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Decreased frequency of CD73$^+$CD8$^+$ T cells of HIV-infected patients correlates with immune activation and T cell exhaustion

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ABSTRACT

Recent studies indicate that murine Tregs highly express the ENTDP1, as well as the 5′-NT and thereby, suppress Teff function by extracellular adenosine production. Furthermore, CD73 seems to play a role as costimulatory molecule for T cell differentiation. In this study, we analyzed the expression of CD73 on peripheral and lymph nodal Teffs and Tregs in a cohort of 95 HIV patients at different stages of disease, including LTNP and ECs. In contrast to murine Tregs, CD73 was only expressed on a small minority (~10%) of peripheral Tregs. In contrast, we see high expression of CD73 on peripheral CD8$^+$ T cells. In HIV infection, CD73 is markedly reduced on all Teffs and Tregs, regardless of the memory subtype. On CD8$^+$ T cells, a positive correlation between CD73 expression and CD4 counts (P=0.0003) was detected. CD73 expression on CD8$^+$ T cells negatively correlated with HLA-DR (<0.0001) and PD1 (P=0.0457) expression. The lower CD73 expression on CD8$^+$ T cells was partially reversible after initiation of ART (P=0.0016). Functionally, we observed that CD8$^+$CD73$^+$ T cells produce more IL-2 upon HIV-specific and unspecific stimulation than their CD73$^-$ counterparts and show a higher proliferative capacity. These data indicate that down-regulation of CD73 on CD8$^+$ T cells correlates with immune activation and leads to functional deficits in HIV infection.

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Introduction

Chronic, untreated HIV infection is characterized by general immune activation, immune dysregulation, high turnover, and gradual decline of CD4$^+$ T cells through infection and bystander-induced apoptotic death [1]. Next to the phenomenon of rapid viral-immune escape of the CD8$^+$ T cell response [2, 3], the adaptive cellular CD4$^+$ and CD8$^+$ T cell response is altogether dysfunctional [4], and T cells show a diminished proliferative capacity with lacking ability to produce IL-2 [5–8]. However, our understanding of the immune dysfunction in HIV infection is still incomplete [9].

Recent studies point toward an important role of Tregs in modulating Teff responses in HIV infection [10–13]. Nevertheless, the exact mechanisms by which Tregs mediate their inhibitory function have not been determined fully so far [14]. Studies in mice show that one of the mechanisms by which Tregs suppress Teff functions is by producing adenosine via the surface co-expression of the ENTPD1 CD39 and the 5′-NT CD73 [15]. Extracellular adenosine can mediate proinflammatory or proinhibitory signals in immune cells through binding to different ARs [16]. Alternatively, adenosine also can be degraded by the enzyme complex CD26/ADA into inosine [17], which in turn, can undergo purinergic recycling or complete degradation for bile excretion.

A higher expression of CD39 has been shown previously on Tregs of HIV patients compared with Tregs of healthy controls

Abbreviations: 5′NT=5′-ectonucleotidase (CD73), A_{2A}=adenosine A, ADA=adenosine deaminase, AR=adenosine receptor, ART=antiretroviral therapy, CD62L=CD62 ligand, CDC=Centers for Disease Control, CM=central memory cells (CD45RA$^-$CD62L$^+$), EC=elite controller, EMRA$^-$=effector memory cells (CD45RA$^-$CD62L$^-$), EMRA$^+$=terminally differentiated effector memory cells (CD45RA$^+$CD62L$^-$), ENTPD1=ectonucleoside triphosphate diphosphohydrolase 1 (CD39), FoxP3=forkhead box P3, ICS=intracytoplasmic staining, LNL=LN lymphocyte, LNMC=LN mononuclear cell, LTNP=long-term nonprogressor, naïve=CD45RA$^+$CD62L$^+$, PD1=programmed death 1, Teff=T effector cell, Treg=regulatory T cell, SEB=Staphylococcus aureus enterotoxin B

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

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[18]. As CD39 degrades ATP and ADP to the point of 5′-AMP, we aimed to elucidate further the exact expression patterns of CD73 on T cells and especially Tregs in HIV infection.

Former studies revealed that in humans, CD73 is expressed by many cell types, including B cells, T cells, and endothelial cells [19, 20]. Furthermore, CD73 has a key role in T cell signaling and homing [21, 22], and there is also evidence of an ectonucleotidase-independent function of CD73 with altogether T cell activating potential, which is partly mediated by the GPI anchor of this protein [23, 24]. Taken together, CD73 seems to be an important T cell differentiation marker, with T cell activating functions on the one hand and with inhibiting functions via the production of adenosine on the other hand [25]. It is noted that reduced CD73 activity has been described in B and T cells of patients with primary immune defects, such as common variable immunodeficiency or X-linked agammaglobulinemia [26–28]. In addition, previous reports described low CD73 expression in patients with zinc deficiency [29] and a (reactive) higher expression of CD73 in $CD4^+$ Teffs in patients with inflammatory bowel disease [30, 31]. In murine models, CD73 deficiency leads to enhanced acute graft-versus-host disease [32] and might have a role in the suppression of antitumor responses [25, 33–35].

Here, we present a comprehensive human study on the expression of CD73 on different peripheral T cell subsets of patients at different stages of HIV infection. In a subset of patients, we also analyzed CD73 expression on lymph nodal T cells [36, 37]. In contrast to murine Tregs [15, 38], we find that only a small minority of human Tregs ($CD4^+CD25^+FoxP3^+$) expresses CD73 on their surface, regardless of the infection status. However, CD73 is highly expressed by $CD8^+$ T cells in humans [19], and a significant down-regulation is observed during HIV infection in all Teff subsets, as well as Tregs. This global disappearance of CD73 correlates with immune activation and a functional impairment of the $CD8^+CD73^-$ T cells compared with $CD8^+CD73^+$ T cells.

MATERIALS AND METHODS

Study subjects and samples

PBMCs (n=95), as well as LNMCs (n=17) of HIV patients and of HIV-uninfected controls (n=5), were collected at the University Medical Center Hamburg-Eppendorf (Germany), University of Cologne (Germany), and the Medizinische Hochschule Hannover (Germany). Healthy individuals (n=27) served as controls and for validation of the immunological tests. Written, informed consent was obtained from all patients enrolled into this study, which was approved by the respective Institutional Review Boards. Active hepatitis C and B virus infection was ruled out serologically in all patients. To correlate immunological results with clinical data and for intergroup comparisons, the cohort was divided into the following subgroups (also see Table 1): (1) EC (n=14), naive to ART treatment, stable CD4, and HIV viral load <50 HIV copies/ml blood, no HIV-associated diseases, CDC Status A1–A2; (2) LTNP (n=16), naive to ART treatment, stable CD4 counts, stable viral load <10,000 HIV copies/ml blood, CDC Status A1–C2; (3) ART (n=35), ART, nondetectable viral load <50 HIV copies/ml blood, CD4 counts variable, CDC Status A1–C3; (4) viremic patients (n=33), patients naive to ART treatment (n=16) or without current ART treatment (n=14) or on ART treatment, according to clinical databank, but still with a high viral load (n=2), CD4 counts, and HIV viral load variable, CDC Status A1–C3. The HIV LTNP and EC cohorts consisted of HIV-infected patients who were recruited from the natural virus controller study group, which is composed of patients selected from a detailed clinical and laboratory database of more than 6000 individuals of a German network of clinical HIV centers [18]. The time and duration of HIV infection and the definition of the different stages of the disease were extracted from the electronic databases of the participating centers and confirmed by the treating physicians, according to standard classifications, and by criteria commonly used in the literature [39]. HIV-1 viral load was determined using COBAS amplicor assays with a limit of detection of 50 RNA copies/ml. HIV CDC status, antiretroviral treatment, and CD4 counts were determined via chart review.

