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Down-regulation of CD73 on B cells of patients with viremic HIV correlates with B cell activation and disease progression

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ABSTRACT

Recently, alterations of the T cell expression of the ectonucleotidases, CD39 and CD73, during HIV infection have been described. Here, peripheral ($n = 70$) and lymph nodal B cells ($n = 10$) of patients with HIV at different stages of disease as well as uninfected individuals were analyzed via multicolor flow cytometry with regard to expression of CD39 and CD73 and differentiation, proliferation, and exhaustion status. Patients with chronic, untreated HIV showed a significantly decreased frequency of CD73-expressing B cells ($P < 0.001$) compared with healthy controls. Decreased frequencies of $CD39^+CD73^+$ B cells in patients with HIV correlated with low $CD4^+$ counts ($P < 0.0256$) as well as increased proliferation and exhaustion status as determined by Ki-67 and programmed death-1 expression. Down-regulation of CD73 was observed in naive and memory B cells as determined by CD27 and CD21. Neither HIV elite controller patients nor antiretroviral therapy–treated patients had significantly lower CD39 and CD73 expression on B cells compared with healthy controls. Of importance, low $CD73^+$ expression on B cells was associated with modulated in vitro B cell function. Further in vivo studies are warranted to evaluate the in vivo role of phenotypic loss of CD73 in B cell dysregulation in HIV. ***J. Leukoc. Biol.*** **101: 1263–1271; 2017.**

Introduction

It is known that B cell functions are essential for the development and maintenance of functional antiviral immune T cell responses [1, 2]. Chronic, untreated HIV infection is characterized by general immune activation, cellular immune dysregulation, high turnover, and gradual decline of $CD4^+$ T cells via infection and bystander-induced apoptotic death [3, 4]; however, it has long been recognized that HIV-1 infection affects not only T cells, but also B cells. B cell hyperactivation and dysfunction, hypergammaglobulinemia as a result of polyclonal Ab production, and poorly inducible pathogen-specific Ab responses were widely reported in patients with HIV [5, 6]. A missing $CD4^+$ T cell help and activation via IL-4, IL-10, and IL-21, and via CD154 are thought to be some of the reason for attenuated Ab production in HIV infection [7, 8]. Recently, it was shown that CD40L-bearing HIV-1 particles lead to activation of B cells via CD40 signaling [9].

Normally, activated B cells—having entered the germinal centers—undergo Ig class switch recombination, substituting IgM or IgD to IgG and IgA. In the germinal center, B cells differentiate into memory B cells and plasma B cells. HIV-1 infection profoundly impairs the generation of IgG and IgA responses to T cell dependent antigens via different indirect mechanisms [7], and direct interactions of HIV viral particles and viral proteins, such as NEF or GP120, have been described to influence B cell function and Ig class switch [10–15].

Recently, ectonucleotidases, CD39 and CD73, became the focus of HIV research as possible immune-modulatory molecules [16–23]. CD39 and CD73 are widely expressed by a variety of immune cells. Their main function is to control the extracellular ratio of ATP/adenosine. In this process, CD39 degrades ATP and ADP to AMP, which serves as substrate for CD73. CD73 is the rate-limiting enzyme in the generation of adenosine. ADO is a potent immunomodulatory molecule for T cells [24, 25]. Several T cell studies were carried out to analyze expression and function of these enzymes in HIV infection: a higher frequency of CD39-expressing T cells was observed in patients with HIV patients

Abbreviations: AMPCP = adenosine 5′-(α,β-methylene)diphosphate, ART = antiretroviral therapy, CVID = common variable immunodeficiency, HCV = hepatitis C virus, LNMC = lymph node mononuclear cell, PD-1 = programmed death-1, PWM = pokeweed mitogen

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compared with healthy controls [18, 20, 22]. Moreover, a distinct role for CD39 has been shown in the suppression of T effector cell functions [18]. Parallel to the expansion of $CD39^+$ regulatory T cells, general down-regulation of CD73 on all T cell subsets was observed in HIV infection, which correlated with immune activation and functional defects of these cells [21, 23, 26, 27].

CD39 and CD73 have also been recognized as B cell differentiation markers for a long time [28–31]. Recently, Saze et al. [2] showed that in vitro activated B cells down-regulated expression of CD73 and slightly up-regulated CD39, as well as inhibited T cell proliferation and cytokine production. Furthermore, a reduced CD73 expression has been described in patients who suffer from CVID for decades [28, 32–34]; however, it has only recently been shown that patients with CVID who have an impaired Ig class switch showed a specific deficiency of CD73 expression of B cells, which suggests that CD73-dependent extracellular adenosine generation critically contributes to the Ig class switch and to pathogenesis of this disease [34]. Of note, in vitro blockade of the CD73 activity of B cells of healthy controls led to an impairment of IgG class switch in this study [34].

Data on B cell expression and function of CD39 and CD73 in HIV infection are sparse. Early studies that reported on the activity of CD39 and CD73 on B cells were not followed-up, to the best of our knowledge [35, 36].

Here, we present the first study, to our knowledge, on the expression of CD39 and CD73 of different peripheral B cell subsets of patients at different stages of HIV infection. In a subset of patients, we also analyzed CD39 and CD73 expression in lymph nodal B cells. Patients with untreated, viremic HIV displayed a low frequency of CD73-expressing B cells that was associated with B cell activation, decreased AMP consumption, and impaired in vitro Ig class switch capabilities. The results of this study warrant further functional studies and might be important for understanding B cell dysfunction in HIV infection.

