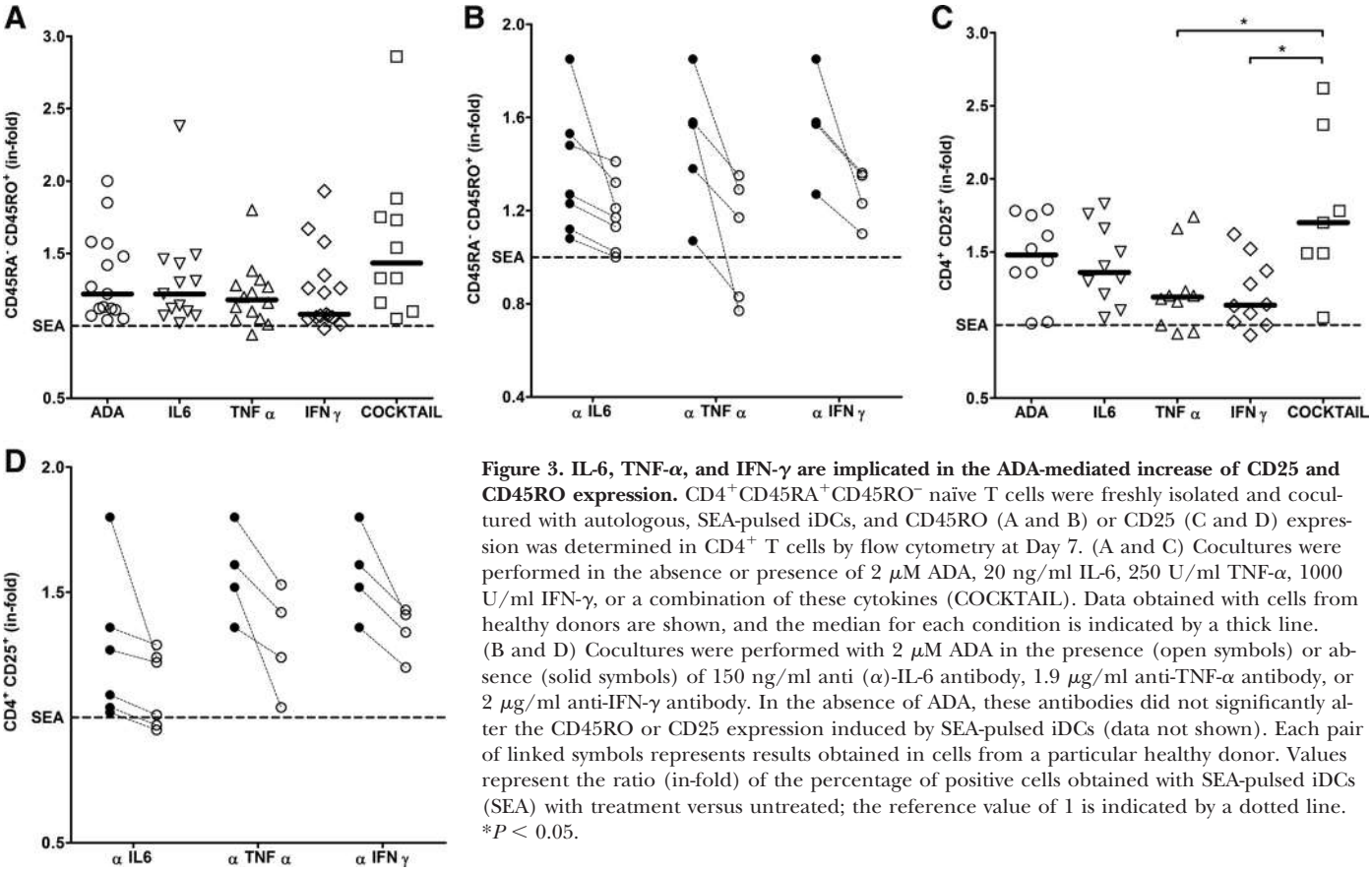

Figure 3. IL-6, TNF-α, and IFN-γ are implicated in the ADA-mediated increase of CD25 and CD45RO expression. $CD4^+CD45RA^+CD45RO^-$ naïve T cells were freshly isolated and cocultured with autologous, SEA-pulsed iDCs, and CD45RO (A and B) or CD25 (C and D) expression was determined in $CD4^+$ T cells by flow cytometry at Day 7. (A and C) Cocultures were performed in the absence or presence of 2 μM ADA, 20 ng/ml IL-6, 250 U/ml TNF-α, 1000 U/ml IFN-γ, or a combination of these cytokines (COCKTAIL). Data obtained with cells from healthy donors are shown, and the median for each condition is indicated by a thick line. (B and D) Cocultures were performed with 2 μM ADA in the presence (open symbols) or absence (solid symbols) of 150 ng/ml anti (α)-IL-6 antibody, 1.9 μg/ml anti-TNF-α antibody, or 2 μg/ml anti-IFN-γ antibody. In the absence of ADA, these antibodies did not significantly alter the CD45RO or CD25 expression induced by SEA-pulsed iDCs (data not shown). Each pair of linked symbols represents results obtained in cells from a particular healthy donor. Values represent the ratio (in-fold) of the percentage of positive cells obtained with SEA-pulsed iDCs (SEA) with treatment versus untreated; the reference value of 1 is indicated by a dotted line. $*P < 0.05$.