Immunophenotypic analysis

For immunophenotypic staining, cryopreserved PBMCs or LNMCs were thawed using standardized techniques. To characterize T cell populations, at least 5×10^5 cells were stained with appropriate fluorochrome-conjugated surface antibodies, including anti-CD4, anti-CD8, anti-CD25, anti-HLA-DR, anti-CD39, and anti-PD1 (all BD Biosciences, Heidelberg, Germany) and anti-CD3, anti-CD73, anti-CD45RA, anti-CD62L, and anti-CD45RO (all BioLegend, Fell, Germany), for 30 min at 4°C in the dark. After surface staining, cells were washed once with 1× PBS, and the intracellular staining of FoxP3 (BD Biosciences and BioLegend) was performed as published previously using the FoxP3 staining buffer set (eBioscience, San Diego, CA, USA) [18]. All samples were resuspended in 0.5% PFA, and data were collected on a BD LSR II machine using FACSDiva version 5 (BD Biosciences).

Dextramer staining of HIV-specific $CD8^+$ T cells

HLA was determined for immunophenotypic staining of HIV-specific $CD8^+$ T cells at the Department of Transfusion Medicine at the University of Hamburg (Germany). The MHC class I dextramers HLA-A*0201(FLGKIWPS) and HLA-A*0201(SLYNTVATL) (Immudex, Copenhagen, Denmark) were used as described previously [40]. Briefly, cryopreserved cells were thawed using standard procedures in the laboratory. After thawing, at least 1×10^6 cells were stained with the Aqua LIVE/DEAD 405-nm staining kit (Invitrogen, Life Technologies, Carlsbad, CA, USA). Cells were then subsequently washed with 2 ml PBS + 5% FCS, and the cell pellet was resuspended in maximum 30 μl staining volume and then stained with HLA class I-specific dextramers for 20 min at room temperature. Cells were then washed twice with 2 ml PBS + 5% FCS. Anti-CD3, anti-CD8, anti-CD45RA, anti-CD62L, anti-CCR7, and anti-CD73 surface antibodies were added for 30 min at 4°C. After staining, cells were washed with 2 ml PBS + 5% FCS and resuspended in 200 μl 0.5% PFA. Cells were analyzed on a BD LSR II machine immediately after staining.

Cell purification and proliferation assays

Fresh PBMCs from healthy volunteers were enriched for $CD8^+$ T cells via magnetic separation (Stemcell Technologies, France) and stained with a proliferation stain (eFluor450; eBioscience). Then, the cells were stained with anti-CD3, anti-CD8, anti-CD45RA, anti-CD62L, and anti-CD73 antibodies and sorted into two populations: $CD3^+CD8^+CD45RA^-CD62L^-CD73^+$ (termed memory $CD8^+CD73^+$) and $CD3^+CD8^+CD45RA^-CD62L^-CD73^-$ (termed memory $CD8^+CD73^-$) on a BD FACSAria (BD Biosciences). The purity of the memory $CD8^+CD73^+$ and memory $CD8^+CD73^-$ populations exceeded 98% (data not shown). To analyze the proliferative features of memory $CD8^+CD73^+$ and memory $CD8^+CD73^-$ cells under physiological conditions, the sorted cells were mixed back into 500,000 full PBMCs of the donor that were put aside before $CD8^+$ enrichment and plated in 24-well plates (TPP, Switzerland) at 30,000–50,000 $eFluor450^+$ cells/well in 1000 μl R10. SEB (Sigma-Aldrich, Germany) was used to stimulate the PBMCs at a final concentration of 10 μg/ml. Cells were incubated at 37°C and 5% CO_2. To determine the percentage of proliferated memory $CD8^+CD73^+$ and memory $CD8^+CD73^-$ cells, the cells were stained for FACS analysis with Aqua LIVE/DEAD 405 nm and anti-CD3, anti-CD8, anti-

CD73, anti-CD62L, and anti-CD45RA on day 4 of incubation. The percentage of proliferated cells was determined by the measurement of the eFluor450 signal.

Intracellular cytokine staining

To characterize memory $CD8^+CD73^+$ and memory $CD8^+CD73^-$ T cells further, ICS was carried out additionally to proliferation assays, as described previously [41]. ICS assays were performed with at least 1,000,000 cells. HLA-specific, HIV-derived peptide pools and PMA (50 ng/ml final concentration)–ionomycin (0.67 M final concentration), as positive control, were used to stimulate cells overnight with the addition of Brefeldin A (10 g/ml). The cells were incubated at 37°C and 5% CO_2. PBMCs were then washed and stained with the Aqua LIVE/DEAD 405 nm and with surface antibodies anti-CD3, anti-CD8, anti-CD45RO, and anti-CD73. After being washed, the PBMCs were fixed and permeabilized (BD Fix & Perm kit; BD Biosciences). The antibodies anti-IFN-γ (BD Biosciences), anti-IL-2 (BD Biosciences), and anti-TNF-α (BioLegend) were added, and the cells were incubated for 45 min at 4°C. Cells were washed with Perm buffer, according to protocol of BD Biosciences, and after the staining, at least 400,000 events were measured on a LSR II machine.

Statistical analysis

All flow cytometric data were analyzed using FlowJo version 9.3 software (TreeStar, Ashland, OR, USA). Statistical analysis was carried out using Prism 5.0 software (GraphPad Software, San Diego, CA, USA). A Gaussian distribution of data was assumed, and parametric Student's unpaired/paired *t*-tests of significance were performed throughout all samples for intergroup comparisons and Pearson's correlation for bivariate correlation analyses. All data are expressed as means with SD or with SEM. $P \leq 0.05$ was considered significant.

RESULTS

Decreased frequency of $CD73^+$ $CD8^+$ T cells correlates with immune activation and disease progression

CD73 expression profiles of the different T cell subsets of 95 HIV patients at different stages of disease, including EC and LTNP, viremic and ART-treated patients, and 27 healthy, uninfected controls were analyzed by multicolor flow cytometry. Virological and immunological characteristics of the cohort are shown in **Table 1**.

In healthy subjects, we observed the highest expression of CD73 on $CD8^+$ T cells of healthy subjects (mean 54.33%, range 82.2–11.8%) and a substantial lower expression on $CD4^+$ non-Tregs (mean 14.16%, range 41.1–3.82%). $CD4^+$ non-Tregs were defined as $CD4^+$ T cells minus the Treg population. In HIV-infected patients, the percentage of $CD73^+$ cells was significantly lower in $CD8^+$ T cells ($P<0.0001$) and $CD4^+$ non-Tregs ($P<0.0001$). In concordance with previous reports [42], we also observed a slight trend toward lower CD73 expression on $CD8^+$ T cells with higher age, but this trend did not reach statistical significance, neither in healthy controls ($r=-0.3051$, $P=0.1908$; data not shown) nor in HIV-infected patients. However, there were significant differences in CD73 expression on $CD8^+$ T cells, according to the HIV disease status. Compared with healthy controls, the $CD8^+$ T cells of HIV-infected patients displayed a significantly lower percentage of $CD73^+$ cells, independent of disease status. Among the HIV patients, EC (mean 36.55%, range 70.7–14.3%)- and ART-treated patients (mean 27.11%, range 72.3–3.86%) showed the highest percentages of $CD73^+$ cells. The viremic patients displayed the lowest expression levels of $CD73^+$ (mean 16.96%, range 47.3–1.71%), followed by LTNP (mean 18.5%, range 45–2.08%). Compared with LTNP and viremic patients, a significantly higher percentage of $CD73^+$ $CD8^+$ T cells was observed in EC- and ART-treated patients (**Fig. 1A** and **B**).

Similar to $CD8^+$ T cells, CD73 was also diminished in $CD4^+$ non-Tregs of HIV-infected patients. However, CD73 is generally expressed only on a small subset of $CD4^+$ T cells. Thus, the decrease of expression and the differences between the patient groups were less pronounced (Fig. 1A and C).

