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Inversed Ratio of CD39/CD73 Expression of γδ T Cells in People Living with HIV Versus Healthy Controls Correlates With the Course of Disease

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Inversed Ratio of CD39/CD73 Expression on γδ T Cells in HIV Versus Healthy Controls Correlates With Immune Activation and Disease Progression

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Background: γδ T cells are unconventional T cells that have been demonstrated to be crucial for the pathogenesis and potentially for the cure of HIV-1 infection. The ectonucleotidase CD39 is part of the purinergic pathway that regulates immune responses by degradation of pro-inflammatory ATP in concert with CD73. Few studies on the expression of the ectoenzymes CD73 and CD39 on human γδ T cells in HIV have been performed to date.

Methods: PBMC of n=86 HIV-1-infected patients were compared to PBMC of n=26 healthy individuals using 16-color flow cytometry determining the surface expression of CD39 and CD73 on Vδ1 and Vδ2 T cells in association with differentiation (CD45RA, CD28, CD27), activation and exhaustion (TIGIT, PD-1, CD38, and HLA-DR), and assessing the intracellular production of pro- and anti-inflammatory cytokines (IL-2, TGF-ß, TNF-α, Granzyme B, IL-10, IFN-γ) after *in vitro* stimulation with PMA/ionomycin.

Results: CD39 and CD73 expression on γδ T cells were inversed in HIV infection which correlated with HIV disease progression and immune activation. CD39, but not CD73 expression on γδ T cells of ART-treated patients returned to levels comparable with those of healthy individuals. Only a small subset (<1%) of γδ T cells co-expressed CD39 and CD73 in healthy or HIV-infected individuals. There were significantly more exhausted and terminally differentiated CD39+ Vδ1 T cells regardless of the disease status. Functionally, IL-10 was only detectable in CD39+ γδ T cells after *in vitro* stimulation in all groups studied. Viremic HIV-infected patients showed the highest levels of IL-10 production. The highest percentage of IL-10+ cells was found in the small CD39/CD73 co-expressing γδ T-cell population, both in healthy and HIV-infected individuals. Also, CD39+ Vδ2 T cells

produced IL-10 more frequently than their CD39+ Vδ1 counterparts in all individuals regardless of the HIV status.

Conclusions: Our results point towards a potential immunomodulatory role of CD39+ and CD73+ γδ T cells in the pathogenesis of chronic HIV infection that needs further investigation.

Keywords: γδ T cells, HIV-1, T cell, CD39, CD73, Vδ2, IL-10, elite controllers

INTRODUCTION

The human immunodeficiency virus-1 (HIV) is a lymphotropic virus that mainly infects and depletes CD4+ T cells, leading to chronic immune activation, immune dysfunction, and, ultimately, immunodeficiency (1–4). Although highly active antiretroviral therapy (HAART) potently suppresses viral replication, no cure is available to date (5, 6).

Lately, the role of unconventional T cells for HIV pathogenesis and HIV cure approaches has come more into focus (7–11). Among these T-cell populations, γδ T cells seem to have important immunomodulatory properties relevant for the disease (4). γδ T cells express a T-cell receptor with gamma and a delta chain (12–17). They are "innate-like" T cells that make up 1-15% of circulating leukocytes and exert a direct cytotoxic activity independently of MHC presentation (12–22). About 30% of γδ T-cells express a CD8+ T cell receptor, less than 1% a CD4+ T-cell receptor and 70% none of the conventional T-cell receptors (23).

γδ T cells recognize stress-induced molecules, non-peptide, and phosphoantigens, self- or MHC-related molecules, and lipids associated with different kinds of pathogens (20, 21, 24–29). γδ T cells are generally seen to be pro-inflammatory and involved in the initiation and propagation of immune responses, but lately, it has been shown that they also act as immunomodulators and can inhibit T- and B-cell responses (20, 21, 30–32). They produce the pro-inflammatory cytokines TNF-α, IL-17, and IFN-γ as well as the anti-inflammatory cytokines IL-10 and TGF-β and IL-2, IL-22, IL-21, IL-4, IL-5, IL-13 (31, 33–38). γδ T cells can alleviate or maximize inflammation in the blood and different tissues and might be used to directly target HIV-infected cells but have also been described as potential targets of HIV (18, 22, 39, 40).

The two main subsets of γδ T cells, Vδ1 and Vδ2 are present in different anatomic compartments (23, 41–44). While Vδ1 cells can be found in the intraepithelial layer of mucosal surfaces, the Vδ2 population is mostly present in the blood and secondary lymphoid tissues of healthy adults (ratio in the peripheral blood Vδ1:Vδ2 3:10) (23, 41–49).

Early during primary HIV infection, an inversion of the Vδ1: Vδ2 ratio can be observed in the blood whereas the frequency of total γδ T cells remains relatively stable (18, 22). Vδ2 cells are depleted while Vδ1 cells expand (4, 18, 50–52). It has been shown that Vδ2 cells express high levels of the HIV co-receptors CCR5 and α4β7, which possibly contributes to their preferential depletion in HIV infection (53–56). Their number, but also functionality remains below that of healthy controls even after successful implementation of ART and restoration of the CD4+ T-cell compartment (51, 52). It has been implied that Vδ1 cells are involved in antiviral immunity and their expansion may be an indirect consequence of viral infection and reflects an increased translocation of stimulatory bacterial products across the gut epithelium in non-human primate studies (57–59). In contrast, direct cytotoxicity towards HIV-infected cells has been demonstrated to be largely restricted to Vδ2 cell clones, and the frequency of cervical Vδ2 cells correlates with SIV viral load (60–62). Interestingly, elite controllers exhibit higher frequencies of Vδ2 cells than untreated or antiretroviral treated HIV progressors (63, 64).

The ectonucleotidases CD39 and CD73, members of the adenosine pathway that are expressed on several lymphocyte populations, convert extracellular pro-inflammatory ATP and ADP to anti-inflammatory Adenosine (ADO) (65–67). In healthy individuals, the level of extracellular ADO is low but can increase 100-1000-fold in situations of strong inflammation and tissue injury (56). ADO strengthens epithelial barrier functions and inhibits leukocyte extravasation by binding to four different receptors: A1R, A2AR, A2BR, and A3R (68, 69). ADO binding to A2AR increases intracellular cAMP and inhibits the production of cytokines and T-cell proliferation (70–74). Importantly, both CD39 and CD73 can work in cis (interaction on the same cell), trans (interaction with enzymes expressed on different cells), and as soluble forms, and detailed knowledge about the respective microenvironment is essential (67, 75).

Over the last decade, multiple roles of CD39 and CD73 in the regulation of inflammation and immune responses have been revealed (75–82). It has also been demonstrated that mutations in the purine system can cause severe primary immunodeficiency diseases (78, 83–85). In HIV-infected untreated individuals, an over-expression of CD39 on and an increased hydrolysis of ATP by lymphocytes has been observed. Also, a variant of the CD39 gene associated with low CD39 expression and a slower progression to AIDS has been described in lymphocytes (86–88). Furthermore, the CD39/CD73/adenosine axis has been linked to inhibition of HIV-1 replication as well as immune suppression by CD39+ regulatory T cells (Tregs) (66, 88, 89).

In viremic HIV patients, the frequency of CD73+ cells in different T-cell subsets, especially Tregs and CD8+ T cells, is markedly reduced, and the function of CD73+ CD8+ T cells is impaired (82, 90). On B cells, low CD73 and CD39 expression are associated with low CD4+ T-cell counts (77). While both CD39 and CD73 are co-expressed on murine Tregs, only a small fraction of human peripheral Tregs expresses CD73 (65, 66, 82, 91).

Higher CD73 levels have been associated with immunosuppression and poor prognosis in e.g. breast or ovarian

cancer (76, 92–95). In mice, suppressive activities of CD73+ γδ T cells *via* adenosine were shown (96).

Only recently it has been shown that γδ T cells can also act in an immunosuppressive manner and that they can infiltrate tumors and suppress dendritic cells and T cells (30, 97, 98). Liang et al. demonstrated that the regulation of γδ T cells in autoimmunity is associated with ADO (96). Hu et al. described $CD39^+$ γδ T cells as capable of suppressing T cells *via* the adenosine-mediated pathway but independent of IL-10 and TGF-beta expression (98). In contrast, Otsuka et al. have reported a potential role of CD39+ γδ T cells with a regulatory phenotype mediated by IL-10 secretion in mice (98, 99).

In HIV infection, the plasma concentration of IL-10 increases over time and limits specific T-cell responses (100). CD39+ NK cells secreting IL-10 also contribute to this increase: Dierks et al. demonstrated that elevated levels of CD39+ NK cells in viremic patients correlated directly with viral load and activation, and negatively correlated with CD4+ T-cell count (101). IL-10 secretion was associated with the expression of CD39 (99, 101–103).

We and others have previously shown that CD39 expression of Tregs correlates with the progression of HIV infection and that Tregs of HIV elite controllers show the lowest levels of CD39 (79, 104). We have also recently identified CD39+ γδ T cells with an immunosuppressive phenotype in the gut (81). Bhatnagar et al. suggested a suppressive activity especially of Vδ2 *via* TGF-β, which is dysregulated in progressed HIV infection (35).

In HIV infection, a comprehensive assessment of the expression of CD39 and CD73 on different γδ subsets including Vδ1 and Vδ2 γδ T cells has never been performed. Therefore, we sought to characterize CD39+ and CD73+ expression on γδ T cells in relation to phenotype and function in a large cohort of healthy individuals and people living with HIV with different disease statuses including HIV elite controllers and long-term non-progressors.

MATERIAL AND METHODS

Study Subjects and Samples

Peripheral blood mononuclear cell (PBMC) samples of chronic, treatment-naïve HIV patients (viremic, n=36), HIV antiretroviral therapy (ART)-treated patients (ART, n=32), HIV elite controllers (EC, n=8), HIV long-term non-progressors (LTNP, n=10) and HIV negative healthy controls (n=26) were collected at the University Medical Center Hamburg-Eppendorf. HIV elite controllers were defined as HIV-infected individuals capable of spontaneously controlling HIV infection (maintaining stable CD4+T-cell counts and viral loads below the level of detection) without the need for antiretroviral medication (105–107). Written informed consent was obtained from all patients who were recruited for this study, which was approved by the local Institutional Review Board of the Ärztekammer Hamburg, Germany (MC-316/14, PV4780, PV5798, PV4081, WF14-09). Active Hepatitis C virus and Hepatitis B virus co-infections were ruled out serologically in the HIV-infected patients studied. $CD4^+$ T-cell counts and plasma viral loads were extracted from the clinical database (**Table 1**). Clinical and virologic data of the HBV and HCV patients can be found in **Supplementary Table 1**.

Immune Phenotypic Analysis for Surface and Intracellular Markers

Cryopreserved PBMC were isolated and used for immunophenotypic staining as previously described (108). Cells were stained with Zombie NIR fixable viability stain (BioLegend, San Diego, USA) and the following anti-human monoclonal fluorochrome-conjugated antibodies: anti-CD45RA, anti-CD4, anti-TCR-γ/δ (BD Biosciences, Heidelberg, Germany), anti-TCR-Vδ2 (Beckman Coulter Life Sciences, Indianapolis, USA), anti–HLA-DR, anti-CD27, anti-CD279 (PD-1), anti-TIGIT, anti-CD8, anti-CD28, anti-CD39, anti-CD38, anti-CD19, anti-CD3, anti-CD73 and anti-CD14 (all BioLegend) (**Supplementary Table 2**). Cells were incubated for 30 minutes at room temperature with the respective antibodies. After washing, cells were fixated with 4% paraformaldehyde. All samples were run on a Becton Dickinson LSR Fortessa flow cytometer with FACS Diva version 8 (BD Biosciences).

Intracellular Cytokine Staining and Kinetic of CD39 Expression After *In Vitro* Stimulation of PBMC

For intracellular staining, cells were stimulated with phorbol 12-myristate 13-acetate (PMA; final concentration 25 ng/mL; Merck, Darmstadt, Germany) and ionomycin (final concentration 1 μg/mL; Merck) for 18 hours. After 2 hours, Brefeldin A (5μg/mL; Merck) and Monensin (1μg/mL; Merck)

TABLE 1 | Demographic, virologic, and immunological basic data of the cohort [**average** (± SD) min – max].

	Healthy	*ART*	*Viremic*	*EC*	*LTNP*
n	**26**	**32**	**36**	**8**	**10**
Sex in % (f/m/d)	**63/37/0**	**23/77/0**	**22/78/0**	**67/33/0**	**38/62/0**
Age (years)	**29,1** (± 10,8) 20- 61	**44,5** (± 14,3) 25 – 75	**39,6** (± 12,5) 22 - 72	**43,5** (± 14,9) 21 - 56	**47** (± 11,9) 32 - 73
CD4+ T-cell count (cells/mL)	**>500**	**531,9** (± 256,1) 125 – 1190	**276,5** (± 300,8) 6 - 1731	**866,3** (± 301,4) 458 - 1219	**596,4** (± 408) 175 - 1485
Viral load (copies/mL)	**n.a.**	**<10**	**312256** (± 566792) 6300 - 3300000	**<10**	**1323,75** (± 973,7) 40 - 2600

For the study, 26 healthy volunteers, 36 virally suppressed HIV patients on ART, 32 HIV-infected viremic individuals, 8 aviremic elite controllers, and 10 long-term non-progressors were included.
Mean values in bold type. N.a. not applicable.

were added. First, surface antigens were stained as described above. Cells were then permeabilized with fixation/permeabilization solution (Cytofix/Cytoperm; BD Biosciences) and stained with fluorochrome-conjugated antibodies for 30 minutes at 4°C. The following anti-human monoclonal antibodies were used: anti-IL-2, anti-CD4, anti-IL-10, anti-TCR-γ/δ (BD Biosciences), anti-TCR-Vδ2 (Beckman Coulter Life Sciences), anti–IFN-γ, anti-TNF-α, anti-CD8, anti-TGF-β, anti-CD39, anti-Granzyme-B, anti-CD19, anti-CD3, anti-CD73, and anti-CD14 (all BioLegend), see also **Supplementary Table 3**.

For kinetic studies of CD39 surface expression, cells were stimulated as previously described with small adaptations (109). Briefly, cryopreserved PBMC were plated into 48-well plates and stimulated with rhIL-2 (20 U/mL; Miltenyi Biotec, Bergisch Gladbach, Germany), PMA (5 ng/mL), ionomycin (0,5 µg/mL), anti-CD3/CD28-Dynabeads (ratio 1:1; ThermoFisher Scientific, Waltham, USA) or combinations thereof. Cells were cultured for up to 6 days before FACS analysis.

Data Analysis and Statistics

Cytometric data were analyzed using FlowJo version 10.7.1 (BD Biosciences). The applied gating strategy and exemplary dot plots are depicted in **Supplementary Figures 2**, **3** and **8**. Statistical analysis was performed using GraphPad Prism version 7.04 (GraphPad Software, Inc., La Jolla, CA). For multiple comparisons, Kruskal–Wallis and Dunn's post-test with an alpha value of 0.05 were performed. All reported *P* values were multiplicity adjusted according to Dunn. To compare ranks, 2-tailed Mann–Whitney and Wilcoxon tests were performed. Pearson correlation and Spearman rank correlation coefficient were applied for bivariate correlation analysis. Multidimensional cytokine analysis was carried out using SPICE 6 (110). Data are expressed as mean with SD. P-values of less than 0,05 were considered significant. Levels of significance correspond to asterisks as follows: ns p≥0,05; * p<0,05; ** p≤0,01; *** p≤0,001; **** p≤0,0001.

RESULTS

The ectonucleotidases CD39 and CD73 have been described as important immunoregulatory molecules on Tregs and T effector cells (67, 75–79, 81, 82, 92, 93, 98, 99, 101, 104, 111). In mice, CD39+ γδ T cells with a regulatory phenotype have already been described (99, 112). In humans, CD39+ immunosuppressive γδ T cells have been described in the context of colon cancer (98). Little is known about the expression of these two molecules on γδ T cells in healthy humans and the context of viral infections. In this study, we aimed at the detailed assessment of the CD39 and CD73 expression pattern on peripheral γδ T cells in healthy and HIV-infected individuals with respect to their differentiation, activation, and exhaustion status and their immunomodulatory properties in terms of their cytokine profiles.

In line with previously published data, we found that the percentage of total γδ T cells was stable during HIV infection regardless of the stage of HIV infection while the ratio between the subsets Vδ1 and Vδ2 was inversed (**Supplementary Figure 1;** see **Supplementary Figures 2** and **3** for the gating strategy) (4, 18, 22, 40). In healthy individuals, the percentage of Vδ2 (68,5%) was significantly higher than that of Vδ1 γδ T cells (31,4%, p=0,0027), whilst it was significantly lower in viremic (Vδ1: 81,6%, Vδ2: 18,4%, p<0,0001) and patients on ART (Vδ1: 73,3%, Vδ2: 26,8%, p<0,0001). Interestingly, in long-term non-progressors, the proportions of Vδ1 (50,34%) and Vδ2 γδ T cells (49,7%) were similar. In elite controllers, we observed a difference between Vδ1 (69,7%) and Vδ2 (30,3%) γδ T-cell proportion that did not reach statistical significance, most likely due to the small patient number.

The Frequency of CD39+ γδ T Cells Increases While the Frequency of CD73+ γδ T Cells Decreases in HIV Infection

To understand the biology of CD39 and CD73 on γδ T cells, we first globally assessed the expression pattern of the two ectonucleotidases on total γδ T cells in healthy individuals and HIV patients who were further sub-stratified according to their disease status (**Figures 1A, B**). The highest frequency of CD39+ γδ T cells was detected in samples from viremic HIV-infected patients. In viremic and individuals on ART, the CD39+ γδ T-cell frequency was significantly increased compared to healthy individuals (viremic: 11,3% vs. 1,4%, p<0,0001; ART: 3,2% vs. 1,4%, p=0,0146). Of interest, in samples from EC, the frequency of CD39+ γδ T cells was similar compared to samples from healthy controls (2,7%), while it was slightly elevated in LTNP (5,1%) compared to healthy controls. The differences between CD39+ γδ T cells in EC/LTNP and healthy controls did not reach statistical significance.