cell response. According to this notion, the ADA-mediated potentiation on the generation of $CD4^+CD25^{high}Foxp3^+$ T cells was not directly related to the ADA-induced IFN-γ, TNF-α, and IL-6 secretion. In fact, when the generation of $CD4^+CD25^{high}Foxp3^+$ T cells was analyzed in cocultures performed in the absence or in the presence of ADA or in the presence of IFN-γ, TNF-α, IL-6, or a cocktail of these cytokines, the ADA-triggered increase of Treg production was not emulated (**Fig. 7**). Therefore, the generation of Tregs seemed to be dependent on the enzymatic activity of ADA.

DISCUSSION

The lack of ADA is deleterious for the development and function of lymphocytes. The pathological mechanism linking ADA deficiency to SCID was first assumed to be largely dependent on dATP accumulation and toxicity [30]. AdoRs were placed into the equation later on, as excess of Ado as a result of an ADA deficit may lead to sustained activation of AdoRs present in lymphocytes [10, 31]. AdoR-mediated regulation of the immune system is mediated by at least three of the four receptor subtypes, A_{2B} [32, 33], A_3 [34], and A_{2A} [12, 35]. As we demonstrated that ADA was on the cell surface of lymphocytes [36, 37], and cell surface ADA-binding proteins started to be identified [38–41], the enzyme-independent, signaling-dependent role of ADA on immune function has been demonstrated. Cell surface ADA may therefore contribute to lymphocyte development, activation, and fate by two mechanisms: by regulating the levels of extracellular Ado available to AdoRs and by interacting with ADA-binding proteins and engaging cAMP-independent signaling pathways. It is here shown that ADA-mediated costimulation, in a deaminase activity-independent way, promotes an enhanced production of Teffs during the effector phase of the T cell response and augmented generation of memory T cells after the effector phase. Furthermore, the enzymatic activity of ADA promotes an augmented generation of Tregs during the effector phase of the T cell response. Importantly, the potentiation in the induction of Teffs, Tregs, and memory T cells was not only observed in cocultures of cells obtained from healthy individuals but also in cultures of cells obtained from HIV-infected patients.

Cellular and molecular mechanisms involved in the generation of memory T cells are not yet well known. The findings here reported point to the idea that the intercellular $A_{2B}R$-ADA-CD26 interaction contributes to the differentiation from naïve $CD4^+CD45RA^+$ T cells toward memory $CD4^+CD45RO^+$ T cells. It has been demonstrated that CD26-triggered signaling occurs through its interaction with CD45 in the plasma membrane of T cells [42, 43]. When CD45RO anchors CD26 in lipid raft microdomains, CD26 engagement evokes stimulatory signals for T cell activation [42]; in contrast, when CD45RA maintains CD26 excluded from lipid rafts, CD26 engagement attenuates T cell activation [43]. This evidence suggests that in our coculture conditions, the interaction of the