We also analyzed the expression of CD73 on the Treg population, which was defined as $CD4^+$ $CD25^+$ $FoxP3^+$ cells [18]. In contrast to murine Tregs, only a small minority of peripheral Tregs was found to express CD73, even in healthy controls (mean 9.78%, range 20.2–3.04%). In the different HIV-infected patient groups, the percentage of $CD73^+$ Tregs was decreased significantly (mean LTNP 6.28%, $P=0.0073$, mean ART 5.14%, $P<0.0001$, mean viremic 5.96%, $P=0.0003$) but EC (mean 8.49%, $P>0.05$) compared with healthy controls (Fig. 1D). These results are in contrast to the current paradigm in the literature, as murine Tregs are characterized by a high degree of surface CD73 expression.

We next tested whether lower expression of CD73 on $CD8^+$ T cells correlates with immune activation and HIV disease progression. First, the frequency of $CD73^+$ cells was significantly correlated with HIV viremia, when compared with treated patients and ECs ($P<0.001$; data not shown). We also noticed a significant positive correlation between the frequencies of $CD73^+CD8^+$ T cells and overall CD4 counts ($r=0.4396$, $P=0.0003$). Significant inverse correlations were found among the percentages of $CD73^+CD8^+$ T cells and the expression of the activation marker HLA-DR ($r=-0.6789$, $P<0.0001$), the

TABLE 1. Patient Characteristics ($n=95$)—PBMCs

Patient classification	Age[a]	Viral load (copies/ml)[a]	CD4 count (cells/μl)[a]	Treatment
Healthy subjects ($n=27$)	28 (22–56)	Not applicable	Not applicable	Not applicable
EC ($n=14$)	45 (29–65)	<50	769 (370–1355)	AN: 14
LTNP ($n=16$)	48 (32–76)	2282 (210–8600)	533 (265–1096)	AN: 16
ART patients ($n=32$)	50 (26–82)	<50	505 (107–1173)	ART: 32
Viremic patients ($n=33$)	39 (23–67)	369,021 (670–1.6×10^6)	278 (1–852)	AN: 16, P: 5, NA: 10, ART: 2

[a]Values are medians (ranges). AN, ART-naïve; P, patients on therapy pause; NA, patients currently not on ART and no information of previous ART treatment.

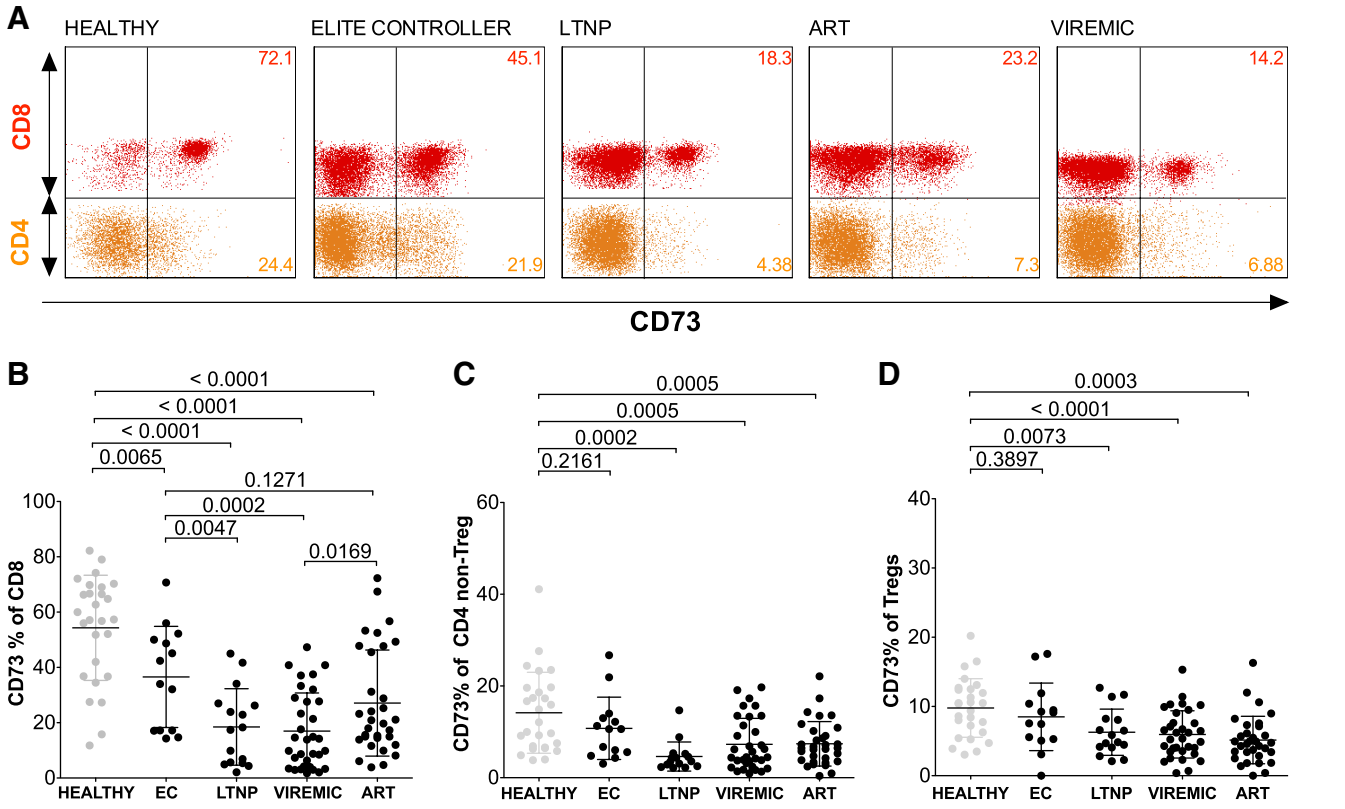

Figure 1. (A–D) Expression of CD73 on T cell populations. (A) Representative FACS plots of CD73 expression on PBMCs of healthy donors and HIV patients at different stages of disease. (B) Cross-sectional data of CD73 expression on $CD8^+$ Teffs; in healthy controls, 54.33% (mean) of the $CD8^+$ T cells expressed CD73. In HIV-infected patients, there was a significant decrease in the frequency of CD73-expressing $CD8^+$ T cells, independent of disease status (EC mean 36.55%, LTNP mean 18.5%, viremic mean 16.96%, ART mean 27.11%). (C) CD73 expression on $CD4^+$ non-Tregs showed a similar pattern, only the EC group (mean 10.76%) showed a comparable frequency of $CD73^+$ cells with healthy controls (mean 14.16%); in all other groups, the frequency of CD73-expressing cells was decreased significantly (LTNP mean 4.61%, viremic mean 7.27%, ART mean 7.39%). (D) In healthy controls, 9.78% (mean) of the Tregs expressed CD73. In all patient groups except the EC (mean 8.49%), a decrease of CD73-expressing cells was detected compared with healthy controls (mean LTNP 6.28%, mean ART 5.14%, mean viremic 5.96%). Data are presented as mean ± SD.

coexpression of the activation markers CD38 and HLA-DR ($r=-0.4547$, $P=0.0293$; data not shown), and the exhaustion marker PD1 ($r=-0.34$, $P=0.0457$). We did not find any direct correlation ($r=-0.2744$, $P=0.2052$) between the frequency of $CD73^+CD8^+$ T cells and the expression of the important homing and differentiation marker [43–46] CCR5 in the patients tested (**Fig. 2A–D**).