Conversely, the frequency of CD73+ γδ T cells was markedly decreased in PBMC from HIV-infected individuals regardless of their infection status compared to healthy individuals (**Figure 1B**). The differences between CD73+ γδ T cells from healthy and HIV-infected individuals were statistically significant (healthy: 20,7% vs. ART: 5,8%, p<0,0001; viremic: 3,7%, p<0,0001; LTNP: 6,8%, p=0,0016). Of note, only the differences between CD73+ γδ T cells from healthy individuals and EC were non-significant (EC: 9,4%, p=0,0525).

Next, we analyzed the co-expression pattern of CD39 and CD73 on γδ T cells. The number of CD39+CD73- γδ T cells was similar to the number of CD39+ γδ T cells in healthy individuals and all HIV patient subgroups (**Figures 1A, C**). Also, the frequency of CD73+CD39- γδ T cells (**Figure 1D**) was similar to the frequency of CD73+ γδ T cells (**Figure 1B**). In contrast to that, the frequency of double-positive CD39+CD73+ γδ T cells was considerably lower in PBMC from all study groups and did not differ significantly between healthy and HIV-infected individuals (healthy: 0,25%; ART: 0,11%; viremic: 0,15%; EC: 0,11%; LTNP: 0,14%) (**Figure 1E**).

To understand if the pattern of CD39 and CD73 expression on γδ T cells was similarly affected in other acute or chronic viral infections, PBMC from patients with acute and chronic hepatitis B (HBV) and chronic hepatitis C (HCV) were also analyzed

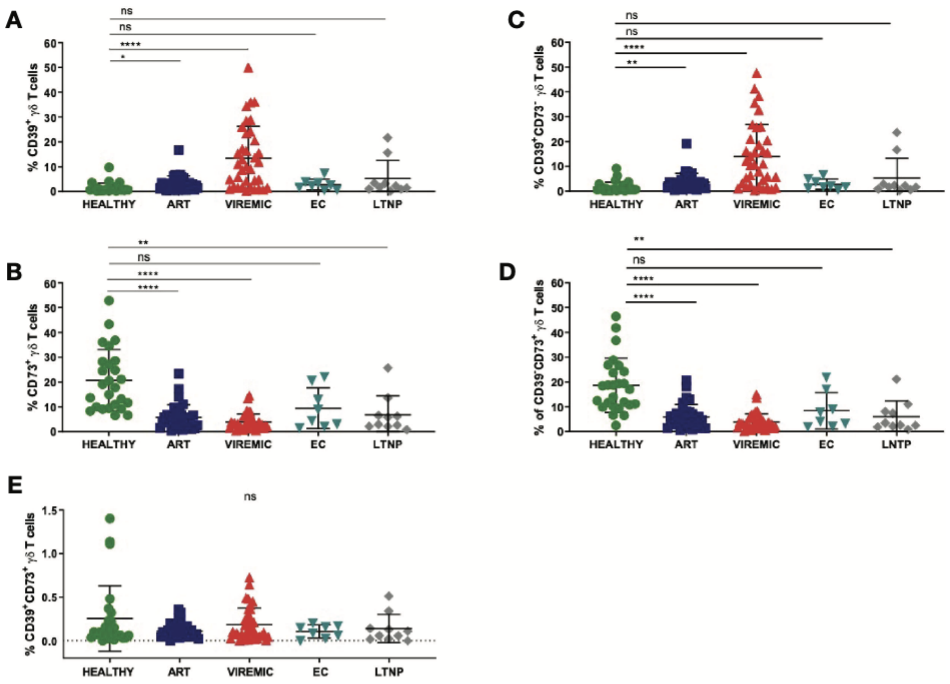

FIGURE 1 | The relative frequency of CD39+ γδ T cells is increased in viremic and HIV patients on ART compared to healthy controls **(A)** while the frequency of CD73+ γδ T cells is decreased in HIV infection regardless of the disease status **(B)**. **(C)** Frequency of CD39+CD73- γδ T cells in PBMC. **(D)** Frequency of CD39-CD73+ γδ T cells in PBMC. **(E)** Frequency of double-positive CD39+CD73+ γδ T cells in PBMC. Data from 26 healthy individuals, 32 viremic, 36 HIV patients on ART, 8 EC, and 10 LTNP. ns, non-significant p≥0,05; *p<0,05; **p≤0,01; ****p≤0,0001.

(**Supplementary Figure 4** and **Supplementary Table 1**). In acute HBV, an increase of CD39+ CD8+ T cells (data not shown) and a decreased frequency of CD73+ γδ T cells could be measured compared to healthy controls (7,8% vs. 23,9%). By contrast, there was no increase of CD39+ γδ T cells in patients with acute HBV, chronic HBV, or chronic HCV compared to healthy individuals. The reasons for the specific expansion of CD39+ γδ T cells in HIV compared to other viral infections are unclear and must be elucidated.

CD39 Expression on γδ T Cells From HIV-Infected Individuals Correlates With Viral Load, CD4+ T-Cell Counts, and Immune Activation

Since we observed divergent expression of CD39 and CD73 on γδ T cells in PBMC from HIV-infected patients compared to healthy individuals, we next examined whether there was a correlation with standard clinical parameters defining the HIV disease course. Indeed, the frequency of CD39+ and CD73+ γδ T cells was significantly correlated with the HIV viral load and CD4+ T-cell counts (**Figure 2**). Furthermore, the frequency of CD39+ γδ T cells correlated with immune activation indicated by the co-expression of HLA-DR and CD38 on CD8+ and total γδ T cells (**Supplementary Figures 5** and **6**).

There was a negative correlation between the frequency of CD39+ γδ T cells and CD4+ T-cell counts (Spearman ρ=-0,44, p<0,0001, **Figure 2A**) and a positive correlation between plasma viral load and the frequency of CD39+ γδ T cells (Spearman ρ=0,43, p<0,0001, **Figure 2B**).

The same analyses for CD73+ γδ T cells yielded the opposite results: the frequency of CD73+ γδ T cells positively correlated with CD4+ T-cell counts (Spearman ρ=0,35, p=0,0013, **Figure 2C**) and negatively with viral load (Spearman ρ=-0,32, p=0,0033, **Figure 2D**).

In samples from individuals with HIV, the frequency of CD39+ γδ T cells correlated with the proportion of activated CD8+ T cells (Spearman ρ=0,26, p=0,0375; **Supplementary Figure 5A**) and activated γδ T cells (Spearman ρ=0,42, p=0,0004; **Supplementary Figure 6**).

Interestingly, in PBMC of healthy individuals, there were non-significant negative correlations between activated CD8+ T cells and the frequency of CD39+ γδ T cells (Spearman ρ=-0,30, p=0,2205) and between activated γδ T cells and CD39+ γδ T cells (Spearman ρ=0,26, p=0,2819; **Supplementary Figures 5B** and **6**). Regardless of the disease status, a significantly higher

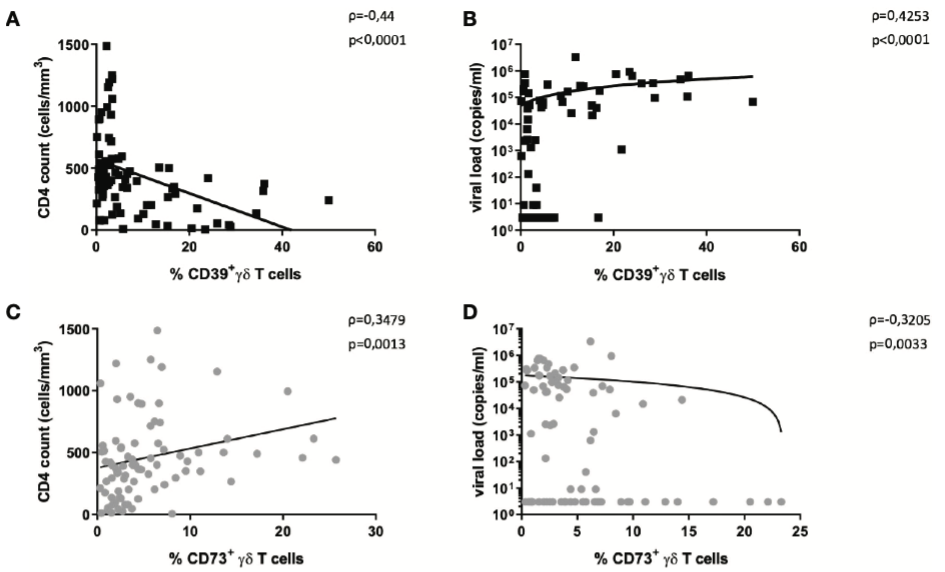

FIGURE 2 | Correlation of CD39+ and CD73+ γδ T cells with disease progression markers. The frequency of CD39+ γδ T cells in HIV correlates negatively with CD4+ T-cell count **(A)** and positively with HIV viral load **(B)**. The frequency of CD73+ γδ T cells in HIV correlates positively with CD4+ T-cell count **(C)** and negatively with HIV viral load **(D)**. Graphs contain pooled data from HIV-infected patients including viremic and individuals on ART, EC, and LTNP.

frequency of activated cells was measured among CD39+ compared to CD39- γδ T cells (**Supplementary Figure 5C**). There was no correlation between the frequency of CD73+ γδ T cells and activated γδ or activated CD8+ T cells in HIV-infected patients (data not shown).

Of note, the frequency of CD39+ γδ T cells increased steadily for 6 days after *in vitro* stimulation with CD3/CD28 or PMA/ionomycin (**Supplementary Figure 7**).

Taken together, the frequency of activated CD8+ and γδ T cells correlated with the frequency of CD39+ γδ T cells in HIV infection, and CD39+ γδ T cells expanded in response to *in vitro* stimulation.

Vδ2 γδ T Cells Are Less Exhausted and Less Differentiated Than Their Vδ1 γδ T-Cell Counterparts But Do Not Differ in Their Activation Status

Vδ1 and Vδ2 T cells differ considerably in their phenotype and functionality (20, 23, 113–115). Higher frequencies of Vδ2 T cells have been found in elite controllers and the frequency of cervical Vδ2 T cells has been correlated with SIV viral load, pointing towards a beneficial, potentially immunomodulatory role of this subset (62, 63, 116).

We aimed to investigate the expression pattern of CD39+ Vδ1 versus CD39+ Vδ2 γδ T cells in healthy individuals and HIV patients, with the idea that CD39+ Vδ2 γδ T cells might have a stronger immunomodulatory function. In general, the expression of CD39 on the Vδ2 T-cell subset was significantly lower than on the Vδ1 γδ T-cell subset in all study groups except EC (**Figure 3A**). Also, there was a marked increase of CD39+ Vδ1 and Vδ2 γδ T cells in PBMC from viremic patients compared to healthy controls. The largest differences in CD39 expression between Vδ1 and Vδ2 γδ T cells were observed in healthy and viremic individuals (healthy: 3,7% vs. 1,0%, p=0,0001; viremic: 14,8% vs. 6,98,3%, p<0,0001). In samples from patients on ART, the expression levels of CD39+ γδ T cells were similar in the Vδ1 and Vδ2 subsets (3,7% vs. 3,0%, p=0,0266). The same was observed in PBMC from EC, where no statistically significant differences were detected between Vδ1 and Vδ2 γδ T cells (3,5% vs. 2,6%, p=0,3125). In samples from LTNP, the frequency of CD39+ cells was also significantly higher among Vδ1 compared to Vδ2 γδ T cells (7,2% vs. 3,0%, p=0,0039) (**Figure 3A**).

Overall, CD39 expression on total γδ T cells correlated with immune activation in HIV patients (**Supplementary Figure 5A**). We thus compared the frequency of activated (HLA-DR+CD38+) CD39+ Vδ1 and CD39+ Vδ2 subsets (**Figure 3B**). We observed similar frequencies in all studied groups except LTNP, where the frequency of activated CD39+ Vδ2 was significantly lower than the frequency of activated CD39+ Vδ1 γδ T cells (CD39+ Vδ1 vs. CD39+ Vδ2: healthy: 11,2% vs. 7,0%; ART: 9,8% vs. 11,0%; viremic: 12,7% vs. 12,8%; EC: 24,8% vs. 16,4%; LTNP: 24,8% vs. 6,0%, p=0,0020) (**Figure 3B**). Interestingly, regardless of disease status, total Vδ1 γδ T were significantly more activated than Vδ2 γδ T cells (data not shown).

In chronic, untreated HIV infections, an increase of terminally differentiated, exhausted, and dysfunctional CD8+

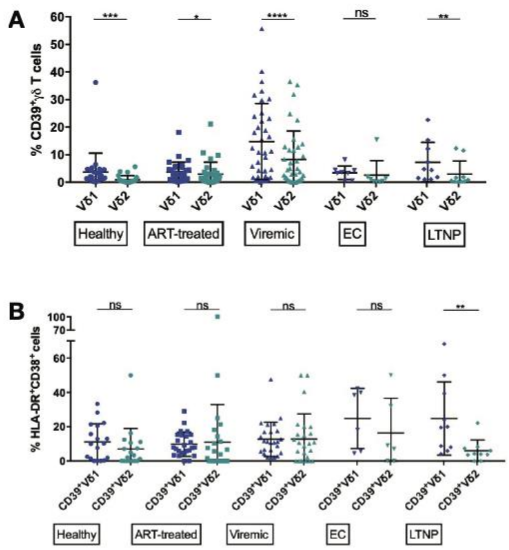

FIGURE 3 | Frequency of CD39+ Vδ1 and Vδ2 cells among total and activated γδ T cells. **(A)** Frequency of CD39+ Vδ1 versus Vδ2 γδ T cells. **(B)** Frequency of activated (HLA-DR+CD38+) CD39+ Vδ1 versus Vδ2 γδ T cells. ns, non-significant p≥0,05; *p<0,05; **p≤0,01; ***p≤0,001; ****p≤0,0001.

and CD4+ effector T cells has been described (82, 117, 118). We thus also assessed the differentiation and exhaustion status of Vδ1 and Vδ2 γδ T cells in conjunction with CD39 expression, taking the co-expression of the exhaustion markers PD-1 and TIGIT as an indicator of exhaustion and the absence of CD27 and CD28 as an indicator for a late stage of differentiation (**Figure 4**) (119).

We found significantly lower levels of exhausted (PD-1+TIGIT+) γδ T cells among CD39+ Vδ2 compared to CD39+ Vδ1 γδ T cells regardless of disease status (healthy: 21,15% vs 2,56%, p<0,0001; ART: 14,90% vs. 8,49%, p<0,0001; viremic: 25,76% vs. 17,22%, p=0,0018; EC: 16,5% vs. 2,67%, p=0,0312; LTNP: 32,21% vs. 5,87, p=0,0020; **Figure 4A**). As observed for activation, a significantly higher frequency of total Vδ1 compared to Vδ2 γδ T cells were exhausted (PD-1+ TIGIT+) in all study groups (data not shown).

We found a higher frequency of cells with late differentiation status (CD27-CD28-) among CD39+ Vδ1 compared to CD39+ Vδ2 γδ T cells in healthy individuals and HIV patients regardless of the disease status (**Figure 4B**). These differences of differentiation between CD39+ Vδ1 and Vδ2 γδ T cells were statistically significant except for the γδ T cells of EC and LTNP

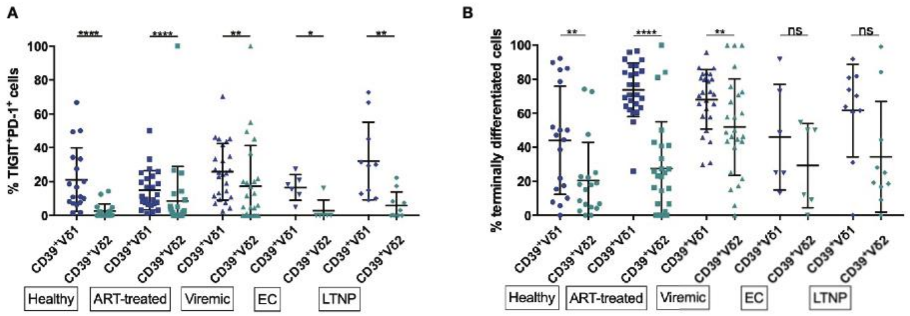

FIGURE 4 | Frequency of exhausted and terminally differentiated cells among CD39+ Vδ1 versus CD39+ Vδ2. **(A)** Frequency of exhausted (TIGIT+PD-1+) CD39+ Vδ1 versus Vδ2 γδ T cells **(B)** Frequency of CD39+ Vδ1 versus Vδ2 γδ T cells at a late stage of differentiation (CD27-CD28-). *p<0,05; **p≤0,01; ****p≤0,0001.

(healthy: 44,02% vs. 20,4%, p=0,0032; ART: 73,71% vs. 27,44%, p<0,0001; viremic: 68,08% vs. 51,86%, p=0,0018; EC: 45,97% vs. 29,24%, p=0,3125; LTNP: 61,56% vs. 34,42%, p=0,0645).

In summary, markers of T-cell exhaustion were more frequently expressed among CD39+ Vδ1 than CD39+ Vδ2 T cells regardless of disease status, and late differentiation of cells was more often present in CD39+ Vδ1 than CD39+ Vδ2 T cells of healthy individuals and HIV progressors.