MATERIALS AND METHODS

Study participants and samples

PBMCs ($n = 70$) and LNMCs ($n = 10$) of patients with HIV and LNMCs of uninfected controls ($n = 4$) were collected at the University Medical Center Hamburg-Eppendorf and the Medizinische Hochschule. PBMCs of patients with chronic HCV infection ($n = 16$), patients with CVID ($n = 3$), and healthy individuals ($n = 21$) served as controls and for validation of immunologic tests. Healthy controls serologically tested negative for HIV, hepatitis B virus, and HCV. Written informed consent was obtained from all patients who enrolled in this study, which was approved by the institutional review board of the Ärztekammer Hamburg, Germany. Active HCV and hepatitis B virus infection were ruled out serologically and by PCR in all patients with HIV. The time and duration of HIV infection and the definition of the different stages of disease were extracted from electronic databases of the participating centers and confirmed by the treating physicians according to standard classifications and by criteria commonly used in the literature and previously published [23]. HIV-1 viral load was determined by using COBAS amplicor assays with a limit of detection of 50 RNA copies/ml. HIV CDC status, ART, and $CD4^+$ T cell counts were determined via chart review.

Immunophenotypic analysis

For immunophenotypic staining, cryopreserved PBMCs or LNMCs were thawed using standardized techniques. To characterize B cell populations, at least 5×10^5 cells were stained with LIVE/DEAD Fixable AQUA Dead Cell Stain 405 nm (Thermo Fisher Scientific, Waltham, MA, USA) and the appropriate fluorochrome-conjugated surface Abs, including anti-CD19, anti-CD20, anti-CD27, and anti-CD21 (all BD Biosciences, Heidelberg, Germany), as well as anti-CD73, anti-CD39, anti-IgG, anti-IgM, and anti–PD-1 (all BioLegend, Fell, Germany), for 30 min at 4°C in the dark. After surface staining, cells were washed once with 1× PBS, and intracellular staining of Ki-67 (BD Biosciences and BioLegend) was performed as previously published [22] by using the FoxP3 staining buffer set (Thermo Fisher Scientific, San Diego, CA, USA). All samples were fixed in 0.5% paraformaldehyde, and data were collected on a BD LSR Fortessa and BD LSRII machine using FACS Diva (version 5; BD Biosciences).

AMP consumption assay

Fresh PBMCs from patients were enriched for $CD20^+$ B cells via magnetic separation (Stemcell Technologies, Grenoble, France) and were resuspended in RPMI 1640 medium plus 10% FCS at a concentration of 5×10^5 cells/ml, and 50 μl were plated into a polystyrene 96-well round bottom plate. AMP (10 μM) was added at 0, 5, 30, 45, 60, and 90 min to separate wells and was incubated at 37° and 5% CO_2. Furthermore, CD73 enzymatic activity was blocked by addition of AMPCP (10 μM/ml end concentration) in healthy controls. After incubation, plates were centrifuged for 5 min at 450 *g* twice to remove all cells. Supernatants were transferred into a solid-white 96-well plate and AMP detection was prepared by using a luciferase-based AMP Glo assay kit according to manufacturer instructions (Promega, Madison, WI, USA). Supernatants were analyzed on a Spark 10 M reader (Tecan, Zürich, Switzerland).

Cell purification, proliferation assays, in vitro Ab switch, and CD73-blocking assay

Human B cells were isolated from PBMCs as described previously and stained with a proliferation stain (eFluor450; Thermo Fisher Scientific, San Diego, CA, USA). To analyze the proliferative features of B cells under physiologic conditions, sorted cells were mixed back into autologous 1×10^6 full PBMCs. As a further control, sorted B cells were tested without PBMCs of the respective patient, which were put aside before and plated in 24-well plates (TPP, Trasadingen, Switzerland) at 1×10^5 $eFluor450^+$ cells per well in 1000 μl R10. CpG 2006 (2.5 μg/ml; Miltenyi Biotech, Bergisch Gladbach, Germany), megaCD40L (6 ng/ml; Enzo Life Sciences, New York, NY, USA), PWM (5 μg/ml), LPS (5 μg/ml), and IL-21 (0.1 μg/ml; all Sigma-Aldrich, St. Louis, MO, USA) were used to stimulate PBMCs. Cells were incubated for 4 d at 37°C and 5% CO_2. To determine the percentage of proliferated B cells and in vitro Ab switched B cells, cells were stained for FACS analysis with LIVE/DEAD Fixable AQUA Dead Cell Stain 405 nm and anti-CD19, anti-CD20, anti-CD27, anti-CD39, anti-CD73, anti-IgG, and anti-IgM on day 4 of in vitro incubation. The percentage of proliferated cells was determined by measurement of eFluor450 as previously described [23]. In addition, for blockade of the enzymatic activity of CD73 AMPCP, 10 μM/ml end concentration (Sigma-Aldrich) was added.

Statistical analysis

All flow cytometric data were analyzed using FlowJo (version 9.2; Treestar, Ashland, OR, USA). Statistical analysis was carried out using Prism 5.0 (GraphPad Software, San Diego, CA, USA). Normality test, followed by ANOVA with Tukey post-test or the Kruskal-Wallis test, with Dunn's post-test for any nonparametric data set were performed throughout all samples for intergroup comparisons. Pearson's correlation was performed for bivariate correlation analyses. All data were expressed as means ± SD or SEM. *P* values ≤ 0.05 were considered significant.