We observed the same correlation patterns for the $CD4^+$ non-Treg population. CD73 expression on $CD4^+$ non-Tregs positively correlated with CD4 counts ($r=0.3866$; $P=0.0017$) and negatively with HLA-DR ($r=-0.4766$, $P=0.0138$) and PD1 ($r=-0.4$, $P=0.0339$) expression (data not shown). Consequently, $CD8^+CD73^-$ T cells express significantly higher levels of HLA-DR (healthy controls $P=0.0268$, mean 26.98%, range 51.5–8.6%; ART-treated $P<0.0001$, mean 41.29%, range 59.1–18.4%; viremic $P<0.0001$, mean 57.32%, range 89.1–35.9%) than $CD8^+CD73^+$ T cells (Fig. 2E). Further analysis with a second activation marker, CD38 (Fig. 2F), revealed that $CD8^+CD73^-$ T cells of viremic patients express significantly higher levels of CD38 ($P<0.0001$, mean 35.28%, range 97.3–6.26%) than their $CD73^+$ counterparts or the $CD8^+CD73^-$ T cells of healthy donors ($P=0.0186$, mean 4.75%, range 10.1–2.89%) or ART-treated patients ($P=0.0005$, mean 6.55%, range 15.2–1.87%). The phenotypic analysis of the expression of the exhaustion marker PD1 showed that the $CD8^+CD73^+$ T cell subset expresses PD1 significantly lower in healthy donors ($P=0.0134$, mean 1.59%, range 2.45–1.24%) and HIV-infected patients (ART-treated $P=0.0002$, mean 4.18%, range 6.57–2.22%; viremic $P<0.0001$, mean 6.87%, range 18–1.14%) than the $CD8^+CD73^-$ T cell population (Fig. 2G). Interestingly, when we analyzed the expression of CCR5 (Fig. 2H) of these T cell subsets, we observed a significantly higher expression of CCR5 on $CD8^+CD73^-$ T cells (healthy $P=0.0194$, mean 21.22%, range 40.4–10.5%; ART-treated $P=0.0073$, mean 14.5%, range 35.3–1.21%; viremic $P=0.0033$, mean 12.59%, range 55.7–0.749%) compared with $CD8^+CD73^+$ T cells. To rule out the possibility that CD73 is a mere marker for resting T cells, which is down-regulated immediately upon T cell activation, we stimulated live-sorted, eFluor450-labeled, stained memory T cells in vitro with the superantigen SEB and recorded CD73 expression longitudinally. After a 4-day period of stimulation, no significant decrease was observed in the overall surface expres-

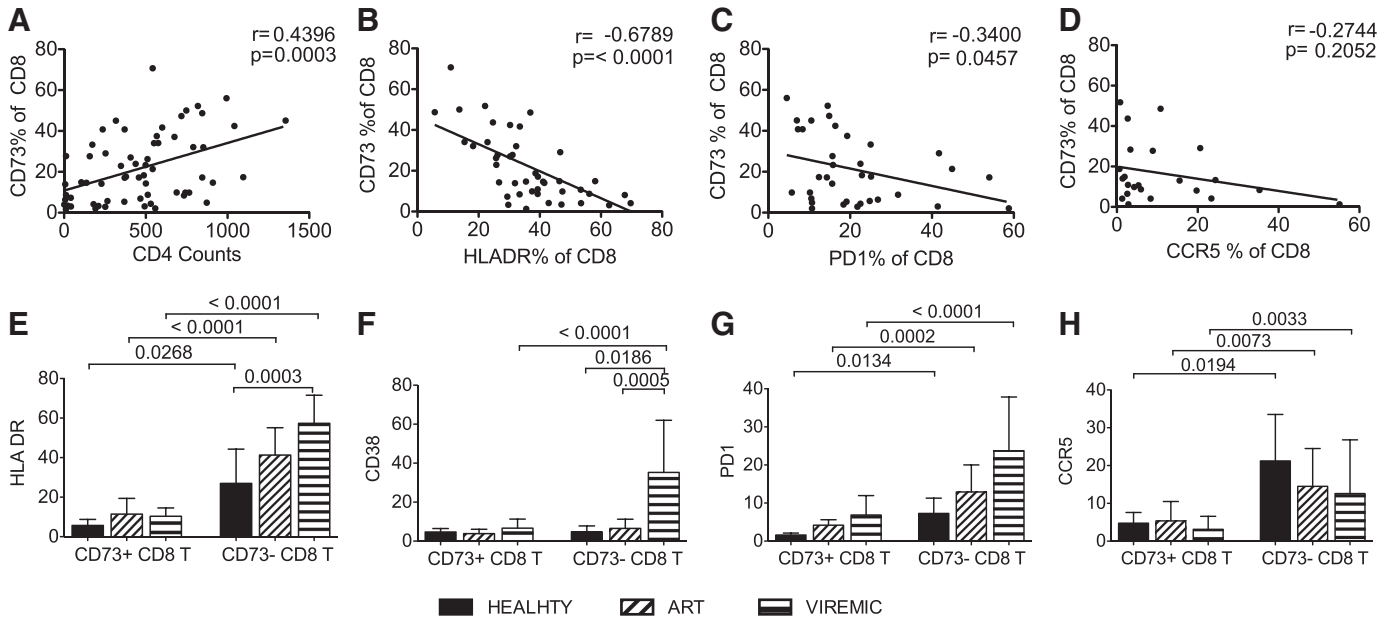

Figure 2. (A–H) Decreased frequency of CD8$^+$CD73$^+$ T cells correlates with disease progression and immune activation. (A) The expression of CD73 on CD8$^+$ T cells showed a strong, positive correlation with rising CD4 counts, which was inversely correlated with (B) activation status, measured by HLA-DR expression, and (C) the exhaustion status, measured by PD1 expression of the CD8$^+$ T cells. (D) There was no significant correlation between CD73 and CCR5 expression on CD8$^+$ T cells. The expression of the activation markers HLA-DR and CD38, the exhaustion marker PD1, and the homing marker CCR5 on CD8$^+$CD73$^+$ versus CD8$^+$CD73$^-$ T cells was measured in five healthy controls, 13 ART patients, and 23 viremic patients. (E) HLA-DR expression was elevated significantly on CD8$^+$CD73$^-$ T cells of healthy controls (mean 26.98%) and HIV patients (mean ART: 41.29%; mean viremic: 57.32%) compared with the CD8$^+$CD73$^+$ T cell population (mean healthy 5.71%; mean ART 11.41%; mean viremic 10.34%). (F) CD38 expression was elevated significantly on CD8$^+$CD73$^-$ T cells of viremic patients (mean 35.28%) compared with their CD8$^+$CD73$^+$ T cells (mean 6.63%) or the CD8$^+$CD73$^-$ T cell populations of healthy controls (mean 4.75%) or ART patients (mean 6.55%). (G) The CD8$^+$CD73$^-$ T cells also showed significantly higher expression levels of the exhaustion marker PD1 (mean healthy 7.28%; mean ART 12.9%; mean viremic 23.74%) than their CD73$^+$ counterparts (mean healthy 1.59%; mean ART 4.18%; mean viremic 6.87%). (H) The same pattern was observed for the chemokine receptor CCR5; CD8$^+$CD73$^-$ T cells showed higher CCR5 expression in healthy controls and HIV patients (mean healthy 21.22%; mean ART 14.5%; mean viremic 12.59%) than their CD73$^+$ counterparts (mean healthy 4.76%; mean ART 5.36%; mean viremic 3.12%). Data are presented as mean ± SD.

sion of CD73 on in vitro-stimulated memory CD8$^+$ T cells, independent of the proliferation status of the cells (data not shown).

CD73 is decreased on naive and memory T cell compartments in HIV infection, as well as on virus-specific CD8$^+$ T cells

We next determined—in a subset of HIV patients with different disease status and of healthy controls—whether HIV infection alters the expression of CD73 on all CD8$^+$ T cell subpopulations (**Fig. 3**). For this purpose, we used the differentiation markers CD45RA and CD62L to define naive and memory (CM, EMRA$^+$, and EMRA$^-$) subsets [47]. We found that the naive CD8$^+$ T cells showed the highest level of CD73 expression in healthy subjects (mean 88.8%, range 96.5–70.8%). The EMRA$^+$, EMRA$^-$, and CM subpopulations of healthy controls displayed CD8$^+$CD73$^+$ T cell percentages between 65.2% and 38.9%, (range of means). The percentage of CD73-expressing cells decreased significantly in the naive as well as in the memory CD8$^+$ T cell subsets of HIV patients compared with healthy controls, with the exception of the naive CD8$^+$ T cell subset of EC patients (mean 76.36%, range 87.2–60.05%, P=0.1991) Altogether, there is a significant decrease of CD73$^+$ T cells in all CD8$^+$ subsets in HIV infection. Thus, this global decrease of CD8$^+$CD73$^+$ T cells does not seem to be a sole result of the contraction and lower proportion of the naive T cell compartment in HIV patients.