While CD39 is upregulated during generalized immune activation in many lymphocyte populations, we and others could also define CD39 as a marker of several immunomodulatory populations in healthy individuals and HIV patients (78, 81, 98, 99, 104, 111). To characterize the functional profile of CD39+ γδ T cells and to assess their potential immunomodulatory effector functions, we performed intracellular cytokine stainings of γδ T cells for IL-10, IL-2, IFN-γ, TNF-α, TGF-β, and Granzyme-B after unspecific stimulation of PBMC with PMA and ionomycin (**Figure 5** and **Supplementary Figure 8**).

Expression of CD39 and CD73 Marks γδ T Cells That Produce IL-10 at High Levels After *In Vitro* Stimulation

In untreated HIV infection, multiple cell types secrete IL-10 that suppresses virus-specific T cells and thereby inhibits virus clearance (100). In mice, a population of regulatory γδ T cells that secrete IL-10 have been described (99). However, there are few data on the IL-10 secretion of peripheral γδ T cells in HIV. Thus, we specifically examined the production of IL-10 by γδ T cells.

The frequencies of IL-10+ cells were generally lower among total γδ T cells than in CD39+ or CD39+CD73+ γδ T cells (**Figure 5A**). There were no statistically significant differences between the study groups. Interestingly, the highest frequency of IL-10+ γδ T cells was found in viremic HIV-infected patients, who also expressed the highest amount of CD39 (**Figure 1A**). We thus compared the frequency of IL-10 producing CD39+ and CD39- γδ T cells (**Figure 5B**). In samples from healthy donors, viremic HIV-infected individuals and patients on ART, the frequency of IL-10 producing γδ T cells was significantly higher among CD39+ than CD39- cells (healthy: 14,8% vs. 0,2%, p=0,0195; ART: 7,4% vs. 0,2%, p=0,0156; viremic: 10,4% vs. 2,3%, p=0,0171). In samples from EC, we detected similar frequencies of IL-10 producing γδ T cells between CD39+ and CD39- cells (1,7% vs. 1,7%, p=0,5556).

Similarly, in all groups but EC, the frequency of IL-10+ cells was significantly higher among CD73+ than CD73- γδ T cells upon *in vitro* stimulation (healthy: 1,5% vs. 0,3%, p=0,0273; ART: 1,4% vs. 0,2%, p=0,0273; viremic: 13,2% vs. 2,2%,

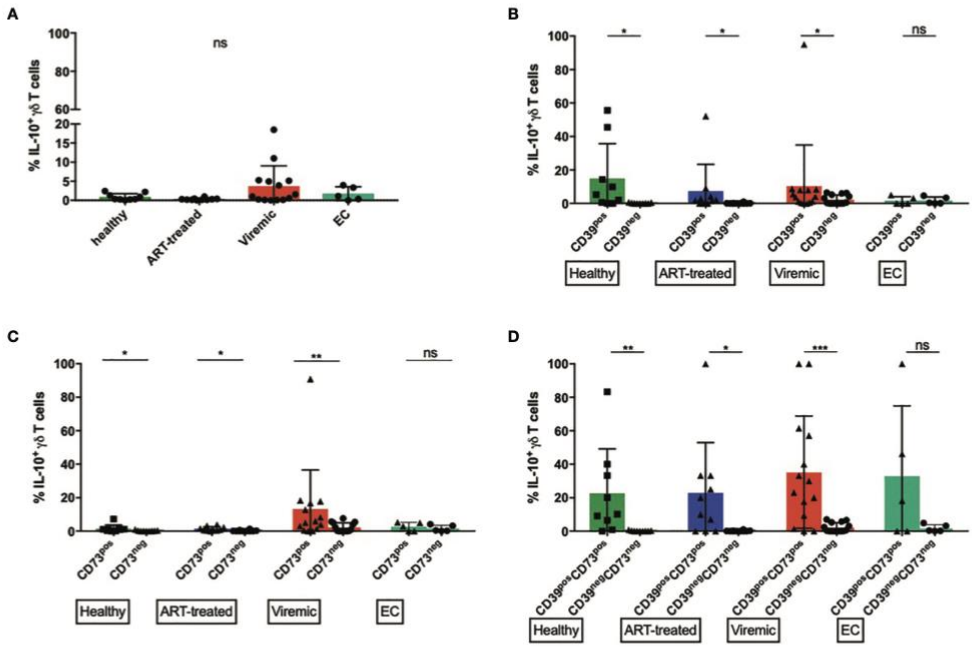

FIGURE 5 | CD39+ γδ T cells produce more IL-10 upon *in vitro* stimulation than CD39- γδ T cells. **(A)** Frequency of IL-10 producing total γδ T cells **(B)** Frequency of IL-10 producing CD39+ versus CD39- γδ T cells. **(C)** Frequency of IL-10 producing CD73+ versus CD73- γδ T cells. **(D)** Frequency of IL-10 producing CD39+CD73+ versus CD39-CD73- γδ T cells. ns, non-significant p≥0,05; *p<0,05; **p≤0,01; ***p≤0,001.

p=0,0034), although the frequency of IL-10+ cells was overall lower than in CD39+ γδ T cells (**Figure 5C**).

We next assessed the capacity of the small population of CD39 +CD73+ versus CD39-CD73- γδ T cells to produce IL-10 (**Figure 5D**). Interestingly, CD39+CD73+ γδ T cells produced more IL-10 than CD39-CD73- γδ T cells regardless of the disease status. In all groups but EC, the differences between CD39+CD73+ and CD39-CD73- γδ T cells reached statistical significance (healthy: 22,6% vs. 0,2%, p=0,0078; ART: 22,9% vs. 0,2%, p=0,0156; viremic: 35,2% vs. 2,2%, p=0,0005; EC: 32,8% vs. 1,7%, p=0,2500).

Lastly, we examined whether there were differences between the IL-10 production of CD39+ Vδ1 versus CD39+ Vδ2 γδ T cells (**Supplementary Figure 9**). The frequency of pooled (combined data from all study groups) IL-10+ CD39+ Vδ2 cells was higher than the frequency of pooled IL-10+ CD39+ Vδ1 γδ T cells (16,2% vs. 3,7%, p=0,01). Comparing the study groups, the frequency of IL-10+ CD39+ Vδ2 γδ T cells was highest in samples from healthy donors and decreased in samples from HIV-infected individuals (31,3% vs. ART: 10,5%; viremic: 15,0%; EC: 4,2%), but these differences did not reach statistical significance.

Taken together, we found that the ability of γδ T cells to produce IL-10 is higher among the CD39+ than the CD39- or the CD73+ subset. The highest frequency of IL-10 producing cells was found among CD39+CD73+ γδ T cells, a subset that is very scarce in PBMC. Also, the percentage of IL-10 producing cells tended to be higher in CD39+ Vδ2 compared to CD39+ Vδ1 γδ T cells and was declined in samples from patients with HIV compared to healthy individuals.

Cytokine Profiles of CD39+ Versus CD39- Vδ2 γδ T Cells

To get a better overview of their functional profile and polyfunctionality in terms of secretion of relevant cytokines, we conducted a multidimensional analysis of the cytokines secreted by CD39+ versus CD39- Vδ2 γδ T cells *via* SPICE analysis (**Figure 6** and **Supplementary Figure 10**). In addition to IL-10, we analyzed the expression of Granzyme B, IFN-γ, IL-2, TGF-ß, and TNF-α after *in vitro* stimulation. The analysis illustrates the differences between CD39+ and CD39- Vδ2 γδ T cells. There are several CD39+ Vδ2 γδ T-cell subpopulations in samples from healthy individuals that co-expressed the anti-inflammatory cytokines IL-10 and TGF-ß (illustrated as red and orange arcs). These subpopulations are found to a lesser extent in the corresponding CD39- Vδ2 γδ T cells but are also strongly reduced in samples from HIV-infected individuals. Also, pro-inflammatory cytokines such as IFN-γ or TNF-α (light blue and green arcs) are distributed differently between CD39+ and CD39- Vδ2 γδ T cells, indicating that these two subtypes might also differ functionally.

To summarize the polyfunctionality of CD39+ versus CD39- Vδ2 γδ T cells, we plotted the number of different cytokines that can be produced by the respective subset (**Supplementary Figure 11**). In samples from healthy controls, all of the CD39+ Vδ2 γδ T cells expressed at least two cytokines, with approximately three-quarters of the CD39+ Vδ2 γδ T cells expressing three cytokines. By contrast, most cells in the respective CD39- subset did not produce any of the analyzed cytokines, and only approximately 15% produced three different cytokines. In samples from patients with HIV, the differences between CD39+ and CD39- Vδ2 γδ T cells were less pronounced. Vδ2 γδ T cells from viremic patients mostly produced none of the analyzed cytokines, regardless of CD39 expression. Similar proportions of the CD39+ and CD39- Vδ2 subsets produced three or fewer of the analyzed cytokines.

In patients on ART, about half of the CD39+ Vδ2 γδ T cells produced none of the analyzed cytokines; however, a considerable fraction produced three different cytokines and two smaller fractions produced one or two cytokines. CD39- Vδ2 T cells of patients on ART had a similar pattern to CD39- Vδ2 γδ T cells from viremic individuals. Interestingly, a small fraction in all subsets of viremic and individuals on ART produced four different cytokines. Finally, the majority of CD39+ Vδ2 γδ T cells from EC expressed one cytokine, followed by one-third that produced three different cytokines and a smaller fraction that produced two different ones. CD39- Vδ2 γδ T cells from EC mostly did not produce any of the analyzed cytokines, a small fraction produced three different ones, followed by minor fractions producing two or one cytokines.

Taken together, we found diverging cytokine profiles of CD39+ and CD39- Vδ2 γδ T cells. In HIV infection, γδ T cells lost their polyfunctionality in parts and produced fewer anti-inflammatory cytokines.

Moderate Changes in the Composition, but Divergent Cytokine Repertoire of γδ T Cells From HIV Elite Controllers

We analyzed samples from HIV elite controllers, where we found striking disparities in the phenotype and functionality of γδ T cells compared to samples from infected individuals who had a high detectable load (viremic) or were on antiretroviral medication (ART). First, the frequency of CD39+ γδ T cells was not elevated compared to samples from healthy controls (**Figure 1**), and there were less pronounced changes in the Vδ1/ Vδ2 ratio (**Supplementary Figure 1**). Also, the frequency of CD73+ γδ T cells was not significantly decreased compared to healthy controls (**Figure 1**). When comparing the features between Vδ1 and Vδ2 γδ T cells, we did not find differences between the frequencies of CD39+ (**Figure 3**) or CD73+ (data not shown) Vδ1 and Vδ2 γδ T cells in samples from EC.

Interestingly, only low frequencies of IL-10 producing CD39+ γδ could be detected compared to HIV progressors (**Figure 5**), and CD39+/- Vδ2 γδ T cells from EC produced a lower number of different cytokines than γδ T cells from healthy controls (**Supplementary Figure 11**).

Long-term non-progressors maintain stable CD4+ T-cell counts despite low to intermediate plasma viremia. The differences between LTNP and HIV progressors were not as pronounced as between EC and the latter (105, 106, 120). By contrast, we observed similar changes in the expression pattern

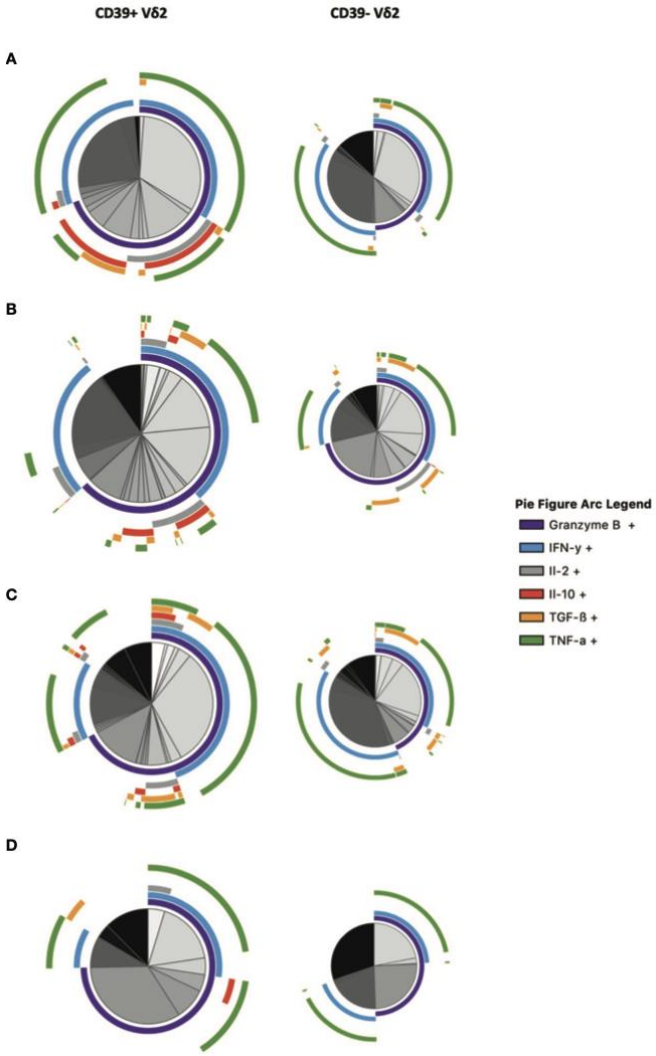

FIGURE 6 | Multidimensional comparison of the cytokine profiles of CD39+/- Vδ2 γδ T cells *via* SPICE analysis. Samples from **(A)** healthy controls **(B)** HIV-infected viremic patients **(C)** HIV-infected patients on ART **(D)** elite controllers. Overlapping arcs denote cell populations that co-express the respective cytokines. See also **Supplementary Figure 10** for a detailed pie figure legend.

of CD39 and CD73 and the phenotype of CD39+/CD73+ γδ T cells compared to patients on ART and viremic individuals.

DISCUSSION

γδ T cells are part of the first-line defense against pathogens since they exert direct cytotoxic functions independent of MHC proteins (15, 17), but also immunomodulatory γδ T cells have been described in different immunological settings such as cancer and inflammatory bowel disease (121, 122). Alterations of CD39 and CD73 expression on several lymphocyte populations have been described and are important for HIV pathogenesis (82, 88, 90, 101, 123). In the current study, a detailed phenotypical and functional characterization of CD39 and CD73 expression on different γδ T-cell populations from healthy individuals and HIV patients with different disease courses was carried out.

Our results show that the expression pattern of these ectoenzymes is associated with distinct functional states and can be used as a marker to identify activated cells. We find

significant differences in CD39 and CD73 expression on total γδ T cells, as well as on Vδ1 and Vδ2 cells between healthy and HIV-infected individuals depending on the clinical status. Importantly, we define a small population of γδ T cells co-expressing CD39 and CD73 that produce IL-10 after *in vitro* stimulation in healthy individuals and HIV patients.

In chronic HIV infection, IL-10 concentrations in the blood plasma were reported to increase over time, mediated by different lymphocyte populations (100, 124). The level of IL-10 production correlates with disease progression and causes reversible T-cell dysfunction to enable a balance between protective responses and immunopathology. IL-10 expression is associated with the expression of CD39 and the frequency of CD39+ cells secreting IL-10 has been correlated with viral load and immune activation (99, 101–103). Furthermore, IL-2 production is inhibited *via* the CD39/ADO pathway in CD39+ Tregs (125, 126).

We and others demonstrate that CD39, PD-1, and IL-10 were increased on γδ T cells in viremic HIV infection and provide an immunosuppressive environment in which the immune system is unable to clear the HI virus. Interestingly, our data show that neither IL-10 nor PD-1 nor CD39 increase strongly in EC, who can control the infection spontaneously.

One way to (partially) revert the function of virus-specific effector T cells (e.g. Vδ1 cells) is by combined checkpoint inhibitor blockade and blockade of adenosine signaling. Interestingly, CD39+ T cells CD8+ often also express PD-1 and other markers of cellular exhaustion. Li et al. demonstrated a reversion of CD8 exhaustion by concomitant blockade of PD-1 and adenosine pathways (127).

(IL-10+CD39+) γδ T cells could be reactivated by blockade of IL-10 or PD-1, CD39, or combinations thereof (80). Restored CD4 T cell function was previously achieved by immune checkpoint blockade of PD-1 and IL-10 in HIV-infected patients and Tang et al. demonstrated an improved function of MAIT cells during HIV/tuberculosis infection (128,129).

Overall, a strong correlation between the frequency of CD39+ and CD73+ γδ T cells and immune activation as well as disease progression (viral load and CD4+ T-cell count) could be determined. Thus, we propose that CD39 expression as well as the down-regulation of CD73 on γδ T cells can be seen as markers of activation, which has also been proposed in other immunological and disease contexts (99, 112, 130). The shifts of CD39 and CD73 have important implications regarding homing, functionality, nucleotide metabolism (that can occur in cis and/or trans), as well as interaction with other lymphocyte populations (131).

In this study, only HIV elite controllers showed an expression of both enzymes on γδ T cells comparable to healthy controls, while the expression pattern of CD39 and CD73 was altered in viremic and not fully normalized in individuals on ART. Viremic HIV patients had the highest CD39 expression on their γδ T cells – and these CD39+ γδ T cells produced the most IL-10 after *in vitro* stimulation. CD39 has lately been defined as a potential marker of immunomodulatory cells like Treg and NK cells, and CD39+ Vδ2 T cells might have a peculiar immunomodulatory role in HIV infection (64, 101, 104). One might interpret the CD39+CD73+IL-10+ γδ T cells as a counter-reaction to viremia to abrogate excessive inflammation, and speculate how this IL-10 production can inhibit HIV-specific immune responses and therefore act detrimentally in HIV pathogenesis (100, 124). Our data from elite controllers, who maintain low levels of CD39 and produce considerably less IL-10 than viremic HIV-infected patients, fit this hypothesis.