RESULTS

Decreased frequency of $CD73^+CD20^+$ B cells correlates with $CD4^+$ counts in the peripheral blood and lymph nodes

We first compared surface expression of CD39 and CD73 in a large cohort of patients with HIV with different disease stages with healthy controls (**Table 1**). Representative flow cytometric

TABLE 1. Patient characteristics: PBMCs (n = 91) and lymph nodes (n = 14)

Patient classification	Age[a]	Viral load (copies/ml)[a]	CD4 count (cells/μl)[a]	Treatment
Healthy (n = 21)	40 (22–69)	n.a.	n.a.	n.a.
EC (n = 7)	52 (35–85)	83 (3–344)	839 (529–1355)	No
LTNP (n = 11)	46 (28–75)	502 (3–2300)	510 (240–824)	No
ART (n = 10)	51 (22–78)	<5	501 (274–889)	Yes
Viremic patients (n = 42)	51 (25–80)	50,000 (3–6.9 × 10^6)	240 (6–955)	No
Lymph node, healthy (n = 4)	62 (48–76)	n.a.	n.a.	n.a.
Lymph node, HIV infected (n = 10)	39 (26–80)	24,883 (3–133,500)	680 (254–1691)	3 yes/7 no

EC, elite controllers, LTNP, long-term nonprogressor, n.a., not applicable. [a]Data are given as median (range).

plots of B cells from an uninfected control and an HIV-infected patient sample are depicted in **Fig. 1A**.

In healthy controls, we observed high expression of both $CD39^+$ and $CD73^+$ on $CD20^+$ B cells in peripheral blood (Fig. 1B–D). These results are in line with data that were presented in previous publications [2, 28, 34, 37].

We next analyzed $CD39^+$, $CD73^+$, and $CD39^+CD73^+$ coexpression on $CD20^+$ B cells in a large cohort of 70 patients with HIV, including HIV elite controllers and long-term nonprogressors [23, 38], viremic, and ART-treated patients. Virologic and immunologic characteristics of the different patient subgroups of the cohort are shown in Table 1.

In HIV-infected untreated patients, frequencies of $CD39^+$ and $CD73^+$ were significantly decreased ($P < 0.001$) on $CD20^+$ B cells compared with that in healthy controls. CD73 expression showed the greatest decrease, whereas the percentage of CD39 expression of B cells showed only a slight decline in patients with HIV (Fig. 1B–D). Of interest, elite controller and long-term nonprogressor patients showed only a slightly lower expression of CD39/CD73 than did healthy controls, and this difference was not statistically significant (Supplemental Fig. 1). CD39/CD73 expression was higher in ART-treated patients with suppressed viremia compared with untreated patients with HIV ($P < 0.01$) but remained slightly below the level of healthy controls, which was suggestive of partial recovery (Fig. 1B–D).

We also analyzed CD39 and CD73 expression of lymph nodal B cells of 10 HIV-infected (3 treated and 7 untreated) and 4 HIV uninfected controls (Fig. 1E–G). Matching the observations of peripheral B cells, we also observed a generally lower expression level of CD39 and CD73 in B cells of lymph nodes in patients with HIV ($P < 0.01$). Again, among HIV-infected patients, 3 ART-treated patients showed the highest frequency of $CD39^+$, $CD73^+$, and $CD39^+CD73^+$ double-positive cells in the lymph node (Fig. 1E–G). As a further control, we analyzed a small cohort of

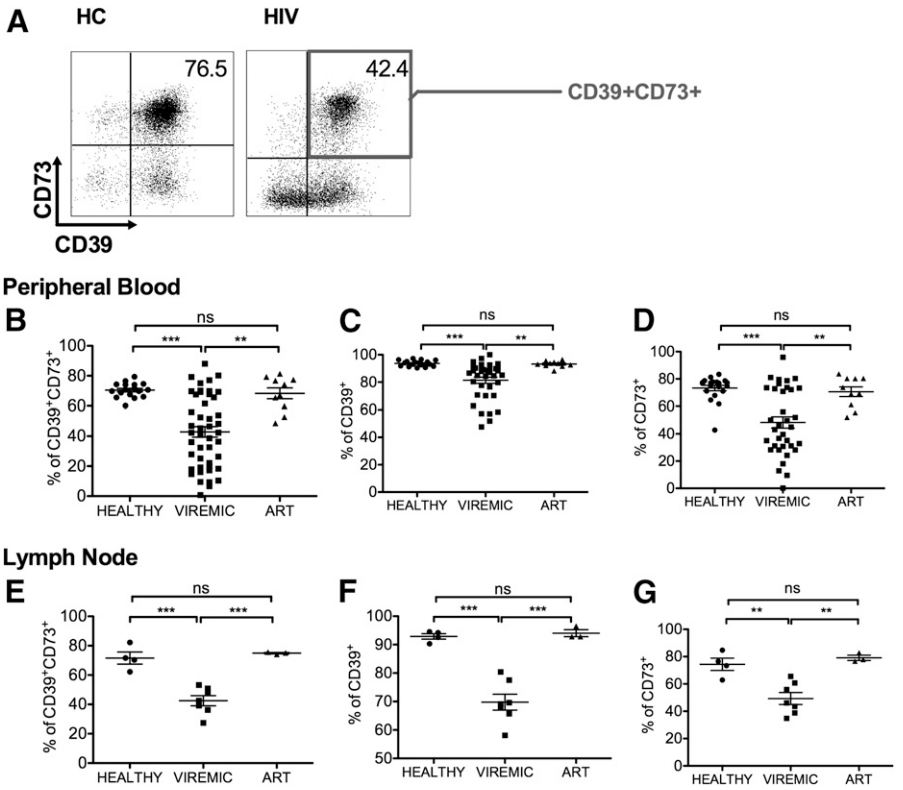

Figure 1. Cross-sectional FACS analysis of CD39, CD73, and CD39/CD73 coexpression of B cells shows reduced frequencies in patients with viremic HIV compared with healthy controls and ART-treated HIV patients in peripheral blood and in lymph nodes. (A) Representative FACS plots of a healthy control and an HIV-infected subject demonstrating the gating strategy for CD39 and CD73 within the $CD20^+$ B cell subset. (B–G) CD39, CD73, and CD39/CD73 coexpression of $CD20^+$ B cells in peripheral blood in healthy controls (n = 18), viremic (n = 42), and ART-treated patients with HIV (n = 10) and lymph nodes (healthy control, n = 4; HIV infected, n = 10). Patients with untreated HIV show lower CD39, CD73, and coexpression compared with healthy controls and ART patients. ns = not significant. Results are expressed as means ± SEM. $^{**}P < 0.01$; $^{***}P < 0.001$.

patients with chronic HCV ($n = 16$) and did not observe an alteration of the frequency of CD39- or CD73-expressing B cells in these patients (Supplemental Fig. 2).