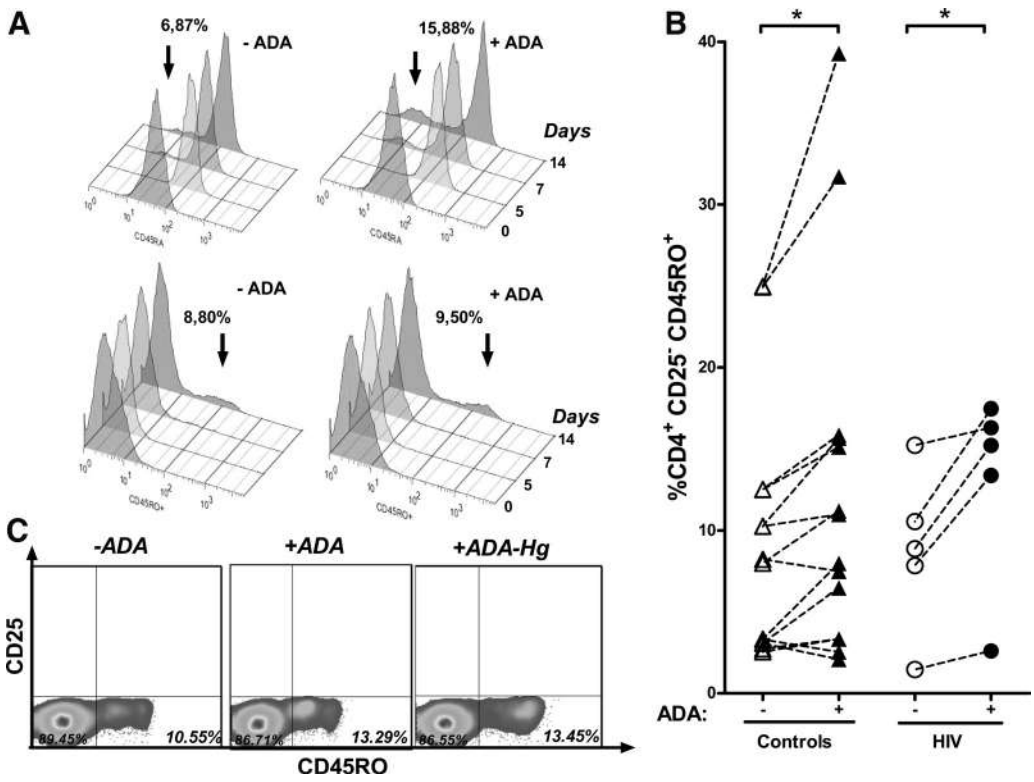

Figure 4. ADA increases the generation of a CD4$^+$ T cell population with memory phenotype. CD4$^+$CD45RA$^+$CD45RO$^-$ naïve T cells were freshly isolated from healthy donors and cocultured with autologous, SEA-pulsed iDCs in the absence or presence of 2 μM ADA, and subsequently, CD45RA, CD45RO, and CD25 expression was determined by flow cytometry on CD4$^+$ T cells. (A) 3D-Overlaid histograms reflecting kinetics of CD45RA (upper images) or CD45RO (lower images) expression in the CD4$^+$CD25$^-$-gated population are shown (representative experiment). Arrows indicate the CD45RA$^-$ or CD45RO$^+$ population at Day 14 of coculture. (B) Percentages of CD4$^+$CD25$^-$CD45RO$^+$ cells induced in healthy (triangles) or HIV-infected (circles) donors after 14 days of coculture in the absence (open symbols) or presence (solid symbols) of ADA. Each pair of linked symbols represents data obtained using cells from a particular individual. *$P < 0.05$. (C) Contour plots of CD45RO expression in the CD4$^+$CD25$^-$-gated population of T cells in cocultures, treated or not with 2 μM ADA or ADA-Hg.

A_{2B}R-ADA-CD26 complex with CD45RA would not, at first, promote any positive effect on the generation of Teffs and memory T cells. Indeed, no ADA-mediated increase of CD25 or CD45RO expression was observed at short incubation times (data not shown). In contrast, when the TCR-triggered switch of CD45RA to CD45RO is induced, the ADA-mediated effect would begin to operate positively in the production of Teffs and later, in the generation of memory T cells.

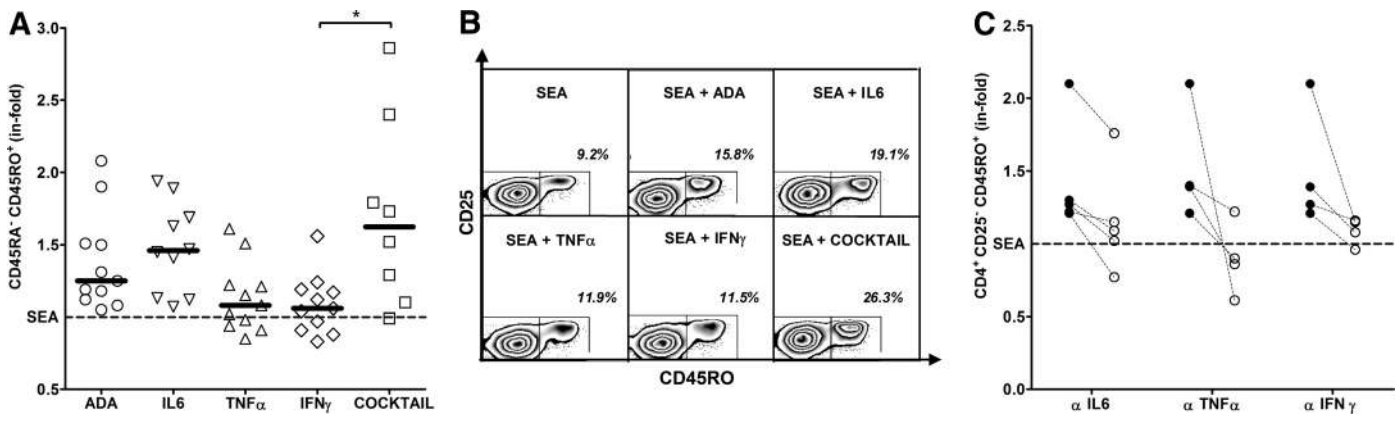

Figure 5. Role of IL-6, TNF-α, and IFN-γ in the ADA-induced increase of memory CD4$^+$ T cell generation. CD4$^+$CD45RA$^+$CD45RO$^-$ naïve T cells were freshly isolated from healthy donors and cocultured with autologous, SEA-pulsed iDCs, and subsequently, CD45RA, CD45RO, and CD25 expression was determined on CD4$^+$ T cells by flow cytometry at Day 14. (A and B) Cocultures were performed in the absence or presence of 2 μM ADA, 20 ng/ml IL-6, 250 U/ml TNF-α, 1000 U/ml IFN-γ, or a combination of these cytokines (COCKTAIL). (A) Expression of the memory phenotype CD45RA$^-$CD45RO$^+$ from healthy donors is shown, and the median for each treatment is indicated by a thick line. (B) Representative contour plots of CD45RO expression in the CD4$^+$CD25$^-$-gated population. (C) Expression of CD4$^+$CD25$^-$CD45RO$^+$ cells in cocultures performed with 2 μM ADA in the absence (solid symbols) or in the presence (open symbols) of 150 ng/ml anti-IL-6 antibody, 1.9 μg/ml anti-TNF-α antibody, or 2 μg/ml anti-IFN-γ antibody is depicted. Each pair of linked symbols represents data obtained using cells from a particular donor. (A and C) Values represent the ratio (in-fold) of the percentage of positive cells obtained with treatment versus untreated; the reference value of 1 is indicated by a dotted line. *$P < 0.05$.