To see whether the observed alterations were specific for HIV infection, we also examined samples from patients with other viral infections, i.e. HCV or HBV. There, no significant increase of CD39+ γδ T cells compared to healthy controls could be measured. Similar to HIV infection, there is a loss of peripheral Vδ2 cells, an expansion of peripheral Vδ1 cells, and strong immune activation in (chronically) HBV-infected subjects (132, 133). The transcriptional pathways and factors (e.g. cytokines) that regulate CD39 and CD73 expression need to be better defined for γδ T cells (see also below), but also other lymphocyte populations. Several cytokines that have also been shown to be altered in HIV infection regulate CD39 on lymphocytes (134). For example, IL-6 and TGF-ß are likely to lead to an up-regulation of CD39 on lymphocytes (134). *In vitro*, TCR engagement and IL-2 increased CD39 expression. In mice, IL-27 signaling triggers CD39 expression in Tregs by a STAT-1-dependent mechanism (134). Another factor that influences CD39 expression on human T cells includes genetic variations (single nucleotide polymorphisms) (134).

Overall, the role of γδ T cells in HIV remains elusive and seems double-edged (41). Pan Vδ2 γδ T cells have been associated with a protective role in HIV: a study in non-human primates identified a relationship between cervical Vδ2 frequency and SIV viral load, and Vδ2 γδ T cells expressing CD16 are capable of mediating potent ADCC (62,135). Another study reported that elite controllers maintain significantly higher frequencies of Vδ2 T cells than untreated patients or those on ART (63, 64). We also find a higher frequency of Vδ2 γδ T cells in samples from long-term non-progressors compared to HIV progressors.

On the other hand, Soriano-Sarabia et al. report that replication-competent HIV could be recovered from purified Vδ2 γδ T cells in 14 of 18 long-term ART recipients and thus concluded that these cells form part of the viral reservoir (55). It has been demonstrated that Vδ2 cells express high levels of the HIV co-receptors CCR5 and α4β7 which contribute to their infectibility (53–56). CD39+ cells are more activated than CD39- cells, thereby inducing transient expression of CD4 on Vδ2 γδ T cells *in vivo* and promoting infection. Follow-up studies should test the hypothesis that CD39+ Vδ2 γδ T cells form part of the viral reservoir, as has been shown for CD39+ Tregs (55, 79). In naïve Tregs, a correlation between HIV DNA and frequency of CD39+ naïve Tregs was demonstrated by Song et al. (79)

We observed a loss of polyfunctionality, defined as cells capable of producing three or more cytokines after *in vitro* stimulation, within the CD39+ Vδ2 γδ T-cell population of viremic HIV patients that was not fully regained in γδ T cells

of HIV-infected individuals under ART. These results are in line with results from Casetti et al., who also measured a reduction of polyfunctionality (cytokine/chemokine production and cytotoxicity) in Vδ2 γδ T cells from ART-treated patients (136, 137).

This first study on the CD39 and CD73 expression pattern and functionality of γδ cells in HIV patients has some limitations. A first one is given by the limited number of parameters that could be measured in a respective panel by flow cytometry analysis. In future studies, the expression of CD16, CD56, and NKG2D, as well as the transcriptional profile [FOXP3, HIF-1, and AhR (138–141)], should be included in the flow cytometry experiments or assessed, e.g. by use of single-cell transcriptional RNA expression analysis (142). NKG2D can activate γδ T cells in an innate TCR-independent manner and is expressed by the vast majority of Vδ2 T cells (29, 43, 143, 144).

We found that the frequency of circulating γδ T cells expressing both CD39 and CD73 is particularly low. However, CD39+CD73+ γδ T cells had the highest frequencies of IL-10-producing cells after *in vitro* stimulation. One could hypothesize that this small subpopulation of CD39+CD73+ γδ T cells that secretes an anti-inflammatory cytokine stays relatively unaffected from HIV infection. By contrast, the majority of γδ T cells show shifts of the CD39/CD73 expression ratio comparable to the changes observed in the effector cell compartment, most likely due to generalized immune activation in HIV (86, 145). It will be interesting to further investigate this scarce population of cells alongside the other γδ T-cell populations, especially regarding their suppressive capacities. Since the frequency of peripheral CD39+, CD73+ γδ T cells is too low for live cell sorting and subsequent co-culture with activated T cells, transcriptional analyses such as single-cell sequencing with regard to the transcriptome will have to be used to understand the capabilities of this and other γδ T-cell populations. Alternatively, γδ T-cell subpopulations could be expanded *in vitro* before life-sorting, co-culture, and flow-based read-out, with the disadvantage that this expansion may alter the phenotype and the function of the γδ T cells.

Previous studies point towards a regulatory role of CD39 in the inflammatory microenvironment of the gut which is caused i.a. by microbial ATP (109). Our group has previously demonstrated that the frequency of mucosa-derived CD39+ γδ T cells is decreased in patients with inflammatory bowel diseases compared to healthy controls (81). Upon stimulation, these cells produced less IL-17 and more IL-10 than CD39- γδ T cells, also pointing towards a regulatory phenotype of these cells. At the same time, the number of Tregs in the mucosal compartment was increased, which could serve as a compensatory mechanism for the loss of the CD39+ γδ T cells (81). The gut is one of the major sites for virus dissemination and formation of the viral reservoir (146–148). It will therefore be worthwhile to examine alterations in the number and function of CD39+ γδ T cells in the gut-associated lymphoid tissue (GALT) from HIV-infected individuals compared to healthy controls.

It will also be interesting to study patients with primary HIV infection. Bhatnagar et al. have demonstrated that Vδ2 γδ T cells transform from an anti-inflammatory phenotype in primary infection into a pro-inflammatory cytokine profile in chronic infection (35).

Taken together, the CD39/CD73 expression ratio on γδ T cells in untreated HIV is inversed and is associated with immune activation and disease progression. We find altered functionality and higher levels of IL-10 production in viremic HIV patients. Also, we defined a small population of CD39+CD73+ γδ T cells producing IL-10 at high frequencies after *in vitro* stimulation.

We hypothesize an immunomodulatory role of CD39+ and CD73+ γδ T cells in the pathogenesis of chronic HIV infection potentially mediated by IL-10 secretion. Similar to the deleterious role of suppressive cells in the microenvironment of tumors, the frequency of CD39+ γδ T cells was correlated with HIV disease progression in this study. This is further supported by our findings in elite controllers, who maintain stable frequencies of (IL-10-producing) CD39+ and CD73+ γδ T cells compared to healthy controls. Also, double-positive CD39+CD73+ produced significantly more IL-10 than γδ T cells expressing only one ectonucleotidase. Finally, a link between CD39 and IL-10 expression and disease progression has already been established in NK cells (90).

Future studies have to understand the role of adenine metabolism for γδ T cell function and elucidate the effects of the alterations of CD39 and CD73 expression on γδ T cells in HIV in more detail.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board, Ärztekammer Hamburg, Hamburg, Germany. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

KK and MW have contributed equally to this work and share the first authorship. KK and JS designed the initial study design. MW and JS wrote the first draft of the manuscript together with KK. JS gave funding and was in charge of the overall research project. KK and MW conducted most of the experiments. A-DH, OD, H-JS, and JS recruited the patients and collected patient data. KK and MW analyzed the data under the supervision of JS, PH, and FH. MW and KK prepared the figures and got input from FH, JS and all other authors. PH and FH aided in interpreting the results. All authors discussed the results and critically reviewed

the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.867167/full#supplementary-material

Supplementary Figure 1 | Stable frequencies of total γδ T cells (left) and inverted ratio (right) between Vδ1 and Vδ2 T cells in HIV infection.

Supplementary Figure 2 | Gating Strategy to define total/pan γδ T cells and Vδ2 γδ T cells. First, dead cells, B cells, and monocytes were excluded. Then, single cells and lymphocytes were selected. After gating for CD3+ T cells, pan γδ T cells were defined. From there, Vδ2 γδ T cells were gated. Vδ1 γδ T cells were defined as Vδ2 negative. Exemplary plots from a long-term non-progressor are shown.

Supplementary Figure 3 | Exemplary dot plots of γδ T cells expressing CD39 (upper panel) and CD73 (lower panel). Data are shown from selected healthy volunteers, HIV patients on ART, HIV-infected viremic individuals, elite controllers (EC), and long-term non-progressors (LTNP).

Supplementary Figure 4 | Changes in the frequency of CD39+ and CD73+ γδ T cells in different viral infections. Left: Frequency of CD39+ γδ T cells. Right: Frequency of CD73+ γδ T cells in samples from healthy and viremic HIV-infected individuals, patients with acute and chronic HBV and patients with chronic HCV.

Supplementary Figure 5 | Correlation between immune activation and frequency of CD39+ γδ T cells. The frequency of CD39+ γδ T cells correlates positively in HIV-infected patients **(A)** and inversely with immune activation in healthy individuals **(B)**. **(C)** Frequency of HLA-DR+CD38+ γδ T cells is higher among CD39+ than CD39- cells of regardless disease status. Graph **A** contains pooled data from HIV-infected patients including viremic and individuals on ART, EC, and LTNP.

Supplementary Figure 6 | Correlation between frequency of activated γδ T cells and frequency of CD39+ γδ T cells. The frequency of activated (HLA-DR+CD38+) CD39+ γδ T cells correlates positively in HIV-infected patients (left) and inversely within healthy individuals (right). The left graph contains pooled data from HIV-infected patients including viremic and individuals on ART, EC, and LTNP.

Supplementary Figure 7 | Kinetics of CD39 frequency after *in vitro* stimulation. Data from *in vitro* stimulated PBMC from healthy individuals.

Supplementary Figure 8 | Exemplary dot plots of CD39+/CD39- Vδ1/Vδ2 γδ T cells expressing IL-10. Data are shown from viremic HIV-infected patients.

Supplementary Figure 9 | Frequency of IL-10 producing total Vδ1 versus total Vδ2 and CD39+ Vδ2 γδ T cells. Data from pooled study subjects (healthy and HIV-infected individuals) and stratified according to disease status.

Supplementary Figure 10 | Category legend to Figure 6. Plus denotes the existence of cells expressing the respective cytokine, minus the absence.

Supplementary Figure 11 | Comparison of the degree of the polyfunctionality of CD39+ and CD39- Vδ2 γδ T cells *via* SPICE analysis. "Number of function" denotes the average number of cytokines produced by the respective subset. Samples from **(A)** healthy controls **(B)** HIV-infected viremic patients **(C)** HIV-infected patients on ART **(D)** elite controllers. Data is presented on a relative scale with median values.

Supplementary Table 1 | Basic demographic and virologic data of the HBV- and HCV-infected patients (average, min - max).

Supplementary Table 2 | Overview of fluorochrome-conjugated antibodies used for phenotypic characterization *via* flow cytometry (surface staining).

Supplementary Table 3 | Overview of fluorochrome-conjugated antibodies used for functional characterization *via* flow cytometry (intracellular cytokine staining).

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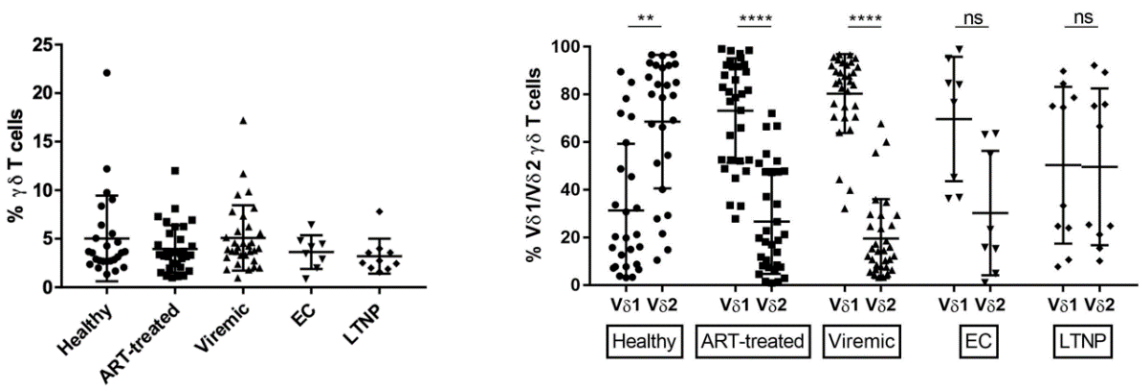
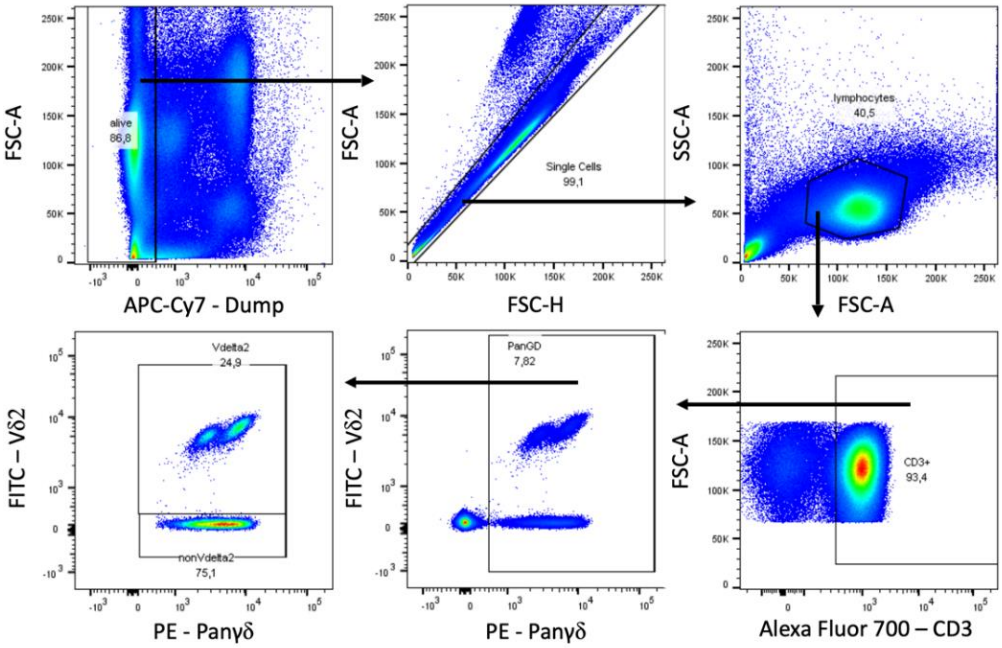
1.1 Supplemental Figures of the Original Article



Suppl. Fig. 1



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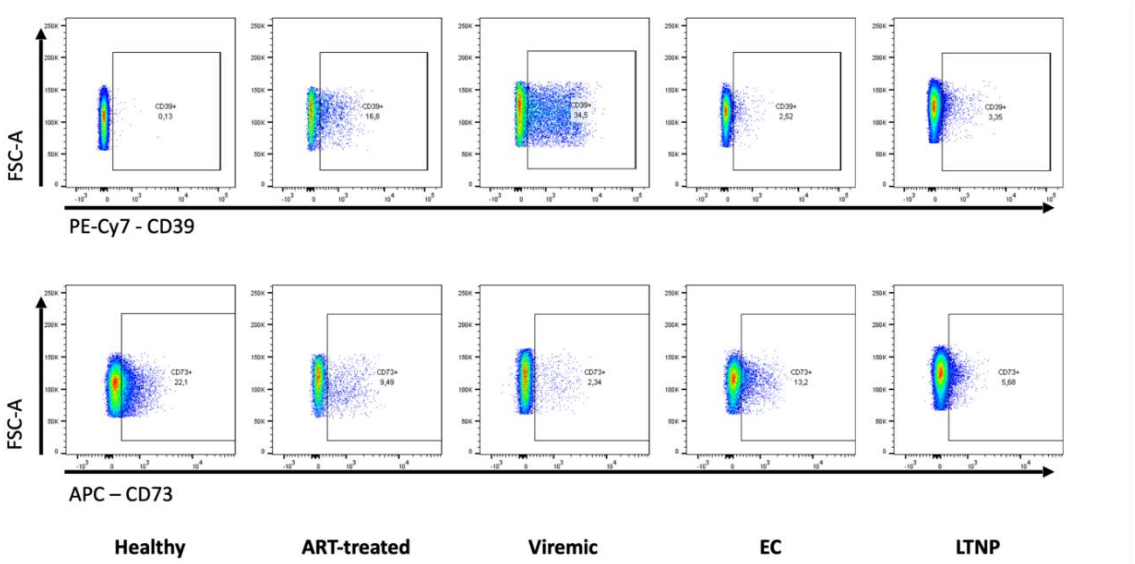

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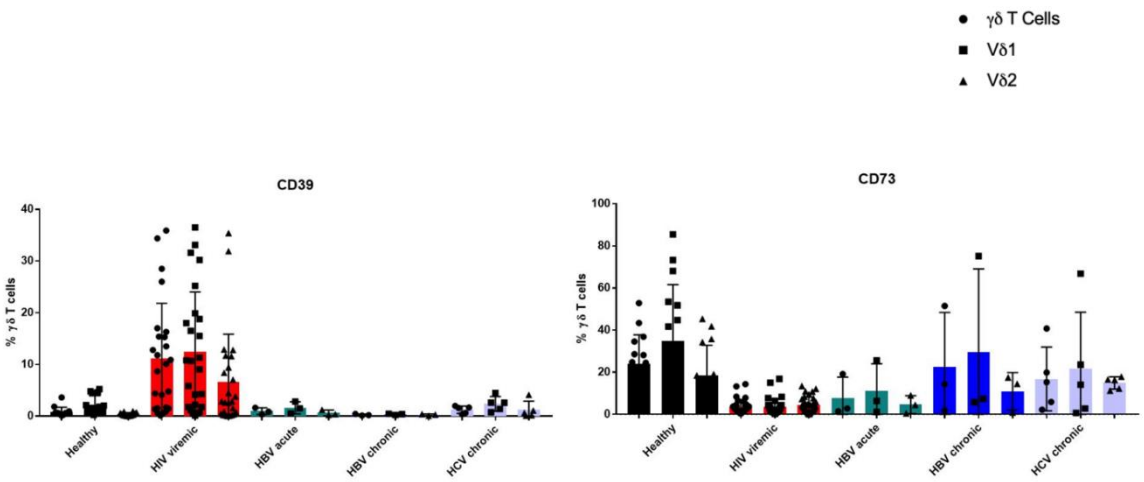