We analyzed a small subset of our samples (healthy, $n = 8$; HIV, $n = 13$) for mean fluorescence intensity and observed a strong trend of decrease in mean fluorescence intensity of CD73 in our HIV cohort compared with healthy controls (data not shown).

To evaluate whether there was a general down-regulation of CD39 and CD73 across all B cell subsets, an extended analysis of CD39 and CD73 expression on naive ($CD21^+CD27^-$) as well as different memory B cell subsets ($CD21^+$,$CD27^+$; $CD21^-$,$CD27^+$; $CD21^-$,$CD27^-$) was performed in blood and lymph nodes (**Fig. 2A–I** and Supplemental Fig. 3) [7, 39–41]. Representative examples of our gating strategy are shown in Fig. 2A.

In line with the literature [41], there was a marked decrease in the frequency of naive B cells ($CD21^+CD27^-$) and a significant increase of the frequency of ($CD21^-$,$CD27^+$) B cells in the HIV viremic population (Fig. 2B and D).

In healthy controls, we observed no major differences in the distribution of $CD73^+$ and $CD73^-$ B cells between the above-mentioned subsets of B cells in the peripheral blood (Supplemental Fig. 3E–H), nor in the lymph nodal compartment (data not shown). There was a general decrease of $CD39^+CD73^+$ B cells in the HIV viremic population throughout all B cell subpopulations, although $CD21^-$,$CD27^-$ B cells generally showed a lower expression level of $CD39^+$,$CD73^+$ (Fig. 2F–I); however, naive B cells measured from the LN nearly retained their CD39/CD73 coexpression in patients with HIV, whereas $CD21^+CD27^+$, $CD21^+CD27^+$, and $CD21^-CD27^-$ subsets also demonstrated down-regulated expression of CD73 (Supplemental Fig. 3I–L). We also analyzed $CD39^+CD20^+$ and $CD73^+CD20^+$ expression for all subsets (Supplemental Fig. 3A–H) and observed no differing trend for any particular B cell subset.

In summary, whereas there were slightly differing levels of $CD39^+CD73^+$ expression in different B cell subsets, overall there was a general down-regulation in HIV viremics throughout all B cell subsets compared with healthy controls.

We then correlated expression levels of $CD39^+$ and $CD73^+$ cells with clinical parameters and, in particular, the viral load (copies/ml) and $CD4^+$ count (cells/μl) in patients with HIV infection (**Fig. 3**). Whereas there was a significant difference of CD73 expression in viremic vs. aviremic patients with HIV ($P < 0.01$; Fig. 1D), we did not find a direct correlation between viral load and changes in the frequencies of $CD39^+CD73^+$ coexpressing cells in the peripheral blood, nor the lymph node (Fig. 3A and B), probably as a result of the relatively low number of samples. Of note, there was a significant direct correlation of CD39/CD73 coexpression of B cells with $CD4^+$ counts in the peripheral blood (CD39/CD73 coexpression $r^2 = 0.116$; $P = 0.0256$). This result also held true for lymph nodal B cells (CD39/CD73 coexpression $r^2 = 0.545$; $P = 0.0231$; Fig. 3C and D). Furthermore, this correlation was detectable for $CD73^+$ cells in the periphery ($r^2 = 0.128$; $P < 0.02$) and in the lymph node ($r^2 = 0.479$; $P < 0.04$; data not shown).

To assess whether CD73 expression on B cells was associated with immune activation, we investigated the ex vivo expression of Ki-67, a marker for proliferative capacity, and PD-1, a marker for activation and exhaustion (**Fig. 4A and B**). $CD73^-$ B cells showed a significantly higher frequency of $Ki\text{-}67^+$ cells than did $CD73^+$ B cells in patients with HIV, but not in healthy controls (Fig. 4A). A similar pattern was observed for PD-1 expression. There was a statistically significant difference ($P < 0.05$) in PD-1 expression on $CD73^-$ B cells compared with $CD73^+$ B cells in healthy controls. This statistical significance ($P < 0.001$) held true in patients with HIV (Fig. 4B).

In summary, we see that the general loss of CD73 expression on B cells coincided with higher expression of markers of cellular proliferation and exhaustion, such as Ki-67 and PD-1 [42, 43].