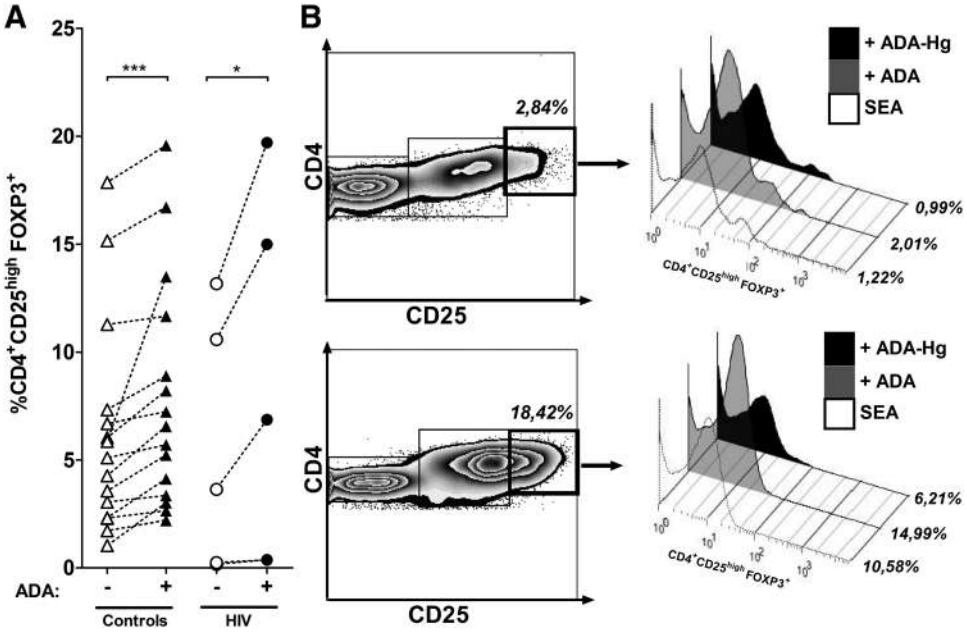

Figure 6. ADA increased the generation of $CD4^+CD25^{high}$ $Foxp3^+$ Tregs. Freshly isolated, naïve $CD4^+CD45RA^+CD45RO^-$ T cells were cocultured for 5 days (see Supplemental Fig. 2) with autologous, SEA-pulsed DCs in the absence or presence of 2 μM ADA. Expression of Foxp3 in the $CD4^+CD25^{high}$ population (gated as indicated in B) was determined. (A) Expression of $CD4^+CD25^{high}$ $Foxp3^+$ T cells in cocultures obtained using cells from healthy (triangles) or HIV-infected (circles) donors in the absence (open symbols) or presence (solid symbols) of ADA. Each pair of linked symbols represents a different donor. $*P < 0.05$; $***P < 0.001$. (B) Contour plots showing $CD25^{high}$ expression in $CD4^+$ T cells and 3D-overlaid histograms representing Foxp3 expression in the $CD4^+CD25^{high}$-gated population in cocultures using cells from a healthy (upper image) or a HIV-infected (lower image) donor, performed in the absence or in the presence of 2 μM ADA or 2 μM ADA-Hg.

CD39 and CD73 have been described as two surface markers specifically expressed on the $Foxp3^+$ population of $CD4^+$ T cells [44, 45]. The coordinated action of these two ecto-enzymes catalyzes the production of pericellular Ado from extracellular nucleotides. As a result of the inhibitory role of $A_{2A}R$ stimulation in the activation and function of Teffs [11–13], the synthesis of extracellular Ado by Tregs constitutes one of the regulatory mechanisms operating in these cells to attenuate Teff responses [44, 45]. In this regard, enhanced expression of ecto-ADA would enhance Ado degradation, thus leading to make Teffs less responsive to the inhibition exerted by Tregs. On the other hand, we observed here that ADA promoted an augmented generation of $CD4^+CD25^{high}Foxp3^+$ Tregs by a mechanism that seemed to be mainly dependent on its enzymatic activity. This constitutes the first study reporting a regulatory role of Ado in the generation of Tregs. Therefore, it seems that the deaminase activity of ADA is involved in potentiating the generation of Tregs.