We also analyzed the CD73 expression of HIV-specific CD8$^+$ T cells using MHC I dextramers with specificities against the HIV epitopes SLYNTVATL and FLGKIWPS (characteristics of analyzed patients in Supplemental Table 1). For the analysis of CD73 expression on virus-specific cells, first we determined their memory status using the markers CD45RA and CD62L. In all patients, >85% of the HIV-specific cells were in the EMRA$^-$ subpopulation. By comparing the CD73 expression of HIV-specific cells with bulk CD8$^+$EMRA$^-$ T cells, we see that the virus-specific cells (mean 1.02%, range 3.49–0.00%) show an even more pronounced down-regulation of CD73$^+$ cells than the matched CD8$^+$EMRA$^-$ bulk cells (P=0.0258, mean 13.52%, range 35.65–3.96%; **Fig. 4**).

CD8$^+$CD73$^+$ memory T cells secrete more IL-2 upon stimulation than their CD73$^-$ counterparts and show stronger proliferative capacity

In addition to the phenotypic studies, we wanted to address the question of whether there are also functional differences

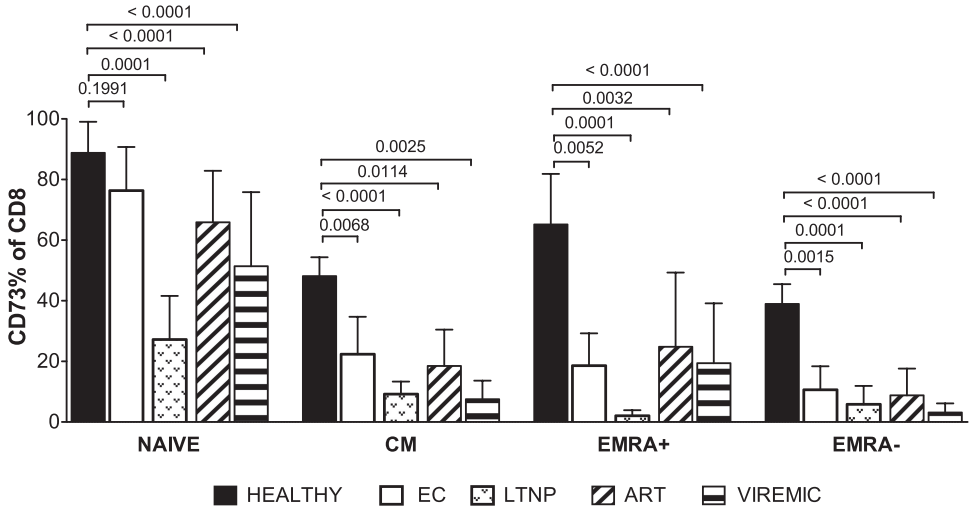

Figure 3. CD73 expression on naive and memory CD8$^+$ T cell subsets. The differentiation markers CD45RA and CD62L were used to analyze the expression of CD73 on CD8$^+$ naive and CD8$^+$ memory T cells of five healthy controls, 15 ART-treated, and 24 viremic HIV patients. Similar to previous reports, in healthy controls, the majority of the naive T cells (mean 88.8%) expresses CD73. The expression of the enzyme was also detectable on memory populations, however at significantly lower rates (mean CM 48.16%, mean EMRA$^+$ 65.2%, mean EMRA$^-$ 38.94%). Compared with healthy controls, in HIV patients, there was a significantly lower percentage of CD73-expressing T cells in all subpopulations, except the naive cell subset of EC (means EC: naive 76.36%, CM 22.37%, EMRA$^+$ 18.6%, EMRA$^-$ 10.62%; means LTNP: naive 27.22%, CM 9.21%, EMRA$^+$ 2.07, EMRA$^-$ 5.82%; means ART: naive 65.87%, CM 18.47%, EMRA$^+$ 24.83%, EMRA$^-$ 8.81%; means viremic: naive 51.42%, CM 7.5%, EMRA$^+$ 19.42%, EMRA$^-$ 3.13%), independent of their differentiation phenotype. Data are presented as mean ± SD.

between CD8$^+$CD73$^+$ and CD8$^+$CD73$^-$ T cells. Therefore, we analyzed CD8$^+$CD73$^+$ versus CD8$^+$CD73$^-$ memory T cells with regards to their cytokine-producing and -proliferating ability. For this aim, we stimulated PBMCs of 10 HIV patients (patient characteristics in Supplemental Table 2) with PMA/ionomycin as a positive control and with HLA-matched CD8$^+$ T cell HIV-peptide pools. After overnight stimulation, we analyzed the frequency of IL-2-, IFN-γ-, and TNF-α-producing cells in the memory subset (defined as CD45RO$^+$) of CD8$^+$CD73$^+$ versus CD8$^+$CD73$^-$ T cells. Upon PMA/ionomycin stimulation (**Fig. 5A**), we observed a significantly higher (P=0.0215) percentage of IL-2-producing cells in the CD8$^+$CD73$^+$ memory subset (mean 33.15%, range 58.1–19.9%) compared with the CD8$^+$CD73$^-$ memory cells (mean 17.75%, range 45.5–5.63%). There were no significant differences between CD8$^+$CD73$^+$ memory (mean IFN-γ 67.83%, range 83.9–21.6%; mean TNF-α 79.5%, range 90.5–68.1%) and CD8$^+$CD73$^-$ memory populations (mean IFN-γ 73.9%, range 89.7–46.3%; mean TNF-α 73.99%, range 92.9–48.1%) regarding the percentage of IFN-γ (P=0.4035)- and TNF-α (P=0.3299)-producing cells. Stimulation of PBMCs with HIV-1-derived peptide pools yielded similar results (Fig. 5B). In detail, we found that 0.27% (range 0.657–0.009%) of the CD8$^+$CD73$^+$ memory cells produced IL-2 upon peptide stimulation, whereas in the CD8$^+$CD73$^-$ memory subset, only 0.057% (range 0.21–0.0061%) of the cells displayed IL-2 production (P=0.0023). Again, we did not observe differences in the percentage of IFN-γ-producing cells (P=0.9222) when CD8$^+$CD73$^+$ memory (mean 1.18%, range 7.8–0.083%) and CD8$^+$CD73$^-$ memory cells (mean 1.24%, range 8.4–0.142%) were compared. However, the CD8$^+$CD73$^+$ memory cells (mean 1.36%, range 3.23–0.39%) showed a significantly higher frequency of TNF-α-producing cells upon peptide stimulation (P=0.001) than their CD73$^-$ counterparts (mean 0.56%, range 1.22–0.244%). Altogether, the CD73$^+$ subset of the memory CD8$^+$ T cells displayed a stronger IL-2- and TNF-α-producing capacity upon HIV-1-specific stimulation, whereas the differences in IFN-γ production were not as obvious.