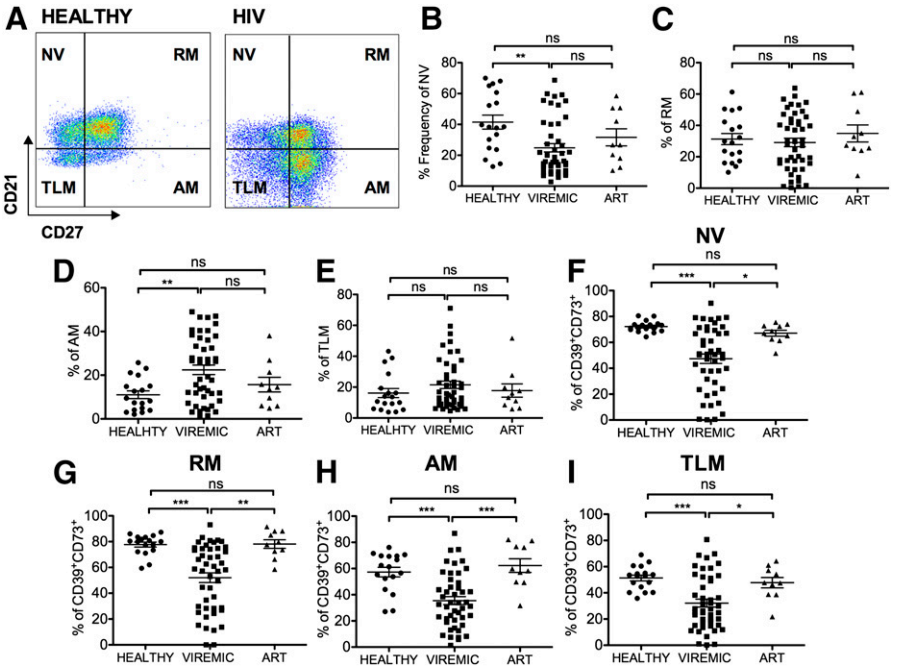

Figure 2. Cross-sectional analysis of naive and different memory B cell subpopulations and their CD39/CD73 coexpression. (A) Resentative FACS plots of a healthy control and an HIV-infected patient demonstrating the gating strategy for CD21 and CD27 within the CD20 B cell subset ($CD21^+CD27^-$, $CD21^+CD27^+$, $CD21^-CD27^+$, and $CD21^-CD27^-$). (B–E) Frequencies of different B cell subsets compared with healthy controls, viremic, or ART-treated patients. In viremic patients, the frequency of $CD21^+CD27^-$ B cells decreased and that of $CD21^-CD27^+$ B cells significantly increased compared with healthy controls. (F–I) CD39/CD73 coexpression on different B cell subsets. CD39/CD73 coexpression is significantly decreased in all B cell subsets in patients with viremic HIV compared with healthy controls. AM = activated memory ($CD21^-$,$CD27^+$), ns = not significant, NV = naive ($CD21^+CD27^-$), RM = resting memory ($CD21^+CD27^+$), TLM = tissue like memory ($CD21^-CD27^-$). Results are expressed as means ± SEM. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

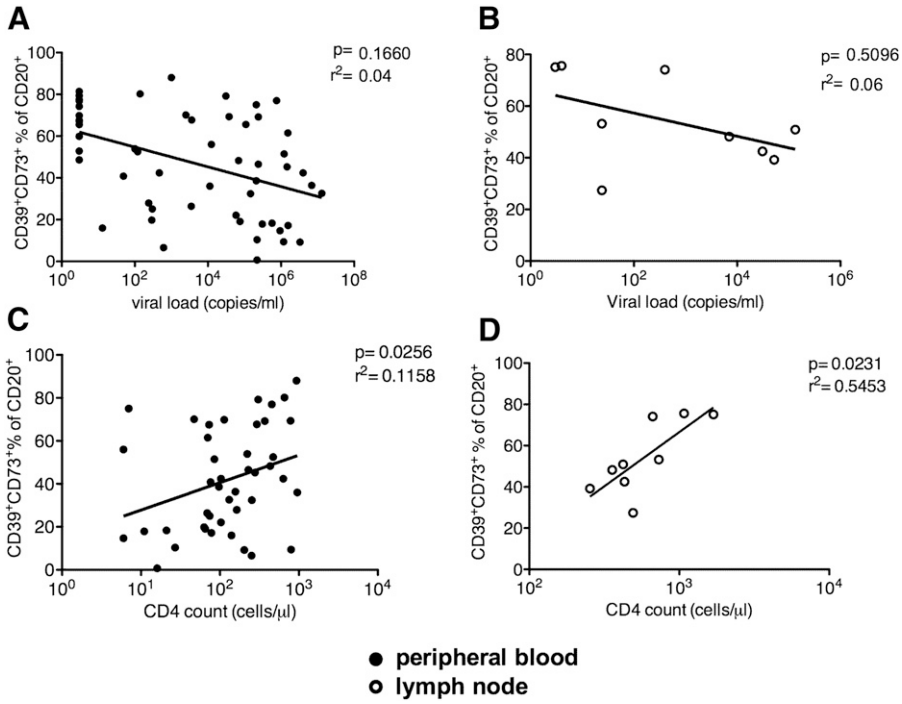

Figure 3. Correlation of CD39/CD73 coexpression and clinical parameters. (A–D) CD39/CD73 coexpression on $CD20^+$ B cells does not correlate with viral load in peripheral blood (A) and lymph nodes (B), but significantly correlates with $CD4^+$ counts in peripheral blood (C) and lymph nodes (D). Pearson's correlation was used. A P value of <0.05 was considered significant.

Finally, we also wanted to elucidate whether CD73 is down-regulated in vitro after B cell stimulation. Indeed, in vitro stimulation of PBMCs and enriched B cells with CPG2006, CD40L, IL-21, PWM, and LPS resulted in down-regulation of CD73, but not CD39, which slightly increased (data not shown) and was as previously published [2, 35].