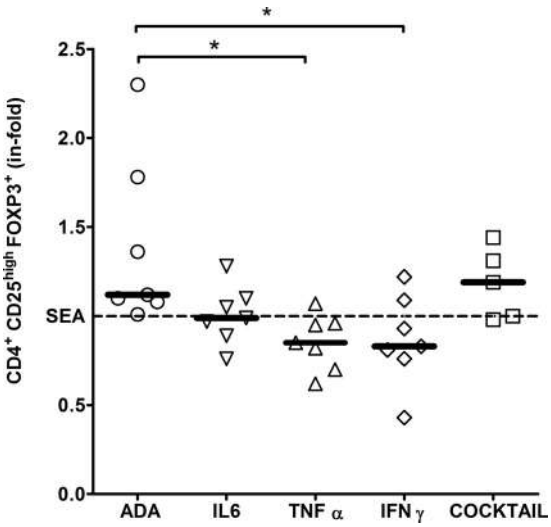

Figure 7. Role of IL-6, TNF-α and IFN-γ in the ADA-induced increase of Tregs. $CD4^+CD45RA^+CD45RO^-$ naïve T cells were freshly isolated and cocultured with autologous, SEA-pulsed iDCs in the absence or presence of 2 μM ADA, 20 ng/ml IL-6, 250 U/ml TNF-α, 1000 U/ml IFN-γ, or a combination of these cytokines (COCKTAIL), and the $CD25^{high}Foxp3^+$ subset in the $CD4^+$ T cell population was quantified by flow cytometry at Day 5. Data obtained using cells from healthy donors are shown, and the median for each treatment is indicated by a thick line. Values represent the ratio (in-fold) of the percentage of $CD4^+CD25^{high}Foxp3^+$ cells obtained with SEA-pulsed iDCs with treatment versus untreated; the reference value of 1 is indicated by a dotted line. $*P < 0.05$.

Several studies have revealed a correlation among depletion of $CD4^+CD26^+$ T cells, increased serum levels of ADA, and the evolution of AIDS in infected individuals [46–48]. Furthermore, it has been described that ADA binding to CD26 is inhibited by soluble rHIV-1 envelope glycoprotein gp120 and by HIV-1 infectious particles [49]. Importantly, whereas ADA-CD26-mediated costimulation of T cell activation is impaired in HIV-1-infected patients [24], we here show that ADA-mediated costimulation promotes potentiation in the generation of Teffs and memory T cells in a similar extent to that observed in cocultures from healthy individuals. Thereby, exogenous addition of ADA could increase effector response and generation of $CD4^+$ memory T cells against HIV. As ADA-mediated enhancement in the production of Teffs is followed by a delayed increase of Tregs, the addition of exogenous ADA to potentiate effector response seems to be a self-limited and promising approach to be used as a complement for immunostimulatory therapies. Another important point to consider is that whereas HIV-infected patients require potentiation of the Th1 reponse [25], their T cell responses are often deviated toward Th2 [25]. We have described previously that ADA-mediated costimulation favors a Th1 pattern in $CD4^+$ T cells [14]. Thus,

an ADA-based therapy could be beneficial for potentiation of Th1 effector responses and generation of immunological memory in HIV-infected individuals. In this regard, we have proposed recently that ADA would be a good adjuvant to be used in a DC-based vaccine for the treatment of AIDS [25]. Similarly, this idea could be applicable to potentiate an antitumor Th1 response in cancer patients.

The present results not only contribute to the knowledge of T cell biology but also provide a rationale for the development of therapeutic strategies aimed at reinforcing a compromised immune system. In fact, an imbalance between Teffs and Tregs has been observed in pathological scenarios ranging from immunosuppression [50, 51] and cancer [52] to autoimmunity [53, 54]. As a result of the fact that development of vaccines relies on the generation of memory T cells [55], our results may also be useful in the formulation of novel vaccines or immunotherapies.

AUTHORSHIP

J.M.M-N., V.C., R.P., T.G., C.L., and R.F. conceived of and designed the experiments; J.M.M-N., V.C., R.P., I.N-M., and N.C. performed the experiments; J.M.M-N., V.C., R.P., F.G., J.M.G., J.M., T.G., C.L., and R.F. analyzed data; and J.M.M-N., V.C., R.P., and R.F. wrote the paper.

ACKNOWLEDGMENTS

This study was supported in part by FIPSE (a nonprofit foundation including Spanish Ministry of Health, Abbott Laboratories, Boehringer Ingelheim, Bristol Myers Squibb, GlaxoSmithKline, Merck Sharp, and Dohme and Roche) 36750/08 (to R.F.) and 36536/05 (to T.G.), HIVACAT (Center for Research and Development of HIV Vaccines in Catalonia), ISCIII-RETIC RD06/006 (Red Temática Cooperativa de Grupos de Investigación en SIDA del Fondo de Investigación Sanitaria de la Seguridad Social; to N.C., F.G., J.M.G., and T.G.), and FIS-PI06-1259 (to T.G.), RIS-173 and USS-5048 (to R.P.), and FONDECYT 1095114 and PFB-16 (to R.P.) from Comisión Nacional de Investigación Científica y Tecnológica. The authors are grateful to all blood donors and to M. C. Pardo, M. Compte, and M. A. López for technical assistance in blood extraction.