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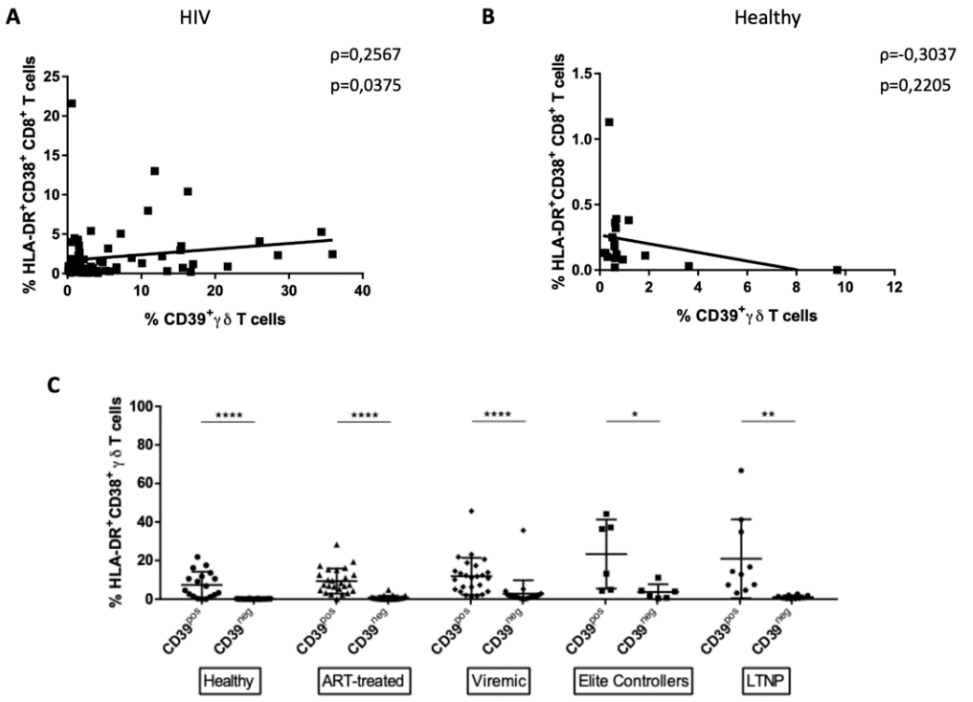

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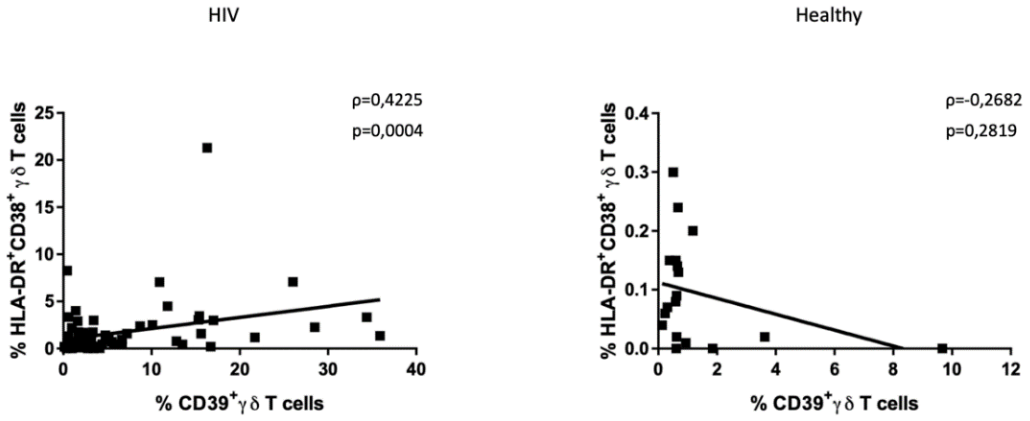

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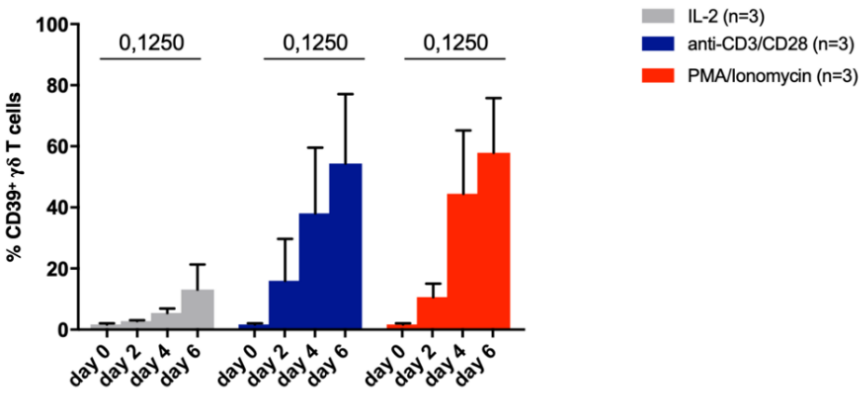

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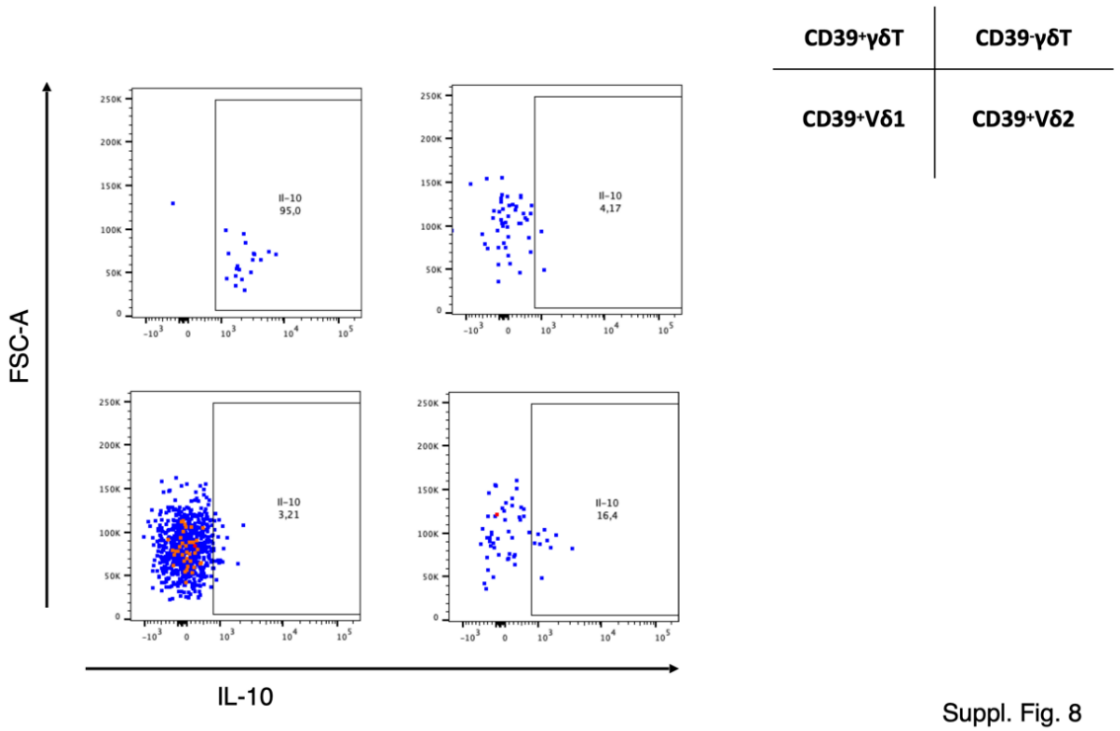

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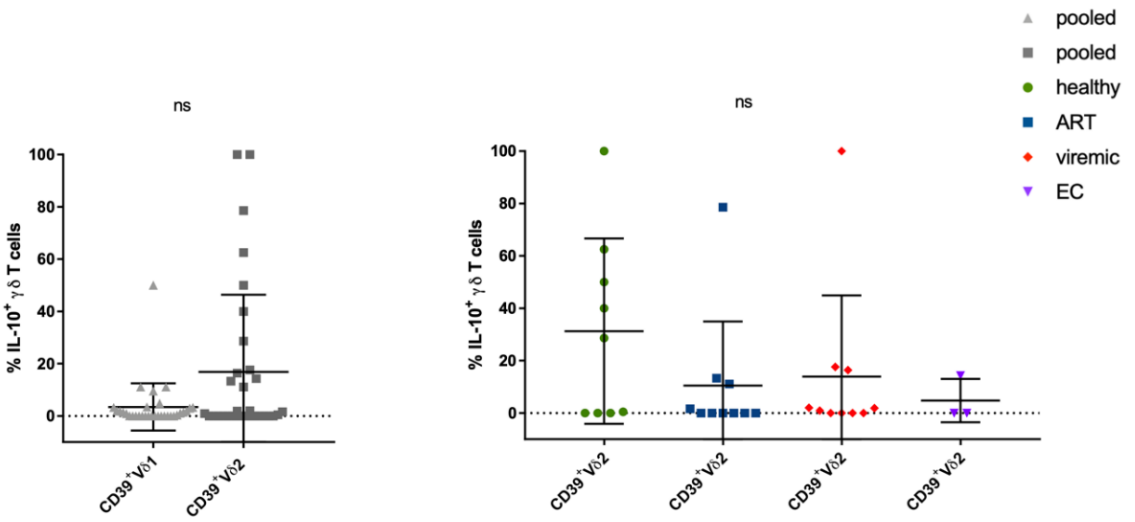

Suppl. Fig. 9

Categories						
#	Granzyme B	IFN-γ	Il-2	Il-10	TGF-ß	TNF-a
1	+	+	+	+	+	+
2	+	+	+	+	+	-
3	+	+	+	+	-	+
4	+	+	+	+	-	-
5	+	+	+	-	+	+
6	+	+	+	-	+	-
7	+	+	+	-	-	+
8	+	+	+	-	-	-
9	+	+	-	+	+	+
10	+	+	-	+	+	-
11	+	+	-	+	-	+
12	+	+	-	+	-	-
13	+	+	-	-	+	+
14	+	+	-	-	+	-
15	+	+	-	-	-	+
16	+	+	-	-	-	-
17	+	-	+	+	+	+
18	+	-	+	+	+	-
19	+	-	+	+	-	+
20	+	-	+	+	-	-
21	+	-	+	-	+	+
22	+	-	+	-	+	-
23	+	-	+	-	-	+
24	+	-	+	-	-	-
25	+	-	-	+	+	+
26	+	-	-	+	+	-
27	+	-	-	+	-	+
28	+	-	-	+	-	-
29	+	-	-	-	+	+
30	+	-	-	-	+	-
31	+	-	-	-	-	+
32	+	-	-	-	-	-

Categories						
#	Granzyme B	IFN-γ	Il-2	Il-10	TGF-ß	TNF-a
33	-	+	+	+	+	+
34	-	+	+	+	+	-
35	-	+	+	+	-	+
36	-	+	+	+	-	-
37	-	+	+	-	+	+
38	-	+	+	-	+	-
39	-	+	+	-	-	+
40	-	+	+	-	-	-
41	-	+	-	+	+	+
42	-	+	-	+	+	-
43	-	+	-	+	-	+
44	-	+	-	+	-	-
45	-	+	-	-	+	+
46	-	+	-	-	+	-
47	-	+	-	-	-	+
48	-	+	-	-	-	-
49	-	-	+	+	+	+
50	-	-	+	+	+	-
51	-	-	+	+	-	+
52	-	-	+	+	-	-
53	-	-	+	-	+	+
54	-	-	+	-	+	-
55	-	-	+	-	-	+
56	-	-	+	-	-	-
57	-	-	-	+	+	+
58	-	-	-	+	+	-
59	-	-	-	+	-	+
[redacted]	-	-	-	+	-	-
[redacted]	-	-	-	-	+	+
[redacted]	-	-	-	-	+	-
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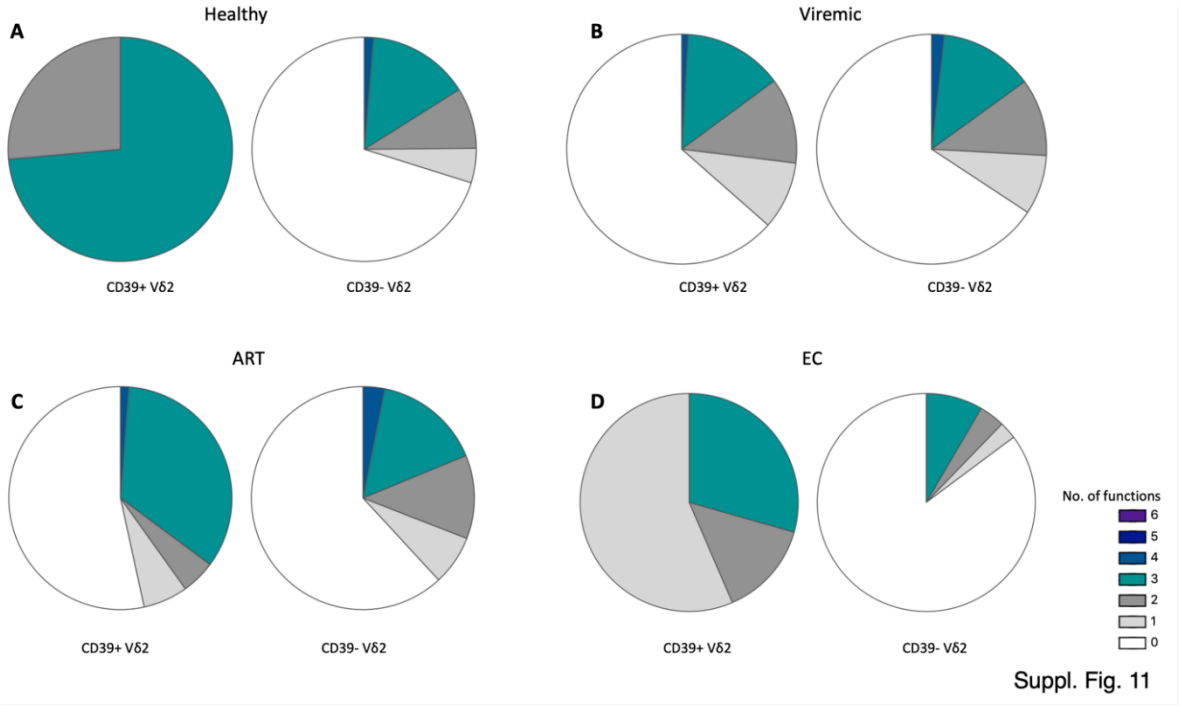
Suppl. Fig. 10


A
Healthy
CD39+ Vδ2
CD39- Vδ2
B
Viremic
CD39+ Vδ2
CD39- Vδ2
C
ART
CD39+ Vδ2
CD39- Vδ2
D
EC
CD39+ Vδ2
CD39- Vδ2
No. of functions
6
5
4
3
2
1
0

Suppl. Fig. 11

1.2 Supplemental Tables of the Original Article

Supplemental Table 1. Basic demographic and virologic data of the HBV- and HCV-infected patients (average, min - max).

	HBV acute	***HBV chronic***	***HCV chronic***
n	**3**	**3**	**5**
Age (years)	**30** 21 – 49	**36** 25 - 50	**51** 40 - 63
Viral load (IU/mL)	**235441333** 24000 - 700000000	**304** 22 - 510	**520009** 15 - 1980000

Supplemental Table 2. Overview of fluorochrome-conjugated antibodies used for phenotypic characterization via flow cytometry (surface staining).

Fluorochrome	***Antigen***	***Clone***	***Supplier***
BUV737	CD45RA	HI100	BD
BUV395	CD4	RPA-T4	BD
BV785	HLA-DR	L243	BioLegend
BV711	CD27	M-T271	BioLegend
BV650	CD279 (PD-1)	EH12.2H7	BioLegend
BV605	TIGIT	A15153G	BioLegend
BV510	CD8	SK1	BioLegend
BV421	CD28	CD28.2	BioLegend
FITC	Vδ2	IMMU389	Beckman Coulter
PE-Cy7	CD39	A1	BioLegend
PE-Texas-Red	CD38	HIT2	BioLegend
PE	Pan γδ	11F2	BD
APC-Cy7	CD19	HIB19	BioLegend
APC-Cy7	CD14	63D3	BioLegend
APC	CD73	AD2	BioLegend
Alexa Fluor 700	CD3	SK7	BioLegend

Supplemental Table 3. Overview of fluorochrome-conjugated antibodies used for functional characterization via flow cytometry (intracellular cytokine staining).

Fluorochrome	***Antigen***	***Clone***	***Supplier***
BUV737	IL-2	MQ1-17H12	BD
BUV395	CD4	RPA-T4	BD
BV785	IFN-γ	4S.B3	BioLegend
BV711	TNF-α	MAb11	BioLegend
BV650	IL-10	JES3-9D7	BD
BV605	TIGIT	A15153G	BioLegend
BV510	CD8	SK1	BioLegend
BV421	CD28	CD28.2	BioLegend
PerCP-Cy5-5	TGF-beta	TW4-2F8	BioLegend
FITC	Vδ2	IMMU389	Beckman Coulter
PE-Cy7	CD39	A1	BioLegend
PE-Texas-Red	Granzyme B	QA16A02	BioLegend
PE	Pan γδ	11F2	BD
APC-Cy7	CD19	HIB19	BioLegend
APC-Cy7	CD14	63D3	BioLegend
APC	CD73	AD2	BioLegend
Alexa Fluor 700	CD3	SK7	BioLegend

2 Presentation of the Publication

2.1 Scientific Background

2.1.1 Human Immunodeficiency Virus

The human immunodeficiency virus-1 (HIV-1), first isolated and discovered in the 1980s, originated from several independent zoonotic transmissions of simian immunodeficiency viruses [1–3]. HIV-1, hereafter referred to as HIV, belongs to the family of *Retroviridae* and hijacks the host cell's machinery to convert viral ribonucleic acid (RNA) into deoxyribonucleic acid (DNA) and then replicate itself, using reverse transcriptases, proteases, and integrases [1]. It predominantly infects, and in the process depletes, the pool of $CD4^+$ T cells[a], leading to chronic immune activation, immune dysfunction, and most eventually lethal Acquired Immunodeficiency Syndrome (AIDS) without sufficient therapy [4–7].