Next, we live-sorted freshly isolated and eFluor450-marked PBMCs of healthy donors into CD3$^+$CD8$^+$CD73$^+$ and CD3$^+$CD8$^+$CD73$^-$ memory populations to analyze their proliferative capacity (during the sort procedure, all CD3$^+$CD8$^+$CD45RA$^+$CD62L$^+$ T cells were discarded). The sorted cells were mixed back to non-eFluor450-labeled, full PBMCs to ensure a full contact environment for the memory subsets, and the cells were stimulated with SEB for 4 days (Fig. 5C). We observed that the CD8$^+$CD73$^+$ memory population possesses a significantly stronger proliferative capacity than

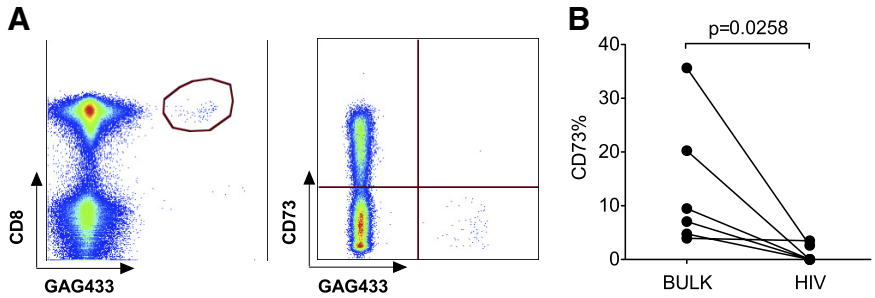

Figure 4. (A and B) CD73 expression on HIV-specific CD8$^+$ T cells. (A) Representative plots of the staining of HIV gag-specific cells with the dextramer HLA-A*0201(FLGKIWPS) and CD73 on T cells. (B) HIV gag-specific and bulk EMRA$^-$ CD8$^+$ T cells were analyzed in three ART and two viremic HIV patients for their CD73 expression. We observed a significantly lower (P=0.0258) expression of CD73 on gag-specific T cells (mean 1.02%) compared with the bulk population (mean 13.52%).

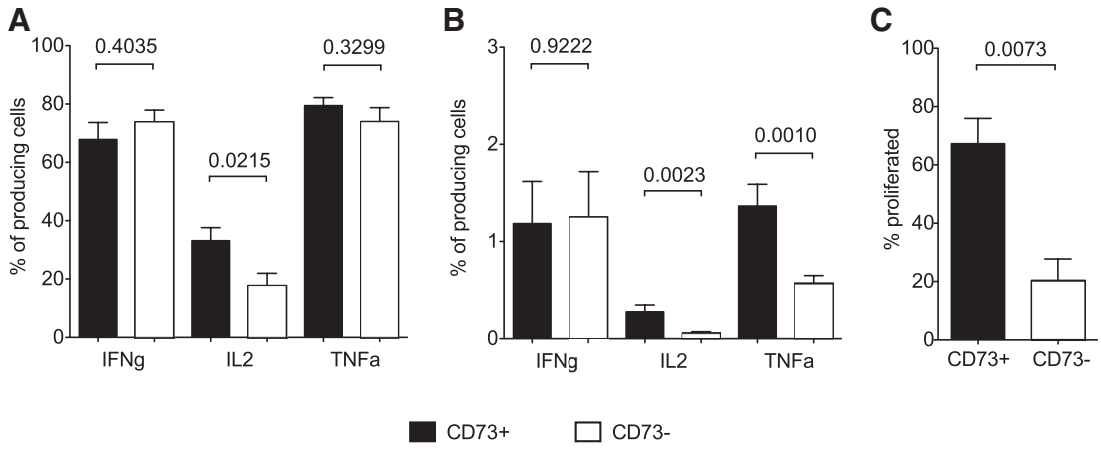

Figure 5. (A–C) Functional analysis of CD8$^+$ CD73$^+$ versus CD8$^+$ CD73$^-$ T cells. PBMCs of 10 ART patients were stimulated overnight with PMA/ionomycin or HIV-derived peptides to analyze the frequency of cytokine-producing cells in CD8$^+$CD73$^+$ versus CD8$^+$CD73$^-$ memory (CD45RO$^+$) T cells. (A) Stimulation of PBMC with PMA/ionomycin showed that CD8$^+$CD73$^+$ T cells produced significantly higher levels of IL-2 than CD8$^+$CD73$^-$ T cells (P=0.0215). The TNF-α and IFN-γ production of the CD8$^+$CD73$^+$ versus CD8$^+$CD73$^-$ T cells did not differ significantly in the positive control. (B) Upon overnight peptide stimulation, the CD8$^+$CD73$^+$ virus-specific T cells showed higher IL-2 production (P=0.0023) and higher TNF-α production (P=0.001) than their CD73$^-$ counterparts, but no difference was observed in the IFN-γ production between CD8$^+$CD73$^+$ and CD8$^+$CD73$^-$ T cells (P=0.9222). (C) In a proliferation assay, sorted CD8$^+$CD73$^+$ T cells showed stronger proliferating capacity than CD8$^+$CD73$^-$ T cells after a stimulation period of 4 days with the superantigen SEB (P=0.0073; n=3; see Materials and Methods). Data are presented as mean ± SEM.

their CD73$^-$ counterparts (P=0.0073). Representative proliferation assay FACS plots of CD8$^+$CD73$^+$ versus CD8$^+$CD73$^-$ cells are shown in Supplemental Fig. 1. These results are in agreement with our PMA/ionomycin and HIV-peptide ICS assays, where CD8$^+$CD73$^+$ memory T cells displayed higher frequencies of IL-2-producing cells.

Decrease of CD73-expressing CD8$^+$ T cells is partly reversible after initiation of ART

To investigate if the initiation of ART affects the frequency of CD73 expression on different T cell populations, we longitudinally investigated patient samples before and after initiation of ART (n=10; patient characteristics in Supplemental Table 3). The CD8$^+$ T cell population indeed showed a significantly higher frequency of CD73$^+$ cells (P=0.0016) after successful suppression of the virus by ART for at least 2 years (**Fig. 6**). In the CD4$^+$ non-Treg population, there was no significant change of CD73 expression upon ART initiation (P=0.2052; data not shown). In the Treg population, we saw a decrease of the percentage of CD73$^+$ Tregs after ART initiation (P=0.0223, data not shown).

Analysis of CD73 expression on T cells and Treg subsets of HIV patients in the LN

CD73 has been described to be an important endothelial and LN homing marker [22]. Therefore, we also conducted preliminary analysis of the expression pattern of CD73 on LNLs of nine viremic and eight ART-treated patients. Unfortunately, no direct matched pairs of blood and LN T cell samples for the same patients were available for this analysis. Furthermore, we analyzed CD73 expression in the LNs of five HIV-uninfected controls (Supplemental Table 4).

In general, CD73 expression on T cells seems to be higher in the LN environment. Similar to the data obtained with PBMCs, we observed the highest CD73 expression on LN CD8$^+$ T cells. LN CD8$^+$ T cells of ART patients showed comparable CD73 expression with HIV-uninfected subjects. However, LN CD8$^+$ T cells of viremic patients showed significantly lower frequencies of CD73$^+$ LN CD8$^+$ T cells when compared with HIV-uninfected subjects (P=0.0285) or ART patients (P=0.0043; **Fig. 7A**). In the LN CD4$^+$ non-Treg population of ART-treated patients, we saw a significantly higher frequency of CD73$^+$ cells (P=0.0278) compared with their peripheral CD4$^+$

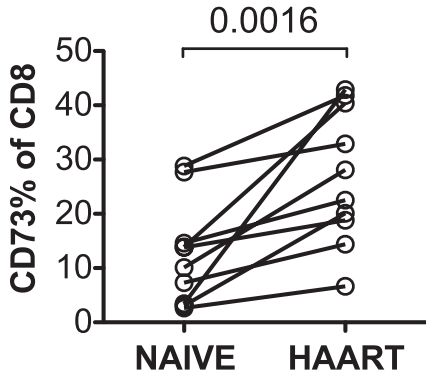

Figure 6. Longitudinal analysis of CD73 expression in HIV patients before and after initiation of ART. The CD73 expression on CD8$^+$ T cells was analyzed in 10 patients before and after initiation of ART. The CD8$^+$ T cells (12.54%; mean) expressed CD73 during the treatment naive status of the disease, and 26.85% (mean) of the CD8$^+$ T cells expressed CD73 on their surface after ART treatment for at least 2 years. HAART, Highly active antiretroviral treatment.