REFERENCES

1. DiPaolo, R. J., Brinster, C., Davidson, T. S., Andersson, J., Glass, D., Shevach, E. M. (2007) Autoantigen-specific TGFβ-induced Foxp3+ regulatory T cells prevent autoimmunity by inhibiting dendritic cells from activating autoreactive T cells. *J. Immunol.* **179,** 4685–4693.
2. Vandenbark, A. A., Offner, H. (2008) Critical evaluation of regulatory T cells in autoimmunity: are the most potent regulatory specificities being ignored? *Immunology* **125,** 1–13.
3. Abbal, C., Jourdan, P., Hori, T., Bousquet, J., Yssel, H., Pene, J. (1999) TCR-mediated activation of allergen-specific CD45RO(+) memory T lymphocytes results in down-regulation of cell-surface CXCR4 expression and a strongly reduced capacity to migrate in response to stromal cell-derived factor-1. *Int. Immunol.* **11,** 1451–1462.
4. Seddiki, N., Santner-Nanan, B., Martinson, J., Zaunders, J., Sasson, S., Landay, A., Solomon, M., Selby, W., Alexander, S. I., Nanan, R., Kelleher, A., Fazekas de St Groth, B. (2006) Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. *J. Exp. Med.* **203,** 1693–1700.
5. Badovinac, V. P., Harty, J. T. (2006) Programming, demarcating, and manipulating CD8+ T-cell memory. *Immunol. Rev.* **211,** 67–80.
6. Farber, D. L. (1998) Differential TCR signaling and the generation of memory T cells. *J. Immunol.* **160,** 535–539.
7. Franco, R., Valenzuela, A., Lluis, C., Blanco, J. (1998) Enzymatic and extraenzymatic role of ecto-adenosine deaminase in lymphocytes. *Immunol. Rev.* **161,** 27–42.
8. Franco, R., Pacheco, R., Gatell, J. M., Gallart, T., Lluis, C. (2007) Enzymatic and extraenzymatic role of adenosine deaminase 1 in T-cell-dendritic cell contacts and in alterations of the immune function. *Crit. Rev. Immunol.* **27,** 495–509.
9. Pacheco, R., Lluis, C., Franco, R. (2005) Role of CD26-adenosine deaminase interaction in the T cell-mediated immunity. *Inmunología* **24,** 235–245 (http://revista.immunologia.org/Upload/Articles/6/5/658.pdf).
10. Hershfield, M. S. (2005) New insights into adenosine-receptor-mediated immunosuppression and the role of adenosine in causing the immunodeficiency associated with adenosine deaminase deficiency. *Eur. J. Immunol.* **35,** 25–30.
11. Ohta, A., Sitkovsky, M. (2001) Role of G-protein-coupled adenosine receptors in downregulation of inflammation and protection from tissue damage. *Nature* **414,** 916–920.
12. Huang, S., Apasov, S., Koshiba, M., Sitkovsky, M. (1997) Role of A2a extracellular adenosine receptor-mediated signaling in adenosine-mediated inhibition of T-cell activation and expansion. *Blood* **90,** 1600–1610.
13. Gessi, S., Varani, K., Merighi, S., Ongini, E., Borea, P. A. (2000) A(2A) adenosine receptors in human peripheral blood cells. *Br. J. Pharmacol.* **129,** 2–11.
14. Pacheco, R., Martinez-Navio, J. M., Lejeune, M., Climent, N., Oliva, H., Gatell, J. M., Gallart, T., Mallol, J., Lluis, C., Franco, R. (2005) CD26, adenosine deaminase, and adenosine receptors mediate costimulatory signals in the immunological synapse. *Proc. Natl. Acad. Sci. USA* **102,** 9583–9588.
15. Panther, E., Corinti, S., Idzko, M., Herouy, Y., Napp, M., la Sala, A., Girolomoni, G., Norgauer, J. (2003) Adenosine affects expression of membrane molecules, cytokine and chemokine release, and the T-cell stimulatory capacity of human dendritic cells. *Blood* **101,** 3985–3990.