B cells of patients with viremic HIV viremia show reduced in vitro AMP consumption and Ig class switch capability

It has recently been shown that neonatal naive B cells demonstrated lower levels of CD73 expression and CD73-mediated ADO generation [44]. To assess to which degree B cells of patients with untreated HIV also show a lower extracellular AMP consumption (and indirectly lower ADO generation), we measured AMP consumption in vitro by using the AMP Glo assay kit [45] in healthy controls and patients with HIV with different levels of CD73 expression. In line with the literature [44], we could show that B cells from HIV-infected patients degraded AMP proportionally less to ADO than healthy controls—directly in correlation to their procentual reduction of CD73 expression ($r^2 = 0.86$; $P = 0.0029$). Not surprisingly, this CD73-dependent AMP degradation could also be blocked by CD73 blockade with AMPCP (**Fig. 5**).

To assess in vitro Ig class switch capabilities of B cells in HIV infection, we performed in vitro studies with B cells from patients with HIV and healthy controls. First, we enriched $CD20^+$ B cells and marked them with the proliferation marker, eFluor 450, then stimulated the cells as described in Materials and Methods. Because B cells died at greater numbers when stimulated on their own, these experiments could only be performed in the presence of unlabeled PBMCs, which suggested an important helper role for T cells in those B cells (data not shown). Representative FACS plots of switched IgG^+CD20^+ B cells in patients with HIV and healthy controls are shown in **Fig. 6A**.

After 4 d of in vitro stimulation with CPG2006, CD40L, IL21, PWM, and LPS using an optimized protocol of a previously published study [34], B cells of healthy individuals ($n = 6$) showed a significant IgG class switch ($P < 0.05$) in accordance with the literature [34], whereas HIV-infected patients did not show any significant change in the frequency of IgG-expressing cells after stimulation (P = not significant), which is suggestive of a malfunction of the Ig class switch properties (Fig. 6B). As a further control, 3 patients with known CVID were included in the class switch assay. As expected [33], these patients also

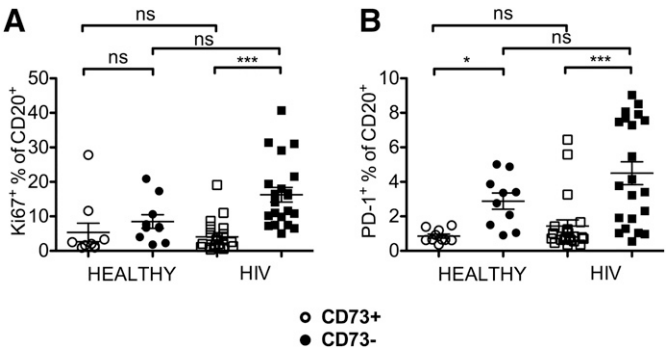

Figure 4. FACS analysis stratified in $CD73^+$ vs. $CD73^-$ B cell subsets regarding proliferation and exhaustion. (A) Frequency of Ki-67 expression is significantly higher in the $CD73^-$ B cell subset of patients with HIV. (B) In healthy controls and patients with HIV, the frequency of PD-1–expressing B cells is significantly higher within the $CD73^-$ B cell subset compared with the $CD73^+$ cells (healthy controls, $n = 10$; viremic HIV patients, $n = 18$). ns = not significant. Results are expressed as means ± SEM. $^*P < 0.05$; $^{***}P < 0.001$.

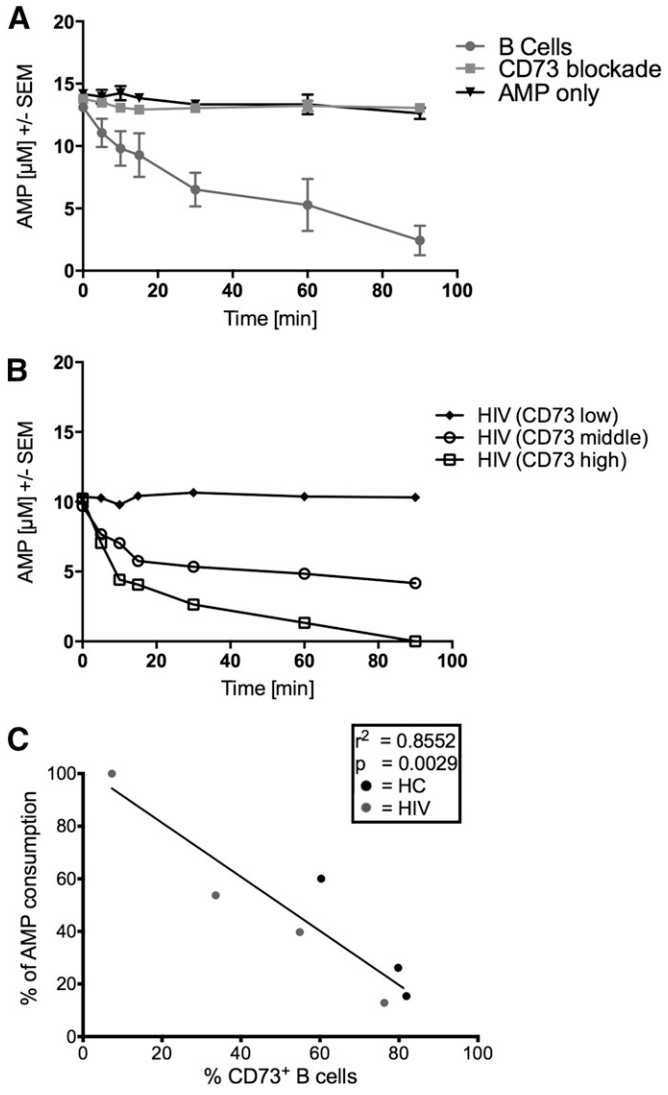

Figure 5. In vitro consumption of AMP by column-purified B cells of healthy controls (HCs; *n* = 3), viremic HIV patients (HIV; *n* = 4), or B cells only at the indicated timepoints. (A) Purified B cells of HCs (CD73 expression mean, 74%) show normal AMP consumption over time, which can be blocked by AMPCP. (B) HIV-infected patients were divided by CD73 expression into high (mean, 76.3%), medium (mean, 44.2%), and low (mean, 7.3%). Lower CD73 expression resulted in a lower AMP consumption. (C) Correlation of CD73 expression on B cells and percentage of AMP consumption at the 60-min time point with HIV-infected patients and HCs.

showed a severe deficiency of Ig class switch properties (Fig. 6B).