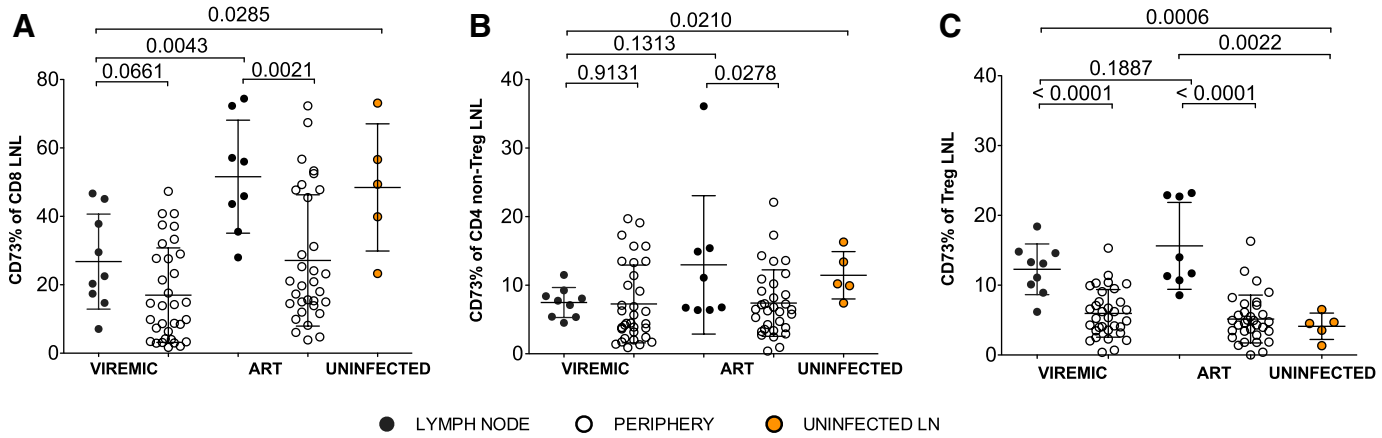

Figure 7. (A–C) Expression of CD73 on T cells is higher in the LN than in the peripheral blood. Staining of LN biopsies revealed a general trend toward higher expression of CD73 on LNL. (A) In ART-treated patients, LN $CD8^+$ T cells show higher expression of CD73 (P=0.0021) than peripheral $CD8^+$ T cells. There is also an increase in the frequency of LN $CD8^+CD73^+$ T cells in viremic patients compared with the peripheral frequency (P=0.661). CD73 expression on LN $CD8^+$ T cells of ART patients is comparable with HIV-uninfected subjects, whereas viremic patients show significantly lower $CD73^+CD8^+$ frequencies when compared with HIV-uninfected subjects (P=0.0285). (B) LN $CD4^+$ non-Tregs of ART-treated patients also show a higher frequency of $CD73^+$ cells than their peripheral counterparts (P=0.0278). Viremic patients do not show a higher frequency of CD73-expressing cells in their LN $CD4^+$ non-Treg population (P=0.9131) compared with the peripheral population. (C) In LN Tregs of ART-treated (P<0.0001) and viremic patients (P<0.0001), we detected a significantly higher frequency of $CD73^+$ cells than in their peripheral counterparts. Compared with HIV-uninfected subjects, LN Tregs of ART (P=0.0022) and viremic patients (P=0.0006) show significantly elevated CD73 expression levels. Data are presented as mean ± SD.

non-Treg counterparts. This difference was not present in LN $CD4^+$ non-Tregs of viremic patients (P=0.9131; Fig. 7B).

Most strikingly and in contrast to the peripheral blood, we found a significantly higher frequency of $CD73^+$ LN Tregs in ART patients and viremic patients compared with their peripheral Tregs (P<0.0001) or to the LN Tregs of HIV-uninfected subjects (P=0.0022 for ART patients; P=0.0006 for viremic patients). There was no significant difference between the frequency of $CD73^+$ LN Tregs of ART-treated patients compared with LN Tregs of viremic patients (P=0.1887; Fig. 7C).

In mice, Tregs are characterized by a constitutive dual expression of CD39 and CD73. However, we and others found that in humans, neither Tregs or $CD4^+$ non-Tregs nor the $CD8^+$ T cells in the periphery coexpress CD39 and CD73 strongly (maximum 3% of T cells; **Fig. 8A**). However, our coexpression analysis in the LN yielded some unexpected find-

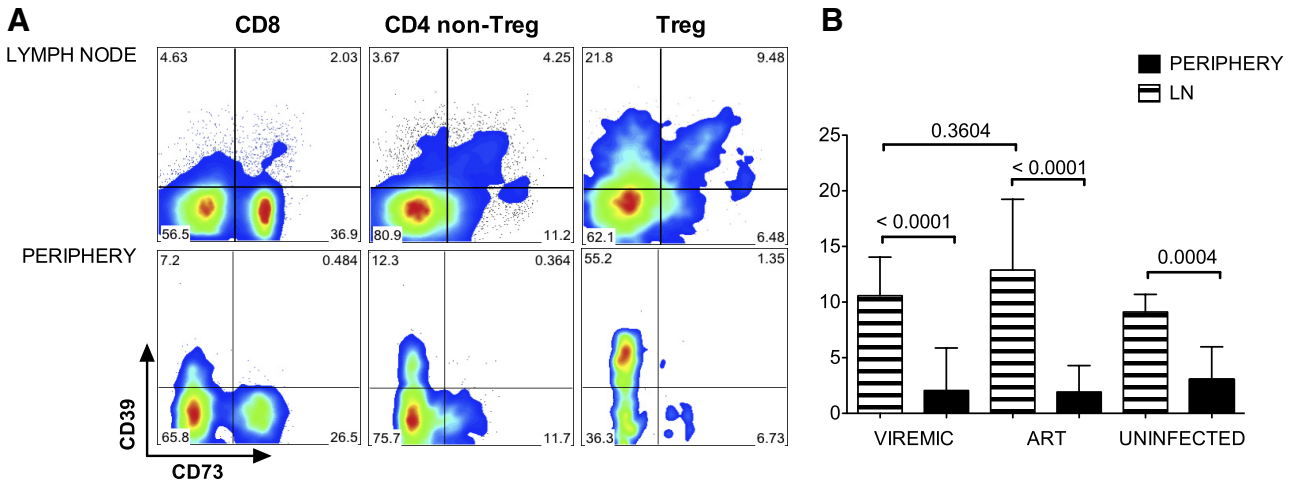

Figure 8. (A and B) Coexpression of CD39 and CD73 on peripheral and LN T cells. (A) Representative plots of CD39 (y-axis) and CD73 (x-axis) staining on peripheral and LN $CD8^+$ T cells, $CD4^+$ non-Tregs, and Tregs. (B) Peripheral Tregs (ART, n=32; viremic, n=33; healthy, n=14) and LN Tregs of HIV patients (ART, n=8; viremic, n=9) and HIV-uninfected subjects (n=5) were analyzed to investigate the coexpression of CD39 and CD73. In the periphery, there was only a minimal percentage of $CD39^+CD73^+$-expressing Tregs (mean viremic 2.056%; mean ART 1.922%; mean healthy 3.05%). However, in the LNs, there was a significant increase in the percentage of $CD39^+CD73^+$ double-positive Tregs in all groups (mean viremic 10.57%; mean ART 12.88%; healthy mean 9.1%). Data are presented as mean ± SD.

ings: in the analyzed LNs, most exclusively among Tregs, there was a significant percentage of $CD39^+CD73^+$ double-positive cells (viremic patients: $P<0.0001$, mean 10.57%, range 15.6–6.11%; ART patients: $P<0.0001$, mean 12.88%, range 21.8–6.9%; HIV-uninfected subjects: $P=0.0003$, mean 9.108%, range 11.5–0.268%) compared with the peripheral $CD39^+CD73^+$ percentages (Fig. 8B).