16. Panther, E., Idzko, M., Herouy, Y., Rheinen, H., Gebicke-Haerter, P. J., Mrowietz, U., Dichmann, S., Norgauer, J. (2001) Expression and function of adenosine receptors in human dendritic cells. *FASEB J.* **15,** 1963–1970.
17. Fossetta, J., Jackson, J., Deno, G., Fan, X., Du, X. K., Bober, L., Soude-Bermejo, A., de Bouteiller, O., Caux, C., Lunn, C., Lundell, D., Palmer, R. K. (2003) Pharmacological analysis of calcium responses mediated by the human A3 adenosine receptor in monocyte-derived dendritic cells and recombinant cells. *Mol. Pharmacol.* **63,** 342–350.
18. Hofer, S., Ivarsson, L., Stoitzner, P., Auffinger, M., Rainer, C., Romani, N., Heufler, C. (2003) Adenosine slows migration of dendritic cells but does not affect other aspects of dendritic cell maturation. *J. Invest. Dermatol.* **121,** 300–307.
19. Schnurr, M., Toy, T., Shin, A., Hartmann, G., Rothenfusser, S., Soellner, J., Davis, I. D., Cebon, J., Maraskovsky, E. (2004) Role of adenosine receptors in regulating chemotaxis and cytokine production of plasmacytoid dendritic cells. *Blood* **103,** 1391–1397.
20. Aandahl, E. M., Moretto, W. J., Haslett, P. A., Vang, T., Bryn, T., Tasken, K., Nixon, D. F. (2002) Inhibition of antigen-specific T cell proliferation and cytokine production by protein kinase A type I. *J. Immunol.* **169,** 802–808.
21. Cone, R. E., Cochrane, R., Lingenheld, E. G., Clark, R. B. (1996) Elevation of intracellular cyclic AMP induces an anergic-like state in Th1 clones. *Cell. Immunol.* **173,** 246–251.
22. Dong, R. P., Kameoka, J., Hegen, M., Tanaka, T., Xu, Y., Schlossman, S. F., Morimoto, C. (1996) Characterization of adenosine deaminase binding to human CD26 on T cells and its biologic role in immune response. *J. Immunol.* **156,** 1349–1355.
23. Hashikawa, T., Hooker, S. W., Maj, J. G., Knott-Craig, C. J., Takedachi, M., Murakami, S., Thompson, L. F. (2004) Regulation of adenosine receptor engagement by ecto-adenosine deaminase. *FASEB J.* **18,** 131–133.
24. Martinez-Navio, J. M., Climent, N., Pacheco, R., Garcia, F., Plana, M., Nomdedeu, M., Oliva, H., Rovira, C., Miralles, L., Gatell, J. M., Gallart, T., Mallol, J., Lluis, C., Franco, R. (2009) Immunological dysfunction in HIV-1-infected individuals by impairment of adenosine deaminase-induced co-stimulation of T-cell activation. *Immunology* **128,** 393–404.
25. Climent, N., Martinez-Navio, J. M., Gil, C., Garcia, F., Rovira, C., Hurtado, C., Miralles, L., Gatell, J. M., Gallart, T., Mallol, J., Lluis, C., Franco, R. (2009) Adenosine deaminase enhances T-cell response elicited by dendritic cells loaded with inactivated HIV. *Immunol. Cell Biol.* **87,** 634–639.
26. Ginés, S., Ciruela, F., Burgueno, J., Casado, V., Canela, E. I., Mallol, J., Lluis, C., Franco, R. (2001) Involvement of caveolin in ligand-induced recruitment and internalization of A(1) adenosine receptor and adenosine deaminase in an epithelial cell line. *Mol. Pharmacol.* **59,** 1314–1323.
27. Franco, R., Canela, E. I., Bozal, J. (1986) Heterogeneous localization of some purine enzymes in subcellular fractions of rat brain and cerebellum. *Neurochem. Res.* **11,** 423–435.
28. Ciruela, F., Saura, C., Canela, E. I., Mallol, J., Lluis, C., Franco, R. (1996) Adenosine deaminase affects ligand-induced signaling by interacting with cell surface adenosine receptors. *FEBS Lett.* **380,** 219–223.
29. Bøyum, A. (1976) Isolation of lymphocytes, granulocytes and macrophages. *Scand. J. Immunol.* **5,** 9–15.