The global incidence of HIV-1 in 2019 was 36.9 million with 5000 new infections occurring every day and an increasing incidence, globally, specifically in Europe and the United States [8]. The Highly active antiretroviral therapy (ART) suppresses viral replication with a combination of at least two drugs to treat HIV-positive patients, but no cure is available to date [1,9,10]. Nearly half of HIV-infected individuals are not receiving ART mainly in sub-Saharan Africa [8,11].

2.1.2 Definition and Role of γδ T Cells

Among $CD4^+$ T cells, unconventional T cells appear to play a role in HIV infection [12–16]. Besides natural killer T cells (NK) and mucosal-associated invariant T cells (MAIT), γδ T cells seem to have important immunomodulatory properties relevant to the disease [7].

γδ T cells are "innate-like" unconventional T cells that makeup 1-15% of circulating leukocytes and that exert a direct cytotoxic activity independent of major histocompatibility complex (MHC) presentation [17–28]. They recognize stress-induced molecules, non-peptide- and phosphoantigens, self- or MHC-related molecules, and lipids and show different effector functions [17,18,21–23,29–38]. They can kill infected or transformed cells by death-inducing pathways and release perforins, granzymes, and other bacteriostatic or bacteriolytic molecules [17,18,21–23,29–38]. In addition, γδ T cells produce pro-inflammatory cytokines but also anti-inflammatory cytokines and have been shown to act as immunomodulators and inhibitors of T and B cell responses [22,23,32,37,39–46].

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[a] CD: cluster of differentiation

While the conventional T-cell receptor is a heterodimer, consisting of an alpha and beta unit, γδ T cells express a T-cell receptor with a gamma and a delta chain [19,20,24,26–28]. The two main subsets of γδ T cells, Vδ1 T cells, and Vδ2 T cells, are present in different anatomic compartments [29–31,33,38]. While Vδ1 T cells can be found in the intraepithelial layer of mucosal surfaces, the Vδ2 T-cell population is mostly detected in the blood and secondary lymphoid tissues (ratio in the peripheral blood Vδ1/Vδ2 T cells: 3:10) [17,29–31,33,38,43,47–51]. About 30% of γδ T cells express a CD8 T-cell co-receptor, less than 1% express a CD4 cell co-receptor, and 70% do not express any of the conventional T-cell co-receptors [31].

2.1.3 γδ T Cells in HIV Infection

In the acute phase of primary HIV infection, an inversion of the Vδ1/Vδ2 T-cell ratio can be observed in blood while the ratio of total γδ T cells remains relatively stable [17,25]. While the pool of Vδ2 T cells is depleted, Vδ1 T cells become more abundant [7,17,52].

γδ T cells can inhibit or stimulate inflammation in the blood and different tissues and might be used to target HIV-infected cells directly but can also become infected by HIV themselves [17,25,53,54].

The observed change in the ratio of Vδ1 and Vδ2 T cells is reminiscent of the inverted CD4/CD8 T-cell ratio observed in untreated HIV infection [52,55]. Vδ1 T cells are suggested to be involved in antiviral immunity and one reason for expansion in peripheral blood is an indirect consequence of viral infection. It reflects the increased translocation of stimulatory bacterial products across the gut epithelium in non-human primate studies [7,55–59].

It has been demonstrated that Vδ2 T cells express high levels of HIV co-receptors CCR5[b] and integrin α4β7, which could contribute to their preferential depletion in HIV infection [60–63]. Another possible mechanism of depletion is the inhibition of Vδ2 T cells by HIV-infected dendritic cells (DCs) [58]. Their abundance, but also activation remains below that of healthy controls even after successful implementation of ART and restoration of the CD4 T-cell compartment [64,65]. Due to the importance of Vδ2 T-cell clones for direct cytotoxicity against HIV-infected and otherwise diseased cells, this rarefication of Vδ2 T cells is unfavorable for people living with HIV, especially in their defense against opportunistic infections [66–69].

[b] CCR5: C-C chemokine receptor type 5

2.1.4 The Ectonucleotidases CD39 and CD73

Immune responses are fine-tuned by a multitude of cell- and molecule-based mechanisms, one of them being the purinergic pathway. Adenosine triphosphate (ATP) can bind to purinergic receptors on the cell surface, increasing influx of Calcium ions and enhancing cellular activation. The dephosphorylated metabolite Adenosine dampens the response of T effector cells by binding to class P1 purinergic receptors (members of the G protein-coupled receptor family) or ATP-gated ion channels [70]. Mutations in the purine system could cause severe primary immunodeficiency diseases [71–73].

The ectonucleotidases CD39 and CD73, members of the Adenosine pathway that are expressed in several lymphocyte subpopulations, convert extracellular pro-inflammatory ATP and Adenosine diphosphate (ADP) to anti-inflammatory Adenosine (ADO) [74–76].

In healthy individuals, the level of extracellular ADO is low but can increase 100-1000-fold in situations of severe inflammation and tissue injury [63]. ADO strengthens epithelial barrier functions and inhibits leukocyte extravasation as well as the production of cytokines and T-cell proliferation [77–83]. Moreover, ADO can induce IL-10[c] production [84–87].

Over the last decade, multiple roles of CD39 and CD73 in the regulation of inflammation have been revealed [72,88–94]. CD39 and CD73 are players of murine regulatory T cells and mediate immune suppression [74,75]. Importantly, both CD39 and CD73 can work in cis (interaction on the same cell), trans (interaction with enzymes expressed on different cells), and as soluble effectors [76,88].

2.1.5 $CD39^+$ and $CD73^+$ γδ T Cells Are Associated With Immune Suppression

Conventional regulatory T cells (Tregs) can control exaggerated or inappropriate immune activation via CTLA-4[d], IL-10, and TGF-β [95,96]. Tregs proliferate in response to the cytokine stimulus of IL-10 and TGF-β[e] and they downregulate excessive immune responses via ADO [97]. However, this effect does not seem to be sufficient to compensate for the overarching immune activation in HIV infection [98]. Human Tregs also express CD39, but only a small amount expresses CD73 [71,72].

[c] IL: interleukin
[d] CTLA-4: cytotoxic T-lymphocyte-associated Protein 4
[e] TGF: transforming growth factor

Interestingly, γδ T cells can also act in an immunosuppressive manner, infiltrate tumors, and suppress dendritic and T cells [46,99,100]. Liang et al. demonstrated that the regulation of γδ T cells in autoimmunity is associated with ADO [101]. Otsuka et al. have reported a potential regulatory role of $CD39^+$ γδ T cells via IL-10 function in mice [102]. In contrast, Hu et al. described $CD39^+$ γδ T cells suppressing T cells through an Adenosine-mediated pathway but independent of IL-10 and TGF-β [100]. Libera et al. have also recently identified $CD39^+$ γδ T cells with an immunosuppressive phenotype in the gut [94].

Higher CD73 levels have been associated with immunosuppression and poor prognosis in e.g. breast or ovarian cancer [89,103–106]. Suppressive activities of $CD73^+$ γδ T cells *via* Adenosine were shown in mice [101].

2.1.6 $CD39^+$ and $CD73^+$ γδ T Cells in HIV Infection

In HIV-infected untreated individuals, an over-expression of CD39 on lymphocytes and an increased hydrolysis of ATP by lymphocytes have been observed [107,108]. Schulze zur Wiesch et al. and others have previously shown that the relative frequency of CD39 expression of $FoxP3^+$ Tregs[f] correlates with the progression of HIV infection and that $CD39^+$ Tregs themselves are targets of the HI virus and absolute numbers were reduced [92,109].

Inversely, a variant of the CD39 gene associated with low CD39 expression on lymphocytes and a slower progression to AIDS has been described [107,108,110].

In HIV infection, the plasma concentration of IL-10 increases over time and limits specific T-cell responses [111]. IL-10 secretion is associated with the expression of CD39 [90,102,112,113].

Dierks et al. demonstrated that elevated levels of $CD39^+$ NK cells in viremic patients, which secrete IL-10, correlated directly with viral load and activation, and negatively correlated with $CD4^+$ T-cell count [112]. In viremic people living with HIV, the frequencies of $CD73^+$ B Cells, $CD73^+$ Tregs, and $CD73^+$ $CD8^+$ T cells are decreased and correlate with progressive HIV disease [72,90,112,114].

2.1.7 Elite Controllers and Long-Term Non-Progressors

HIV elite controllers (EC) were defined as people living with HIV capable of spontaneously controlling HIV infection (maintaining stable $CD4^+$ T-cell counts and viral loads below the

[f] FoxP3: forkhead box protein P3

level of detection) without the need for antiretroviral medication [115–117]. Interestingly, elite controllers exhibit higher frequencies of Vδ2 T cells than untreated or ART-treated HIV progressors, begging the question if Vδ2 T cells are involved in immune control [118,119]. Furthermore, Tregs of elite controllers showed the lowest levels of CD39 [92,109]. Bhatnagar et al. suggested a suppressive activity of γδ T cells, especially of Vδ2 T cells via TGF-β, which is dysregulated in progressed HIV infection [42]. Other authors also report an immunosuppressive Vδ2 T-cell phenotype [120,121]. Little is known yet about the combination of CD39 and γδ T cells in EC.

The definition criteria for HIV long-term non-progressors (LTNP) are to remain asymptomatic and present $CD4^+$ T-cell counts in peripheral blood higher than 500 cells/μL despite low to intermediate plasma viremia [122–124]. Little is known about the behavior of γδ T cells or CD39 and CD73 in LTNP patients.

To sum up, the ectonucleotidases CD39 and CD73 have been described as important immunoregulatory molecules on Tregs and T effector cells [72,76,88–92,94,100,102–104,109,112,125]. In mice and human colorectal cancer, $CD39^+$ γδ T cells with a regulatory phenotype have already been described [100,102,126]. However, the role of $CD39^+$ and $CD73^+$ expression on γδ T cells in healthy humans and the context of viral infections like HIV infection is still largely unknown.

2.2 Working Hypothesis

This dissertation hypothesizes that $CD39^+$ γδ T cells and notably $CD39^+$ Vδ2 T cells might play a role in the immune regulation of HIV through adenine nucleotide signaling.

In the present thesis, a comprehensive assessment of the expression of CD39 and CD73 on different γδ T-cell subsets including Vδ1 and Vδ2 T cells concerning phenotype and function within a large cohort of uninfected individuals and people living with HIV with different disease statuses including HIV elite controllers and long-term non-progressors was performed.

Using sensitive surface antigen and intracellular cytokine staining and subsequent analysis via fluorescence-activated cell sorting (FACS), the following aims were addressed:

- Assessment of the CD39 and CD73 expression pattern on peripheral γδ T cells and their subsets in uninfected and HIV-infected individuals with different disease courses
- Comparison of the expression levels of the ectonucleotidases concerning differentiation, activation, and exhaustion status of peripheral γδ T cells
- Evaluation of the immunomodulatory properties of γδ T cells, specifically their cytokine profiles in relation to the expression of CD73 and CD39

2.3 Methods

2.3.1 Study Subjects and Samples

Peripheral blood mononuclear cell samples (PBMC) of people living with HIV and uninfected, hereafter referred to as healthy, volunteers were collected and cryopreserved. For better readability, the terms patient, HIV-positive, and HIV-infected are also used in this dissertation. PBMCs of HIV patients with different disease statuses were included: treatment-naive HIV patients (viremic), HIV antiretroviral-treated patients (ART-treated), HIV elite controllers (EC), and HIV long-term non-progressors (LTNP).

Written informed consent was obtained from all people who were recruited for this study and co-infections like hepatitis B and C were ruled out serologically.

PBMCs from patients suffering from acute and chronic hepatitis B or C infections were also stained using the flow cytometry surface panel to compare them with people living with HIV.

2.3.2 Immune Phenotypic Analysis for Surface Markers

For the immune phenotypic analysis of surface markers PBMCs of 18 healthy individuals, 25 ART-treated, 25 viremic, 6 EC, and 10 LTNP were isolated and stained with anti-human monoclonal fluorochrome-conjugated antibodies. The flow cytometry panel developed for phenotypic characterization contained the markers CD19, CD14, and viability stain to sort out dead cells, monocytes, and B-cells; CD3, CD4, CD8, Panγδ, and Vδ2 to distinguish between the different types of γδ T cells; CD45RA, CD27, and CD28 for differentiation status; HLA-DR[g] and CD38 for activation status; PD-1[h] and TIGIT[i] for exhaustion status as well as CD39 and CD73.

2.3.3 Immune Phenotypic Analysis for Surface and Intracellular Markers After *In Vitro* Stimulation of PBMCs

For the analysis of released cytokines, intracellular staining with anti-human monoclonal fluorochrome-conjugated antibodies was performed. After *in vitro*-stimulating PBMCs with phorbol 12-myristate 13-acetate (PMA)/Ionomycin for 18 hours, the surface staining was performed with CD4, CD8, CD28, Panγδ, Vδ2, CD39, CD19, CD14, CD73, CD3, and viability stain. Surface characterization data of 8 healthy volunteers, 7 ART-treated, 11 viremic, and 2 EC could be added to previous phenotypic analyses. Moreover, PBMCs were permeabilized and stained for assessing the intracellular production of pro- and anti-

[g] HLA: human leukocyte antigen
[h] PD-1: programmed cell death protein 1
[i] TIGIT: T cell immunoreceptor with Ig and ITIM domains

inflammatory cytokines (IL-2, TGF-ß, TNF-α[j], Granzyme B, IL-10, and IFN-γ[k]). The intracellular flow cytometry panel contained samples of 10 healthy controls, 10 ART-treated, 10 viremic, and 3 EC.

2.3.4 Kinetic of CD39 Expression After *In Vitro* Stimulation of PBMCs

For kinetic studies of CD39 surface expression, cells were stimulated with PMA/Ionomycin, anti-CD3/CD28 dynabeads, rhIL-2[l], or combinations thereof. They were cultured for up to six days before staining with the previously described surface panel and FACS analysis.

2.3.5 Data Analysis and Statistics

All samples were run on a 16-color flow cytometer and cytometric data were analyzed using FlowJo version 10.7.1 (BD Biosciences).

Statistical analysis was performed using GraphPad Prism version 7.04 (GraphPad Software, Inc.). Multiple comparisons were performed using Kruskal-Wallis and Dunn's post-test. For two-way comparisons, the Mann-Whitney test and for correlations the Pearson correlation and the Spearman Rank correlation were used.

For further multidimensional comparisons of FACS data, Simplified Presentation of Incredibly Complex Evaluations (SPICE; version 6, written by J. Nozzi and Dr. M. Roederer) analyses were performed.

[j] TNF: tumor necrosis factor
[k] IFN: interferon
[l] Rh: recombinant

2.4 Results

2.4.1 The Frequency of CD39$^+$ γδ T Cells Increases While the Frequency of CD73$^+$ γδ T Cells Is Decreased in People Living With HIV

First, the results of previously published data were confirmed, as the percentage of total γδ T cells was stable during HIV infection regardless of the stage of HIV infection while the ratio between the subsets Vδ1 and Vδ2 T cells was inverted [7,17,25,54].

In long-term non-progressors, the proportions of Vδ1 and Vδ2 T cells were similar. In elite controllers, an inversion of the Vδ1/Vδ2 T-cell ratio was also observed which, however, was not statistically significant.

Subsequently, the expression pattern of the ectonucleotidases CD39 and CD73 of γδ T cells in different stages of HIV disease was determined. The highest frequency of CD39$^+$ γδ T cells was detected in samples from viremic HIV-infected patients. In viremic individuals and individuals on ART, the CD39$^+$ γδ T-cell frequency was significantly increased compared to healthy individuals (viremic: 11,3% vs. 1,4%, p<0,0001; ART: 3,2% vs. 1,4%, p=0,0146). In samples from EC, the frequency of CD39$^+$ γδ T cells was similar compared to samples from healthy controls (2,7%), while it was slightly elevated in LTNP compared to healthy controls (5,1%). The differences between CD39$^+$ γδ T cells in EC/LTNP and healthy controls did not show statistical significance.

Inversely, the frequency of CD73$^+$ γδ T cells was markedly decreased in PBMCs from HIV-infected individuals regardless of their infection status compared to healthy individuals. The lowest expression level was observed in viremic individuals, followed by ART-treated and LTNP (healthy: 20,7% vs. viremic: 3,7%, p<0,0001; ART: 5,8%, p<0,0001; LTNP: 6,8%, p=0,0016). The difference between healthy controls and EC was the smallest and non-significant (9,4%).

Next, the expression behavior of CD39 and CD73 on γδ T cells as a combination was observed. The frequency of double positive CD39$^+$CD73$^+$ γδ T cells was extremely low in PBMCs from all study groups and did not differ significantly between uninfected and people living with HIV (healthy: 0,25%; ART: 0,11%; viremic: 0,15%; EC: 0,11%; LTNP: 0,14%).

Because hardly any double positive γδ T cells were detected in all study groups, the number of CD39$^+$CD73$^-$ γδ T cells was similar to the number of CD39$^+$ γδ T cells in healthy individuals and all subgroups of HIV patients. Also, the frequency of CD73$^+$CD39$^-$ γδ T cells did not differ from the frequency of CD73$^+$ γδ T cells.