DISCUSSION

Here, we present the first comprehensive study of CD73 expression on different T cell subsets at different stages of HIV infection. Altogether, we see a markedly reduced percentage of CD73-expressing cells in different T cell subsets during HIV infection. Our findings extend the results of some early and less comprehensive studies [48–50].

Another important finding of our comprehensive study is that CD73 is only expressed by less than 10% (mean) of peripheral Tregs in healthy controls and even less in HIV-infected patients. Our results confirm and extend the results of smaller, previous human Treg studies [51–53]. Furthermore, we saw that neither $CD73^+$ peripheral Teffs nor $CD73^+$ Tregs coexpressed CD39 on their surface.

The absence of coexpression of these two molecules on the large majority of peripheral human Tregs is somewhat surprising, as in mice, surface coexpression of CD39 and CD73 is a molecular signature of Tregs. Therefore, it has been hypothesized that one of the most important suppressory mechanisms of Tregs is extracellular adenosine production. Whereas there has been one previous outlier report of coexpression of CD39 and CD73 in human Tregs [54], we and others clearly demonstrate phenotypic differences between the surface coexpression of these molecules on human and murine Tregs [51]. However, these differences have not been fully taken into account by the literature so far [34, 54–57].

Importantly, we and others demonstrated previously that CD39 is up-regulated on Tregs in HIV infection [18, 58]. However, CD39 only degrades ATP/ADP to the point of AMP. Thus, if human Tregs deliver their inhibitory function through the production of adenosine, as murine Tregs do, then CD73 is transiently up-regulated, or the production of adenosine is promoted by other phosphatases or by soluble CD73. Our preliminary analyses of LN Tregs could point partly toward the first theory, as we did find slightly increased levels of $CD39^+CD73^+$ double-positive Tregs (~10%) in LN tissues of HIV-infected patients and HIV-uninfected subjects.

However, further experiments will have to determine how far these Treg populations differ functionally from $CD39^+$ single-positive or $CD39^-$ Tregs. More importantly, it needs to be investigated whether these two enzymes can work together, although they are not present on the same T cells subsets.

Another aspect that needs consideration is the role of CD73 as a costimulatory molecule with synergistic effects on the CD3/TCR interaction, independent from its enzymatic activity [24, 59], and how the loss of this molecule possibly alters T cell functions. Analyzing the expression of CD73 on different lymphocyte populations shows that the majority of $CD8^+$ T cells expresses CD73 in healthy subjects. In the course of HIV infection, we confirm lower percentages of $CD73^+$-expressing cells in the naive and memory subsets of $CD8^+$, as well as $CD4^+$ non-Tregs in HIV patients. Moreover, this down-regulation correlates with the presence of HIV viremia and immune activation.

Again, ECs differ from all other HIV patient groups, as they showed the least decrease of CD73 expression; however, they still display significantly lower percentages of $CD73^+CD8^+$ T cells than healthy controls. These data are in agreement with previous findings describing that the group of ECs displays the least increase of general immune activation among HIV patients [18, 60]. Furthermore, the initiation of ART increased the percentage of $CD8^+CD73^+$ cells in HIV patients. Thus, the loss of CD73 on $CD8^+$ T cells seems to be correlated directly with the chronic immune activation driven by HIV replication, as short-term in vitro TCR superantigen (SEB)-induced activation of T cells did not result in a significant decrease of $CD8^+CD73^+$ T cells (data not shown). Clearly, while in the mouse model, there are several different transcription factors that regulate CD73 expression [31, 61], the specific mechanisms of lower CD73 surface expression in HIV infection or the possible expansion of the $CD73^-$ T cell subset remain to be elucidated. Notably, we find functional differences between $CD8^+CD73^+$ and $CD8^+CD73^-$ T cells regarding the cytokine profile and the proliferative capacity. $CD73^+$ T cells have a higher ability to produce IL-2 and a greater proliferative capacity. Thus, the disappearance of CD73 on the (virus-specific) memory populations of $CD8^+$ T cells may contribute to the T cell dysfunction that is observed during progressive HIV infection.

Two other aspects that could result from the dysregulation of CD73 also have to be considered: first, there might be disturbances of the ATP/adenosine ratio in the extracellular milieu of T cells. Adenosine exerts proinhibitory functions by binding to A_{2A}Rs that are expressed by Teff cells. Upon adenosine binding, A_{A2}Rs are able to mediate the majority of (anti-) inflammatory cascades that results in the suppression of cytokines and chemokines in multiple cell subsets. For example, hypoxia-induced adenosine accumulation has tissue-protecting effects, as A_{2A}Rs trigger switch-off signals in immune cells. Hence, the down-regulation of CD73 on T cells might be a major contributor to the chronic immune activation that occurs during HIV infection. Secondly, it is intriguing to speculate whether $CD39^+$ Tregs preferentially suppress $CD73^+$ but not $CD73^-$ Teffs via jointly generated extracellular adenosine [15]. Furthermore, CD73 has been described to be an important homing marker, and we indeed see a higher proportion of $CD73^+$ T cells in the LNs. Hence, a down-regulation of CD73 during HIV infection might have an impact on homing of the $CD73^-CD8^+$ T cells to lymphoid tissue and other tissues [21, 22].

The functional role of CD73 for T cell activation in humans is poorly understood so far, and further comprehensive studies will have to evaluate the global role of the entire anti-inflammatory ATP-adenosine axis for T cell suppression [57]. To achieve this aim, information is needed about the kinetics and balance of extracellular nucleosides in general and about enzymatic surface activities of CD39 and CD73, in particular. Moreover, intracellular expression studies [34] and quantification

of soluble CD73, as well as of CD26/ADA expression and activity, should be performed [17]. Furthermore, to the best of our knowledge, few studies have evaluated the T cell expression of AR receptor subtypes in HIV infection.

In summary, we find that in contrast to murine models, peripheral human Tregs generally do not express CD73 on their surface, but CD73 is highly expressed on human $CD8^+$ T cells. In healthy controls, there is a significantly higher percentage of CD73-expressing $CD8^+$ T cells (as well as $CD4^+$ non-Tregs to a lower extent) than in HIV-infected patients. The global decrease of CD73 expression on T cells and Tregs in HIV infection correlates well with immune activation driven by viral replication. This is at least partially reversed by ART, indicating the specific effects of uncontrolled viral replication. In turn, low CD73 expression is associated with functional, proliferative defects of $CD8^+$ T cells in progressive HIV disease that may again lead to uncontrolled viral replication.

The results of our study are not only important for the understanding of immune dysfunction in HIV but also for the differences of surface signatures of human and murine Tregs. As well, possible effects of CD73 blockade on $CD8^+$ T cells have to be tested before considering the design of novel immunomodulatory interventions aiming at blocking CD73, as proposed in the field of cancer immunology [22, 35, 62, 63].

AUTHORSHIP

The work presented here was carried out in collaboration among all authors. J.S.z.W. and J.v.L. defined the research theme. J.S.z.W. provided most of the funding. Institutional support was given by A.W.L. and J.H. The clinical cohort was founded, organized, and maintained by A.T., C.L., C.B., O.D., G.F., D.M-O., J.v.L., and J.S.z.W. P.B. and M.B. carried out the surgical procedures. T.E. contributed the HLA determination analysis for our patients. I.T., A.Q.L., V.M., and C.S. carried out most of the laboratory experiments. I.T., P.H., and J.S.z.W. carried out the data analysis and interpreted most of the results. I.T. and J.S.z.W. wrote the first draft. P.H., J.S.z.W., J.v.L., D.M-O., and J.H. gave important input at later stages of the manuscript. All authors read, helped to revise, and approved the final manuscript.

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DISCLOSURES

The authors declare that they have no competing interests.

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