As a further control and to rule out the possibility that our results did not stem only from proliferation of IgG^+ B cells, we live-sorted IgG^-CD27^- naive B cells of 3 uninfected controls and subjected them to the same Ig class switch assay (Supplemental Fig. 4A). Furthermore, we demonstrated that Ig class switch depended on expression of CD73 by repeating the proliferation assay with sorted naive B cells in the presence of anti-CD73 [37, 46]. In line with the literature, there was a statistical trend of Ig class switch impairment after addition of nucleotide analogue AMPCP to the culture (as previously described; Supplemental Fig. 4B) [32, 34, 47, 48].

We also analyzed the frequency of IgG expression on B cells at different stages of HIV infection (**Fig. 7A**). Of interest, the frequency of IgG-expressing $CD20^+$ B cells was significantly higher in ART patients compared with viremic patients or with healthy controls. In general, HIV infection was associated with an increased frequency of activated mature B cells [7], which we could confirm in our cohort (data not shown). Thus, the lower frequency of IgG^+ cells that we detected did not merely result from a lower frequency of memory cells in patients with HIV.

Of note, $CD73^+$ expression was significantly lower in IgG^- compared with IgG^+ B cells in healthy controls as well as in viremic HIV patients (Fig. 7B and C).

DISCUSSION

In HIV-1 infection, exhaustion and malfunction of B cells, as well as changes in the frequency of B cell subsets, have been described (reviewed in [41]). B cells play a central role in the priming of cellular immune responses and vice versa. Thus, a better understanding of the mechanisms that control B cell functionality is needed to understand B cell dysfunction possibly contributes to HIV disease pathogenesis [49].

Here, we present the first broader study of CD39 and CD73 expression in naive and different memory B cell compartments at different stages of HIV infection.

CD39 and CD73 are 2 important B cell differentiation markers. Recently, it has been demonstrated that these 2 molecules play a central role in B cell–mediated suppression of T cell functions [2] and in the initiation of Ig class switch [34]. Results from subsequent studies—for example, in neonates and in juvenile arthritis—could suggest that this decrease in CD73 expression on B cells has functional relevance and might have great clinical implications, leading to a delayed and decreased IgG response as a result of an impaired IgG isotype switch in HIV infection [44, 50–52].

As a main result of our study, we detected a general reduction in the percentage of $CD39^+CD73^+$ cells in different B cell memory subsets in HIV infection. This decrease of both enzymes on the B cell surface correlated with the presence of HIV viremia, low $CD4^+$ counts, and increased B cell activation. In addition, B cells of patients with untreated HIV seem to have an in vitro impairment of AMP consumption and Ab class switch.

Our findings extend the results of early, preliminary, and partially contradicting studies that analyzed only the activity of these enzymes on T cell and non-T cell subsets in patients with chronic HIV >30 y ago [36, 53]. We also confirmed results of a recent study that demonstrated that low CD73 expression on neonatal B cells was associated with lower ADO generation [44]. Not surprisingly, B cells from patients with untreated HIV also showed significantly lower in vitro enzymatic activity and lower ADO generation (Fig. 5B). In summary, we have now shown another link between CD73 down-regulation, reduced ADO production, and in vitro impairment of B cell class switch in chronic, untreated HIV infection that needs to be further mechanistically elucidated and followed-up in vivo (Fig. 6B and Supplemental Fig. 4B). One technical limitation of the

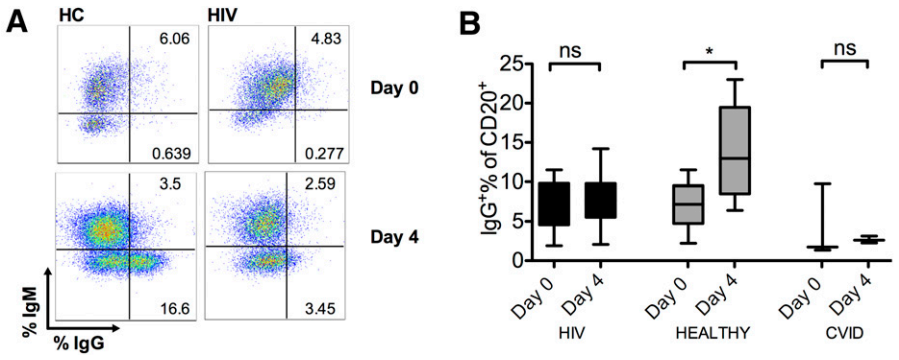

Figure 6. Only healthy controls (HCs) show a significant increase in IgG expression on purified eFluor450-labeled CD20$^+$ B cells after 4 d of in vitro stimulation with 2.5 μg/ml CpG 2006, 6 ng/ml mCD40L, 5 μg/ml PWM, 5 μg/ml LPS, and 0.1 μg/ml IL-21. (A) Gating strategy for the Ig class switch of CD20$^+$ B cells in a representative healthy individual and an HIV-infected patient before (day 0) and after stimulation of B cells (day 4). (B) Ig class switch capabilities of B cells were analyzed in 6 healthy controls, 9 viremic HIV-infected patients, and 3 patients with CVID as a control. ns = not significant. Results are expressed as means ± SEM. *$P < 0.05$.

experimental setup was that in vitro Ig switch assay was performed in the presence of autologous full PBMCs and potential functional T cell defects in HIV-infected patients may have also played a role.