To compare the findings with other viral infections, the same analyses were conducted with PBMCs from patients with acute and chronic hepatitis B (HBV) and chronic hepatitis C (HCV). No increase of $CD39^+$ γδ T cells was found in patients with acute HBV, chronic HBV, or chronic HCV compared to healthy individuals. In contrast, a decreased frequency of $CD73^+$ γδ T cells could be observed compared to healthy controls (7,8% vs. 23,9%) in acute HBV, PBMCs of chronically infected HBV, and HCV patients showed the tendency of decreased CD73 levels. Differences were not significant.

2.4.2 CD39 Expression on γδ T Cells From People Living With HIV Correlates With Viral Load, $CD4^+$ T-Cell Counts, and Immune Activation

The expression results for CD39 and CD73 on γδ T cells in PBMCs from people living with HIV compared to uninfected individuals were subsequently correlated, with standard clinical parameters defining the HIV disease course and immune activation indicated by the co-expression of HLA-DR and CD38 on $CD8^+$ and total γδ T cells.

A positive correlation between the frequency of $CD39^+$ γδ T cells and plasma viral load (Spearman r=0,43, p<0,0001) and a negative correlation between $CD4^+$ T-cell counts and the frequency of $CD39^+$ γδ T cells (Spearman r=-0,44, p<0,0001) was observed. Furthermore, the frequency of $CD39^+$ γδ T cells correlated with the proportion of activated $CD8^+$ T cells (Spearman r=0,26, p=0,0375) and activated γδ T cells (Spearman r=0,42, p=0,0004). Regardless of disease status, a significantly higher frequency of activated cells was measured among $CD39^+$ compared to $CD39^-$ γδ T cells.

The same analyses for $CD73^+$ γδ T cells revealed opposite findings: the frequency of $CD73^+$ γδ T cells positively correlated with $CD4^+$ T-cell counts (Spearman r=0,35, p=0,0013) and negatively with viral load (Spearman r=-0,32, p=0,0033). No correlation between the frequency of $CD73^+$ γδ T cells and activated γδ T cells or activated $CD8^+$ T cells in HIV-infected patients could be detected.

In addition, the frequency of $CD39^+$ γδ T cells increased continuously for 6 days after *in vitro* stimulation with CD3/CD28 or PMA/Ionomycin. Taken together, the frequency of activated $CD8^+$ and γδ T cells correlated with the frequency of $CD39^+$ γδ T cells in HIV infection, and $CD39^+$ γδ T cells expanded in response to *in vitro* stimulation.

2.4.3 Vδ2 T Cells are Less Exhausted and Less Differentiated Than Their Vδ1 T-Cell Counterparts but Do Not Differ in Their Activation Status During HIV Infection

Next, the expression pattern of $CD39^+$ Vδ1 versus $CD39^+$ Vδ2 T cells in uninfected individuals and HIV patients was examined. Vδ1 and Vδ2 T cells differ in their phenotype and functionality and there are indications that $CD39^+$ Vδ2 T cells might have a stronger immunomodulatory function [42,52,55–57,60–63,66–69,92,109,120,121].

Similar to total γδ T cells, there was a marked increase of $CD39^+$ Vδ1 and Vδ2 T cells in PBMCs from viremic patients compared to healthy controls.

The expression of CD39 on the Vδ2 T-cell subset was significantly lower than on the Vδ1 T-cell subset in all study groups except EC. The largest differences in CD39 expression between Vδ1 and Vδ2 T cells were observed in healthy controls, viremic individuals, and LTNP (healthy: 3,7% vs. 1,0%, p=0,0001; viremic: 14,8% vs. 6,98,3%, p<0,0001; LTNP: 7,2% vs. 3,0%, p=0,0039). In samples from patients on ART and EC, the expression levels of $CD39^+$ γδ T cells were similar in Vδ1 and Vδ2 T-cell subsets (ART: 3,7% vs. 3,0%, p=0,0266; EC: 3,5% vs. 2,6%, p=0,3125).

CD39 expression on total γδ T cells correlated with immune activation in HIV patients. Total Vδ1 T cells were significantly more activated than Vδ2 T cells, regardless of disease status.

Thus the frequency of activated ($HLA\text{-}DR^+CD38^+$) $CD39^+$ Vδ1 and $CD39^+$ Vδ2 T-cell subsets were compared and similar frequencies in all studied groups except LTNP were noticed, where the frequency of activated $CD39^+$ Vδ2 T cells was significantly lower than the frequency of activated $CD39^+$ Vδ1 T cells ($CD39^+$ Vδ1 vs.$CD39^+$ Vδ2 T cells: healthy: 11,2% vs. 7,0%; ART: 9,8% vs. 11,0%; viremic: 12,7% vs. 12,8%; EC: 24,8% vs. 16,4%; LTNP: 24,8% vs.6,0%, p=0,0020).

Then the differentiation and exhaustion status of Vδ1 and Vδ2 T cells in the context of CD39 expression were assessed, using co-expression patterns of the exhaustion markers PD-1 and TIGIT as an indicator of exhaustion and the absence of CD27 and CD28 as an indicator for a late stage of differentiation [127–132].

As observed for activation, a significantly higher frequency of total Vδ1 compared to Vδ2 T cells was exhausted ($PD\text{-}1^+TIGIT^+$) in all study groups.

Significantly lower levels of exhausted ($PD\text{-}1^+TIGIT^+$) γδ T cells were found among $CD39^+$ Vδ2 T cells compared to $CD39^+$ Vδ1 T cells regardless of disease status (healthy: 21,15%

vs 2,56%, p<0,0001; ART: 14,90% vs. 8,49%, p<0,0001; viremic: 25,76% vs. 17,22%, p=0,0018; EC: 16,5% vs. 2,67%, p=0,0312; LTNP: 32,21% vs. 5,87, p=0,0020).

A higher frequency of cells with late differentiation status ($CD27^-CD28^-$) was found among $CD39^+$ Vδ1 compared to $CD39^+$ Vδ2 T cells in healthy individuals and HIV patients regardless of the disease status (healthy: 44,02% vs. 20,4%, p=0,0032; ART: 73,71% vs. 27,44%, p<0,0001; viremic: 68,08% vs. 51,86%, p=0,0018; EC: 45,97% vs. 29,24%, p=0,3125; LTNP: 61,56% vs. 34,42%, p=0,0645).

In summary, markers of T-cell exhaustion were more frequently expressed among $CD39^+$ Vδ1 than $CD39^+$ Vδ2 T cells regardless of disease status, and late differentiation of cells was more often detected in $CD39^+$ Vδ1 than $CD39^+$ Vδ2 T cells of healthy individuals and HIV progressors.

2.4.4 Expression of CD39 and CD73 Marks γδ T Cells That Produce IL-10 at High Levels After *In Vitro* Stimulation

After that, the functional profile of $CD39^+$ γδ T cells was characterized and their potential immunomodulatory effector functions were assessed by performing intracellular cytokine staining of γδ T cells for IL-10 after unspecific stimulation of PBMCs with PMA and Ionomycin.

The highest frequency of IL-10^+ γδ T cells was found in viremic HIV-infected patients, which also expressed the highest amount of CD39 on γδ T cells, compared to healthy controls, ART-treated, and EC. Comparing the frequency of IL-10-producing $CD39^+$ and $CD39^-$ γδ T cells within each study group, the samples from healthy donors, viremic HIV-infected individuals and patients on ART showed a significantly higher frequency of IL-10 producing γδ T cells among $CD39^+$ than $CD39^-$ cells (healthy: 14,8% vs. 0,2%, p=0,0195; ART: 7,4% vs. 0,2%, p=0,0156; viremic: 10,4% vs. 2,3%, p=0,0171). In PBMCs from EC, similar frequencies of IL-10 producing γδ T cells between $CD39^+$ and $CD39^-$ cells were detected (1,7% vs. 1,7%, p=0,5556).

Similarly, in all groups but EC, the frequency of IL-10^+ cells was significantly higher among $CD73^+$ than $CD73^-$ γδ T cells upon *in vitro* stimulation (healthy: 1,5% vs. 0,3%, p=0,0273; ART: 1,4% vs. 0,2%, p=0,0273; viremic: 13,2% vs. 2,2%, p=0,0034), although the frequency of IL-10^+ cells was overall lower than in $CD39^+$ γδ T cells.

Next, the capacity of the small population of $CD39^+CD73^+$ versus $CD39^-CD73^-$ γδ T cells to produce IL-10 was assessed and it was found that $CD39^+CD73^+$ γδ T cells produced more IL-10 than $CD39^-CD73^-$ γδ T cells regardless of the disease status. In all groups but EC, the

differences reached statistical significance (healthy: 22,6% vs. 0,2%, p=0,0078; ART: 22,9% vs. 0,2%, p=0,0156; viremic: 35,2% vs. 2,2%, p=0,0005; EC: 32,8% vs. 1,7%, p=0,2500).

Due to the small sample size and cell number, the differences between the IL-10 production of $CD39^+$ Vδ1 versus $CD39^+$ Vδ2 T cells were examined as pooled data (combined data from all study groups). A higher frequency of $IL\text{-}10^+$ $CD39^+$ Vδ2 T cells compared to the frequency of pooled $IL\text{-}10^+$ $CD39^+$ Vδ1 T cells was observed (16,2% vs. 3,7%, p=0,01). Comparing the study groups, the frequency of $IL\text{-}10^+$ $CD39^+$ Vδ2 T cells was highest in samples from healthy donors and decreased in samples from HIV-infected individuals (31,3% vs. ART: 10,5%; viremic: 15,0%; EC: 4,2%), however, observed differences were not statistically significant.

2.4.5 Cytokine Profiles of $CD39^+$ Versus $CD39^-$ Vδ2 T Cells

Next, the cytokine profiles for IL-2, IFN-γ, TNF-α, TGF-β, and Granzyme-B were assessed after *in vitro* stimulation and a multidimensional analysis of the cytokines secreted by $CD39^+$ Vδ2 T cells versus $CD39^-$ Vδ2 T cells was conducted via SPICE analysis.

There are several $CD39^+$ Vδ2 T-cell subpopulations in samples from healthy individuals that co-expressed the anti-inflammatory cytokines IL-10 and TGF-ß, which are found to a lesser extent in the corresponding $CD39^-$ Vδ2 T cells. These subpopulations are greatly reduced in samples from HIV-infected individuals across all disease stages.

To summarize, the polyfunctionality of $CD39^+$ versus $CD39^-$ Vδ2 T cells, i. e. the number of different cytokines that can be produced by the respective subset, was plotted.

In samples from healthy controls, all the $CD39^+$ Vδ2 T-cell subsets expressed at least two cytokines, with approximately three-quarters of them expressing three cytokines. In contrast, most cells in the $CD39^-$ Vδ2 T subset did not produce any of the analyzed cytokines, and only approximately 15% produced three different cytokines.

In samples from patients with HIV, the differences between $CD39^+$ and $CD39^-$ Vδ2 T cells were less pronounced. Most of the Vδ2 T cells from viremic patients produced none of the analyzed cytokines, and about one-third produced three or fewer of the analyzed cytokines, regardless of CD39 expression.

$CD39^-$ Vδ2 T cells of patients on ART had a similar pattern to $CD39^-$ Vδ2 T cells from viremic individuals. The fraction of the $CD39^+$ Vδ2 T cells producing three different cytokines of

patients on ART was larger than the fraction of the CD39^{+} Vδ2 T cells from viremic patients and the CD39^{-} Vδ2 T-cell fractions of both study groups.

In EC, the majority of CD39^{+} Vδ2 T cells expressed one cytokine, followed by one-third that produced three different cytokines and a smaller fraction that produced two different ones. CD39^{-} Vδ2 T cells from EC mostly did not produce any of the analyzed cytokines.

In summary, Vδ2 T cells from viremic HIV patients lost their polyfunctionality partially and produced fewer anti-inflammatory cytokines. This development seems to be more pronounced in CD39^{-} Vδ2 than in CD39^{+} Vδ2 T cells.

2.4.6 Moderate Changes in the Composition, but Divergent Cytokine Repertoire of γδ T Cells From HIV Elite Controllers Compared to Healthy Controls

Finally, the differences in phenotype and functionality of γδ T cells were evaluated by comparing samples from HIV elite controllers and those of viremic and ART-treated patients.

Initially, the frequency of CD39^{+} γδ T cells was not increased compared with samples from healthy controls, and there were less pronounced changes in the Vδ1/Vδ2 T-cell ratio. Also, the frequency of CD73^{+} γδ T cells was not significantly decreased compared to healthy controls. There were no differences in the expression of CD39 and CD73 between Vδ1 and Vδ2 T cells. EC showed only low frequencies of IL-10 producing γδ T cells across all subsets except for CD39^{+}CD73^{+} γδ T cells. In addition, CD39^{+} Vδ2 T cells lost polyfunctionality like the other HIV cohorts but appear to assume an intermediate position between samples of other HIV-infected and healthy individuals concerning the number of secreted cytokines.

2.5 Discussion

γδ T cells are regarded as unconventional T cells associated with different effector functions such as recognizing various antigens and direct cytotoxic activity in viral infections [17–38]. Their subsets Vδ1 and Vδ2 T cells differ in their phenotype and functionality and behave differently in HIV infection [52,55–57,60–63,66–69,92]. The ectonucleotidases CD39 and CD73 are part of the purinergic pathway and have been described as important immunoregulatory molecules on Tregs and T effector cells [72,76,88–92,94,100,102–104,109,112,125]. They are expressed in several lymphocyte populations and seem to be important for HIV pathogenesis [72,108,112,114,133].

Interestingly, the protective HIV-specific proliferative response of $CD8^+$ T cells, restricted by human leukocyte antigen system (HLA) alleles B27 and B57, which are associated with delayed HIV disease progression, are not suppressible by Tregs [134–136]. This indicates that there must be other immunomodulatory cell groups or molecules to control HIV infection.

γδ T cells are associated with immunosuppression and infiltrating tumors for creating an anti-inflammatory environment and suppressing cell proliferation [46,99–101]. CD39 has recently been defined as a potential marker for immunomodulatory cells such as Treg and NK cells, and $CD39^+$ Vδ2 T cells may have a particular immunomodulatory role in HIV infection [42,91,94,100,102,109,112,119,125]. $CD39^+$ Tregs have been described to inhibit IL-2 production via the CD39/ADO pathway [137,138]. Otsuka and Hu described a possible regulatory role of $CD39^+$ γδ T in mice and human cancer cells, whereas the mediator (Adenosine pathway, IL-10, or TGF-β) remains unclear [100,102].

It is hypothesized that $CD39^+$ and $CD73^+$ γδ T cells might play a role in immunomodulatory functions in HIV infection that are mediated by the ATP-Adenosine signaling pathway. To test this hypothesis, a detailed phenotypical and functional characterization of CD39 and CD73 expression on different γδ T-cell populations from people living with HIV at different disease stages in comparison with healthy controls was assessed.

A significant increase in the frequency of $CD39^+$ γδ T cells in samples of viremic HIV patients compared to healthy controls was observed, positively correlating with disease progression (higher viral load, lower $CD4^+$ T-cell count) and immune activation ($HLA\text{-}DR^+CD38^+$ expression of γδ T cells as well as of $CD8^+$ T cells). CD39 expression on γδ T cells of ART-treated patients returned to levels comparable with those of healthy individuals. These results are consistent with those of other groups that have analyzed CD39 expression on other cell populations in HIV infection [92,107–109,112].

Inversely, the frequency of $CD73^+$ γδ T cells decreases in samples of viremic patients compared to healthy controls and correlates negatively with disease progression. As with CD39, Tóth et al. and others have recently noted comparable dynamics in other cell populations in HIV infection that also correlate with progressive HIV disease [72,90,112,114]. Higher CD73 levels have been associated with immunosuppression and poor prognosis, for instance in breast or ovarian cancer [89,103–106]. In contrast, low levels of CD73 are associated with lower T-cell activation and immunosuppression mediated by Adenosine and/or TGF-β [71–73,101,114,139,140].

A very small population of γδ T cells co-expressing CD39 and CD73 that produce more IL-10 than other examined subsets after *in vitro* stimulation in healthy individuals and HIV patients was observed. A potential interpretation is that this subpopulation of $CD39^+CD73^+$ γδ T cells, which secretes an anti-inflammatory cytokine, has immunomodulatory potential but is unable to inhibit HIV-specific immune response, may be due to small frequency. The frequency of $CD39^+CD73^+$ γδ T stayed relatively unaffected by HIV infection. Bastid et al. demonstrated inhibition of other T-cell subsets by $CD39^+CD73^+$ cancer cells, which could be abrogated by CD39 inhibitors, improving proliferation and cytotoxicity [141].

CD39 and CD73 transform extracellular pro-inflammatory ATP and ADP to anti-inflammatory ADO, which is able to induce IL-10 production; IL-10 secretion is also directly associated with CD39 expression [74–76,84–87,102,112,113,142]. It was found that the ability of γδ T cells to produce IL-10 is higher among the $CD39^+$ than the $CD39^-$ or the $CD73^+$ subset. The highest frequency of IL-10-producing cells was found among viremic γδ T cells and $CD39^+CD73^+$ γδ T cells. In addition, the proportion of IL-10-producing cells tended to be higher among $CD39^+$ Vδ2 compared with $CD39^+$ Vδ1 T cells.

In this dissertation, only HIV elite controllers showed an expression of CD73 and CD39 on γδ T cells comparable to healthy controls, while the expression pattern of CD39 and CD73 was altered in viremic patients and not fully normalized in individuals on ART. Chaudhry et al. reported that the T-cell receptor (TCR) repertoire also does not appear to fully recover under ART [143]. Carrière et al. also found high CD73 frequencies in blood samples of elite controllers but on $CD8^+$ T cells [144].