30. Hirschhorn, R. (1995) Adenosine deaminase deficiency: molecular basis and recent developments. *Clin. Immunol. Immunopathol.* **76,** S219–S227.
31. Resta, R., Thompson, L. F. (1997) SCID: the role of adenosine deaminase deficiency. *Immunol. Today* **18,** 371–374.
32. Mirabet, M., Herrera, C., Cordero, O. J., Mallol, J., Lluis, C., Franco, R. (1999) Expression of A2B adenosine receptors in human lymphocytes: their role in T cell activation. *J. Cell Sci.* **112,** 491–502.
33. Gessi, S., Varani, K., Merighi, S., Cattabriga, E., Pancaldi, C., Szabadkai, Y., Rizzuto, R., Klotz, K. N., Leung, E., Mac Lennan, S., Baraldi, P. G., Borea, P. A. (2005) Expression, pharmacological profile, and functional coupling of A2B receptors in a recombinant system and in peripheral blood cells using a novel selective antagonist radioligand, [3H]MRE 2029-F20. *Mol. Pharmacol.* **67,** 2137–2147.
34. Gessi, S., Varani, K., Merighi, S., Cattabriga, E., Avitabile, A., Gavioli, R., Fortini, C., Leung, E., Mac Lennan, S., Borea, P. A. (2004) Expression of A3 adenosine receptors in human lymphocytes: up-regulation in T cell activation. *Mol. Pharmacol.* **65,** 711–719.
35. Csóka, B., Himer, L., Selmeczy, Z., Vizi, E. S., Pacher, P., Ledent, C., Deitch, E. A., Spolarics, Z., Nemeth, Z. H., Hasko, G. (2008) Adenosine A2A receptor activation inhibits T helper 1 and T helper 2 cell development and effector function. *FASEB J.* **22,** 3491–3499.
36. Aran, J. M., Colomer, D., Matutes, E., Vives-Corrons, J. L., Franco, R. (1991) Presence of adenosine deaminase on the surface of mononuclear blood cells: immunochemical localization using light and electron microscopy. *J. Histochem. Cytochem.* **39,** 1001–1008.
37. Martín, M., Aran, J. M., Colomer, D., Huguet, J., Centelles, J. J., Vives-Corrons, J. L., Franco, R. (1995) Surface adenosine deaminase. A novel B-cell marker in chronic lymphocytic leukemia. *Hum. Immunol.* **42,** 265–273.
38. Kameoka, J., Tanaka, T., Nojima, Y., Schlossman, S. F., Morimoto, C. (1993) Direct association of adenosine deaminase with a T cell activation antigen, CD26. *Science* **261,** 466–469.
39. Saura, C., Ciruela, F., Casado, V., Canela, E. I., Mallol, J., Lluis, C., Franco, R. (1996) Adenosine deaminase interacts with A1 adenosine receptors in pig brain cortical membranes. *J. Neurochem.* **66,** 1675–1682.
40. Herrera, C., Casado, V., Ciruela, F., Schofield, P., Mallol, J., Lluis, C., Franco, R. (2001) Adenosine A2B receptors behave as an alternative anchoring protein for cell surface adenosine deaminase in lymphocytes and cultured cells. *Mol. Pharmacol.* **59,** 127–134.
41. Gracia, E., Cortes, A., Meana, J. J., Garcia-Sevilla, J., Herhsfield, M. S., Canela, E. I., Mallol, J., Lluis, C., Franco, R., Casado, V. (2008) Human adenosine deaminase as an allosteric modulator of human A(1) adenosine receptor: abolishment of negative cooperativity for [H](R)-pia binding to the caudate nucleus. *J. Neurochem.* **107,** 161–170.
42. Ishii, T., Ohnuma, K., Murakami, A., Takasawa, N., Kobayashi, S., Dang, N. H., Schlossman, S. F., Morimoto, C. (2001) CD26-mediated signaling for T cell activation occurs in lipid rafts through its association with CD45RO. *Proc. Natl. Acad. Sci. USA* **98,** 12138–12143.
43. Kobayashi, S., Ohnuma, K., Uchiyama, M., Iino, K., Iwata, S., Dang, N. H., Morimoto, C. (2004) Association of CD26 with CD45RA outside lipid rafts attenuates cord blood T-cell activation. *Blood* **103,** 1002–1010.
44. Deaglio, S., Dwyer, K. M., Gao, W., Friedman, D., Usheva, A., Erat, A., Chen, J. F., Enjyoji, K., Linden, J., Oukka, M., Kuchroo, V. K., Strom, T. B., Robson, S. C. (2007) Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *J. Exp. Med.* **204,** 1257–1265.
45. Mandapathil, M., Hilldorfer, B., Szczepanski, M. J., Czystowska, M., Szajnik, M., Ren, J., Lang, S., Jackson, E. K., Gorelik, E., Whiteside, T. L. (2010) Generation and accumulation of immunosuppressive adenosine by human CD4+CD25highFOXP3+ regulatory T cells. *J. Biol. Chem.* **285,** 7176–7186.
46. Vanham, G., Kestens, L., De Meester, I., Vingerhoets, J., Penne, G., Vanhoof, G., Scharpe, S., Heyligen, H., Bosmans, E., Ceuppens, J. L., et al. (1993) Decreased expression of the memory marker CD26 on both CD4+ and CD8+ T lymphocytes of HIV-infected subjects. *J. Acquir. Immune Defic. Syndr.* **6,** 749–757.
47. Blazquez, M. V., Madueno, J. A., Gonzalez, R., Jurado, R., Bachovchin, W. W., Pena, J., Munoz, E. (1992) Selective decrease of CD26 expression in T cells from HIV-1-infected individuals. *J. Immunol.* **149,** 3073–3077.
48. De Paoli, P., Zanussi, S., Simonelli, C., Bortolin, M. T., D'Andrea, M., Crepaldi, C., Talamini, R., Comar, M., Giacca, M., Tirelli, U. (1997) Effects of subcutaneous interleukin-2 therapy on CD4 subsets and in vitro cytokine production in HIV+ subjects. *J. Clin. Invest.* **100,** 2737–2743.
49. Valenzuela, A., Blanco, J., Callebaut, C., Jacotot, E., Lluis, C., Hovanessian, A. G., Franco, R. (1997) Adenosine deaminase binding to human CD26 is inhibited by HIV-1 envelope glycoprotein gp120 and viral particles. *J. Immunol.* **158,** 3721–3729.
50. Robertson, S. J., Hasenkrug, K. J. (2006) The role of virus-induced regulatory T cells in immunopathology. *Springer Semin. Immunopathol.* **28,** 51–62.
51. Zeiser, R., Negrin, R. S. (2008) Interleukin-2 receptor downstream events in regulatory T cells: implications for the choice of immunosuppressive drug therapy. *Cell Cycle* **7,** 458–462.
52. Quezada, S. A., Peggs, K. S., Curran, M. A., Allison, J. P. (2006) CTLA4 blockade and GM-CSF combination immunotherapy alters the intratumor balance of effector and regulatory T cells. *J. Clin. Invest.* **116,** 1935–1945.
53. Korn, T., Oukka, M., Kuchroo, V., Bettelli, E. (2007) Th17 cells: effector T cells with inflammatory properties. *Semin. Immunol.* **19,** 362–371.
54. Oukka, M. (2008) Th17 cells in immunity and autoimmunity. *Ann. Rheum. Dis.* **67** (Suppl. 3), iii26–iii29.
55. Seder, R. A., Darrah, P. A., Roederer, M. (2008) T-cell quality in memory and protection: implications for vaccine design. *Nat. Rev. Immunol.* **8,** 247–258.

KEY WORDS:
dendritic cells · CD26 · T cell activation · Th1 · CD45RA/CD45RO · Foxp3