Apart from its important enzymatic activity, CD73 has also been described as a migration marker and as being important for adhesion of B cells to follicular dendritic cells in the germinal center [54]. Hence, down-regulation of CD73 during HIV infection might have an impact on the migration of B cells to different tissues or interaction of those B cells with the endothelium [54–57].

At this point, it is not clear whether down-regulation of CD73 is the cause or the effect of HIV viremia or subsequent general immune activation [27]; however, lower CD73 expression in B cells was only observed in HIV infection, and we did not see an alteration of the frequency of CD39- or CD73-expressing B cells in patients with chronic HCV infection (Supplemental Fig. 2). Moreover, HIV elite controllers and ART-treated patients showed a nearly normal expression of CD39 and CD73 on their B cells compared with healthy controls (Supplemental Fig. 1).

Future studies should aim to understand the combined effects of CD39 and CD73 modulation for B cell–T cell interactions [2]. As stated by Saze et al. [2] and Toth et al. [23], to achieve this aim, further information is needed about the kinetics and balance of extracellular nucleosides in general, as well as the enzymatic surface activities of CD39 and CD73 on all lymphocyte subpopulations in healthy humans and in patients who were infected with HIV. Future studies should also evaluate B cell expression of adenosine receptor subtypes in HIV infection [46, 58, 59]. The Ab-independent dual regulatory activity of B cells is most likely mediated by the products of ATP hydrolysis, 5′-AMP and ADO. Thus, down-regulation of CD73 on B cells might tip the balance toward higher AMP concentrations and lower ADO concentrations. It is important to note that different studies have shown that a disturbance in the adenosinergic pathway can lead to an imbalance of pro- and anti-inflammatory effects depending on the local environment and ADO concentration [58, 60–62]; therefore, our data add to the hypothesis that lower concentrations of ADO might be detrimental by causing the increased chronic immune activation observed during chronic HIV infection [2, 63].

In summary, we find that patients with chronic, untreated HIV show a significant down-regulation of CD73 on B cells, which is correlated with low CD4$^+$ counts, increased immune activation, and decreased in vitro enzymatic activity and Ig class switch ability. CD73 expression seems to be partially restored after successful initiation of ART. Results of this study warrant further studies on the role of CD73 for B cell function and might be important for the understanding of B cell pathology in HIV infection.

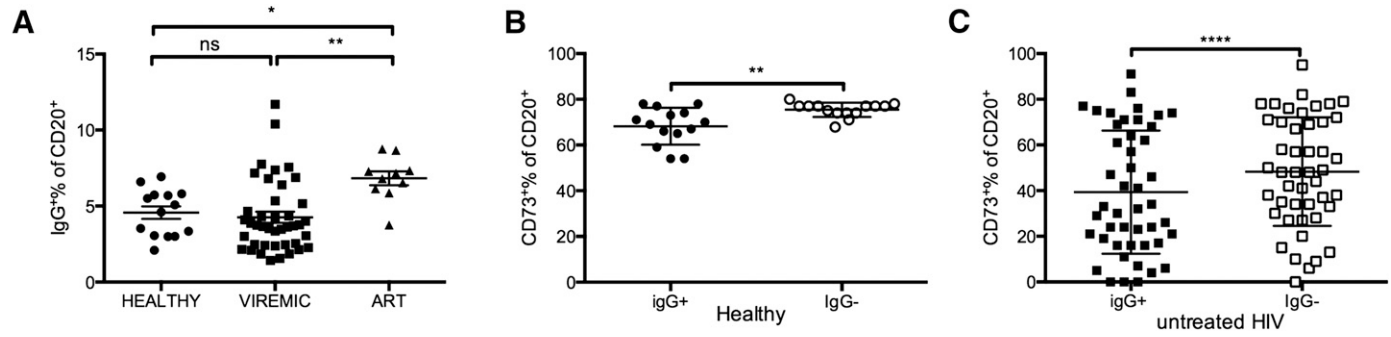

Figure 7. FACS analysis of healthy controls ($n = 18$), viremic ($n = 42$), and ART-treated HIV patients ($n = 10$) of IgG surface expression on B cells. (A) Only ART-treated patients show a significant increase of IgG expression compared with healthy controls and patients with viremic HIV. (B and C) CD73 expression on IgG$^+$ vs. IgG$^-$ CD20$^+$ B cells in healthy controls as well as in patients with untreated HIV. IgG$^-$ B cells show higher frequencies of CD73 expression in healthy controls and in patients with HIV. ns = not significant. Results are expressed as means ± SEM. *$P < 0.05$; **$P < 0.01$; ****$P < 0.001$.

AUTHORSHIP

J.S.z.W. defined the research theme. J.S.z.W. provided most of the funding. C.B., J.M.E., R.E.S., M.B., and J.v.L. founded, organized, and maintained the clinical cohort. M.B. carried out the surgical procedures. E.-S.K., C.A., I.T., P.D., R.W., F.S., and F.H carried out most of the laboratory experiments. M.G. supported with experimental reagents. E.-S.K., I.T., and J.S.z.W. carried out the data analysis and interpreted most of the results. E.-S.K. and J.S.z.W. wrote the first draft. I.T., J.M.E., C.B., F.H., J.v.L., and J.S.z.W. gave input later stages of the manuscript. All authors read, helped to revise, and approved the final manuscript.

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DISCLOSURES

The authors declare no conflicts of interest.

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KEY WORDS:
CD39 · 5′-ectonucleotidase · elite controller · ADO · Ig class switch · CD39