The CD39 gene polymorphism matches the low CD39 frequency on γδ T cells of elite controllers: a gene variant with low CD39 expression has been described, which is associated with a slower progression to AIDS indicating that CD39 plays a role in HIV pathogenesis [116,117,145].

In the polyfunctionality of $CD39^+$ γδ T cells, elite controllers seem to occupy an intermediate position between healthy and HIV-infected individuals which needs further investigation.

In chronic HIV infection, IL-10 and TGF-β concentrations in the blood plasma were reported to increase over time and to correlate with disease progression [111,146,147]. In addition, the frequency of $CD39^+$ cells secreting IL-10 correlates with viral load and immune activation [102,111–113,142,146]. The data from elite controllers, who maintain low levels of CD39 and produce considerably less IL-10 than viremic HIV-infected patients, fit these observations. IL-10 appears to either play a pathogenetic role in HIV infection or is upregulated reactively. Nevertheless, it could not be confirmed that Vδ2 T cells will not be depleted at EC as described by Riedel et al. [118].

In general, the role of γδ T cells in HIV, especially Vδ2 T cells, remains not entirely clear. Vδ2 T cells have been associated with a protective role in HIV and peripheral blood of elite controllers tend to show higher frequencies of Vδ2 T cells than untreated patients or those on ART [69,118,119,148]. Interestingly, Vδ2 T cells have been associated with the formation of part of the viral reservoir, notably because of their infectivity via the high surface expression of CCR5 and α4β7 [61–63,92,149]. Follow-up experiments should investigate $CD39^+$ Vδ2 T in relation to the HIV reservoir.

A significantly lower expression of CD39 on the Vδ2 T-cell subset than on the Vδ1 T-cell subset in all study groups except for elite controllers was observed. The activation level of $CD39^+$ Vδ1 and $CD39^+$ Vδ2 T cells was similar while there were significantly more exhausted ($PD\text{-}1^+TIGIT^+$) and terminally differentiated ($CD27^-CD28^-$) $CD39^+$ Vδ1 compared to $CD39^+$ Vδ2 T cells. A loss of polyfunctionality, defined as cells capable of producing three or more cytokines after *in vitro* stimulation, within the $CD39^+$ Vδ2 T-cell population of viremic HIV patients was observed that was not fully restored under ART. These results are in line with observations made by Casetti et al., who measured a reduction of polyfunctionality (cytokine/chemokine production and cytotoxicity) in Vδ2 T cells from ART-treated patients [150,151]. Others also showed that the functionality as well as the number of Vδ2 T does not recover after a prolonged successful ART and restoration of the $CD4^+$ T-cell compartment [64,65]. In contrast, some studies indicate a partial functional and cell level recovery with using ART for a sufficiently long time [64,152,153].

The data for this dissertation in combination with other results lead to the hypothesis that better virus control could be achieved by restoration of the inverse ratios of Vδ1/Vδ2 T cells and CD39/CD73. This is supported by the similarity to the ratio reversal of CD4/CD8 and

by fewer changes in CD39 expression and Vδ2 T-cell counts in individuals capable of controlling the virus without medication [116–118,145].

Fittingly, Bhatnagar et al. show a transformation of a suppressive phenotype in primary HIV infection of Vδ2 T cells into a pro-inflammatory phenotype with participation in the sustenance of immune activation in chronic HIV infection [42]. Others also report a shift of viremic Vδ2 T cells to an activated and terminally differentiated phenotype with damage to the TCR-chain repertoire [118,154–156]. Pauza et al. hypothesize that HIV-mediated depletion of Vδ2 T cells is part of the mechanism for HIV evasion of host defenses and establishment of chronic, persistent infection with progressing disease [66].

The goal of targeting γδ T cells for immunotherapy is the prevention or reversal of damage to the Vδ2 T-cell subset and regain antiviral functions [66]. Garrido et al. demonstrated Vδ2 T cells from ART-suppressed HIV-infected individuals are capable of targeting and killing reactivated autologous HIV-infected $CD4^+$ T cells *in vitro* [16]. The group also showed a correlation between γδ T-cell cytotoxic capacity with lower recovery of replication-competent HIV cultures of resting $CD4^+$ T cells from ART-suppressed HIV-seropositive individuals indicating the capacity of Vδ2 T cells to be used in immunotherapeutic approaches towards an HIV cure [157]. Clinical studies demonstrate the potential of Vδ2 T cells for cancer immunity by autologously or allogeneically – for treating HIV with CCR5 delta32 mutation - targeting diverse cancer cells after stimulation with phosphoantigens, thereby partially slowing progression or inducing remission [15,33,158–174]. A lot of cytokines and combinations thereof as well as Vitamin C have been tried out for expansion of Vδ2 T cells [166]. The amino bisphosphonate zoledronate was shown to be able to increase Vδ2 T-cell numbers and function both ex vivo and directly in HIV-positive patients [175–179]. Recent trials have not shown sufficient success with cell transfer or zoledronate treatment in humanized mouse models and HIV patients [58,152,178–182]. Stimulation and expansion of Vδ2 T cells was also successfully achieved by the monoclonal antibody BTN3, which results from the involvement of transmembrane butyrophilin (BTN) in intracellular phosphoantigen signaling [166].

In recent years, checkpoint blockades of human cells have become increasingly important in both research and clinical applications. PD-1 and PD-L1 inhibitors have been in clinical use for several years and have shown unprecedented effects on cancer patient survival, even in the metastatic setting [183,184]. Pre-clinical *in vitro* studies have shown improved T-cell function and better recognition of the latent reservoir in simian immunodeficiency virus (SIV) and HIV infection during PD-1 inhibitor therapy [185–190].

The blockade of IL-10 signaling at the time of therapeutic vaccine immunization improves the clearance of chronic viral infection and shows recovery of T-cell effector functions in *in vitro* experiments with HIV-infected cells [191–195].

Ectonucleotidases are also considered new checkpoint inhibitor targets, and an anti-CD73 antibody is currently in pivotal clinical trials [76,196–199]. With the monoclonal antibody and protein kinase inhibitor Ceritinib, only one indirect CD39 inhibitor exists to date [200–202].

Immune checkpoint inhibitor blockade and blockade of Adenosine signaling could be an opportunity to revert the function of virus-specific effector T cells. Li et al. demonstrated a reversion of $CD8^+$ T-cell exhaustion by concomitant blockade of PD-1 and Adenosine pathways in HIV infection *in vitro* while PD-1 blockade alone showed only limited efficacy [203–205]. Schachter et al. showed impaired HIV infection of macrophages by inhibiting ecto-ATPase activities [206].

Interestingly, $CD39^+$ T cells often also express PD-1 and other markers of cellular exhaustion [203,207,208]. The data of this dissertation and others demonstrate that CD39, PD-1, and IL-10 were increased on γδ T cells and other cell types in viremic HIV infection, which leads to the question why an adequate immunosuppressive environment is not established that can eliminate the HI virus [92,108,109,111,112,146,203,209,210]. It must be deciphered whether these increases are a reaction of insufficient immune control, or whether CD39, PD-1, and IL-10 have pro-inflammatory properties and a pathogenic role in HIV infection. Interestingly, the data reveal that neither IL-10 nor PD-1 nor CD39 increases strongly in EC, who can control the infection spontaneously. Brockman et al. suggest an immunosuppressive environment through IL-10 which impairs immunity and virus clearance in HIV infection and shows enhanced HIV-specific T-cell responses after IL-10 blockade in mice [111]. ($IL\text{-}10^+CD39^+$) γδ T cells could be reactivated by blockade of IL-10 or PD-1, CD39, or combinations thereof, and restored $CD4^+$ T-cell function was previously achieved by immune checkpoint blockade of PD-1 and/or IL-10 in HIV-infected patients [93,195,211].

Some human cancer cell types create an immunosuppressive microenvironment to evade the immune system's defense and thereby protect themselves. In colorectal and breast cancer, $CD39^+$ tumor-infiltrating cells are associated with poor prognosis [100,212]. CD39 appears to play a role in tumor immune escape by being expressed on $CD8^+$ T cells, γδ T cells, and Tregs, either via direct suppressive activity or indirectly as an exhaustion marker [100,140,202,212–215]. Li et al. described the use of a CD39 antibody in the humanized mouse model, which enhances intratumor T-cell effector function and overcomes anti-PD-1 resistance via eATP-P2X7-inflammasome-IL18 axis [216].

This brings out the question of whether the elevated frequencies of CD39$^+$ γδ T cells in viremic patients also result in an inadequate immune response to HIV infection. Low CD39 expression on γδ T cells from elite controllers and slower disease progression in individuals with genetically lower CD39 expression indicates this. In contrast, solid tumors with solid cell clusters do not compare well with peripheral blood, so an examination of the alterations in the number and function of CD39$^+$ γδ T cells in lymphoid organs would be informative. Because the gut is one of the major sites of virus dissemination and formation of the viral reservoir, it will be worthwhile to explore the gut-associated lymphoid tissue (GALT) from primary and chronic HIV-infected individuals compared to healthy controls [217–219]. Libera et al. detected CD39$^+$ γδ T cells with an immunosuppressive phenotype in the intestine [94].

Nevertheless, in human autoimmune diseases where the immune system shows pathological overactivity, CD39 expression on Tregs in peripheral blood appears to be lower than in healthy controls [220–222]. This is associated with reduced suppressive activity, and lower levels of IL-10 and ADO, which can be partially remedied with immunosuppressants [220–222]. In a mouse model of rheumatoid arthritis, the blockade of CD39 abrogated the antiarthritic effect of methotrexate (MTX) treatment [222]. Suitable for this, Libera et al. have previously demonstrated that the frequency of mucosa-derived CD39$^+$ γδ T cells is decreased in patients with inflammatory bowel diseases compared to healthy controls [94].

However, this raises the question of whether virus clearance is reduced by the reversal of these conditions, as the virus creates an immunosuppressive environment, or whether this is due to the inadequate containment of the virus and the body counter-regulates or whether the increase in CD39 expression is merely reactive. Chevalier and Weiss suggest a similar theory for the ambivalent role of Tregs in HIV infection and name it ‘the split personality of Tregs in HIV infection’ [134]. The majority of γδ T cells show changes in the CD39/CD73 expression ratio comparable to the changes observed in the effector cell compartment, most likely to a general immune activation in HIV, suggesting CD39 to be an activation and exhaustion marker [102,110,126,207,214,223,224]. Plus, a strong correlation between the frequency of CD39$^+$ and CD73$^+$ γδ T cells and immune activation as well as disease progression (viral load and CD4$^+$ T-cell count) was determined. In support of this hypothesis, TCR engagement, IL-6, IL-2, and IL-27 can promote the expression of CD39 but also TGF-β [225–227]. A proportional increase in CD39 expression on γδ T cells after ex vivo stimulation for several days was shown.

The behavior of CD39 expression on γδ T cells in other viral infectious diseases does not support the hypothesis of CD39 increase being solely due to immune activation and resulting exhaustion. No significant increase in $CD39^+$ γδ T cells was measured in HCV or HBV compared with healthy controls. Similar to HIV infection, (chronically) HBV-infected individuals experience loss of peripheral Vδ2 T cells, proliferation of peripheral Vδ1 T cells, and strong immune activation [228,229]. The reasons for the specific expansion of $CD39^+$ γδ T cells in HIV compared to other viral infections are unclear and must be unraveled. Elsaghir et al. combine both hypotheses while dividing $CD39^+CD4^+$ T cells into two populations, T-effector lymphocytes and T-regulatory lymphocytes [230].

This dissertation is the first study on the CD39 and CD73 expression pattern and functionality of γδ T cells in HIV patients and this work has some limitations. Firstly, the frequency of circulating γδ T cells expressing both CD39 and CD73 is particularly low as well as the subsets of the γδ T-cell population per se. Another limitation is the limited number of parameters that could be measured in a respective panel by flow cytometry analysis. In further studies, the expression of CD16, CD56, and NKG2D[m], which is expressed by the vast majority of Vδ2 T cells and can activate γδ T cells in an innate TCR-independent manner, should be included [33,231–233]. CD16 mediates antibody-dependent cell-mediated cytotoxicity (ADCC) in the early phase of infection and chronic infection and is associated with slower disease progression [16,148,157]. It would merit investigating NKG2A on Vδ2 T cells, which is associated with inhibitory signals and highly cytotoxic potential [166,234–236]. In the context of cytotoxicity, it would be interesting to evaluate the expression pattern of CD244, DNAM-1[n], and CD107a on γδ T cells and additionally CD94/NKG2C, NKp44[o], NKp30, NKp4 on γδ1 T cells as well as the production of perforin [59].

In addition, attention should be paid to the transcription profile, if necessary by use of single-cell transcriptional RNA expression analysis, of interest would be FoxP3, HIF-1[p], and AhR[q] [237–241].

It will be interesting to further investigate this scarce population of $CD39^+$ γδ T cells and their subpopulations, especially regarding their suppressive capacities. Additional research

[m] NKG2D, NKG2A, NKG2C: natural killer cell receptors
[n] DNAM-1: DNAX accessory molecule-1
[o] NKp44, NKp30, NKp4: natural cytotoxicity receptors
[p] HIF-1: hypoxia-inducible factor-1
[q] AhR: aryl hydrocarbon receptor

should distinguish between acute and chronic viremic HIV infection, as immune activation may still be controlled in acute infection [98].

Further studies should include suppression assays with $CD39^+$ γδ T cells, $CD39^+$ Vδ2 T cells, or $CD39^+CD73^+$ γδ T cells to demonstrate the immunosuppressive potential, such as Figueiró et al. performed with human B cells [242].

Additional experiments could be blockading assays with CD39, CD73, IL-10, PD-1, Adenosine, or combinations thereof. For other cell types, a possible reinvigorated HIV-specific immune response has already been demonstrated *in vitro* when one or more of these molecules are blocked [111,195,203,242].

A major problem, besides technical difficulties, in these assays will be cell quantity, since both γδ T cells and its subgroups are scarce in peripheral blood, especially when evaluating blood samples from HIV patients. Since live cell sorting and subsequent co-culture with activated T cells is difficult concerning low cell numbers, transcriptional analyses such as single-cell sequencing must be used to understand the capabilities of these and other γδ T-cell populations. Alternatively, γδ T-cell subpopulations could be expanded *in vitro* before life-sorting, co-culture, and flow-based read-out, with the disadvantage that this expansion may alter the phenotype and the function of the γδ T cells.

Another limitation of this work is the slightly different gender and age distribution between HIV-positive and HIV-negative people. The healthy controls were on average 15 years younger than the HIV-positive people (healthy: 29.1 years vs. HIV-infected individuals: 44.4 years) and predominantly female (healthy: 63% female vs. HIV-infected individuals: 28% female), while the HIV-infected people, except the subgroup of elite controllers, were predominantly male.

The results of the current dissertation show that the expression pattern of these ectoenzymes is associated with different functional states and can be used as a marker to identify activated and exhausted cells. Significant differences in the expression of CD39 and CD73 on total γδ T cells and Vδ1 and Vδ2 T cells between healthy and HIV-infected individuals were observed depending on clinical status. Overall, the CD39/CD73 expression ratio on γδ T cells is reversed in untreated HIV patients and is associated with immune activation and disease progression. Altered functionality and higher IL-10 production were found in viremic HIV patients. In addition, a small population of $CD39^+CD73^+$ γδ T cells that produce IL-10 at high frequency after *in vitro* stimulation was defined.

An immunomodulatory role of $CD39^+$ and $CD73^+$ γδ T cells in the pathogenesis of chronic HIV infection potentially mediated by IL-10 secretion is hypothesized. Similar to the deleterious role of suppressive cells in the microenvironment of tumors, the frequency of $CD39^+$ γδ T cells and inversely $CD73^+$ γδ T cells was correlated with HIV disease progression in this dissertation. This is further supported by the findings in elite controllers, who maintain stable frequencies of (IL-10-producing) $CD39^+$ and $CD73^+$ γδ T cells compared to healthy controls. Also, double-positive $CD39^+CD73^+$ produced significantly more IL-10 than γδ T cells expressing only one ectonucleotidase. Future studies will have to determine the role of Adenosine metabolism for γδ T-cell function and elucidate the effects of alterations of CD39 and CD73 expression on γδ T cells in HIV in more detail.

Currently, there is neither a vaccination nor a causal therapy to cure HIV infection. In some cases, it has been possible to eliminate the HI virus with an allogeneic hematopoietic stem cell transplant with CCR5 deletion. Based on these observations, other groups are pursuing similar strategies for curing HIV utilizing gene editing [243–248]. The ‘shock and kill’ strategy is also very promising, using latency-reversing agents (LRAs) to reactivate viral replication and subsequently eliminate it with an enhanced immune response [249–254]. With the help of public health measures and the World Health Organization's 90-90-90 target, and improvements in antiretroviral therapy such as the dual therapy regime or long-acting injectables, the HIV pandemic has been mitigated in parts of the world [247].

It is hypothesized that $CD39^+$ Vδ2 T cells have an anti-inflammatory function that is attenuated in HIV infection, as demonstrated by decreased frequency of Vδ2 T cells. Therefore, $CD39^+$ Vδ2 T cells were proposed as a promising therapeutic target in HIV infection. The mechanisms leading to Vδ2 T-cell dysfunction should be evaluated and further attempts with checkpoint blockades and stimulation of Vδ2 T cells will have to be explored to pave the way for novel therapeutic approaches.

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3 Summaries

3.1 English Summary

Background: HIV infection leads to chronic immune activation and dysfunction in humans, without a causal cure currently existing. γδ T cells (γδT) are unconventional T cells that have been shown to play a significant role in the pathogenesis and immune response of viral infections in general and in HIV infection in particular. In addition to conventional T cells, HIV also affects γδT and inverts the ratio of its subsets Vδ1 T cells (Vδ1) and Vδ2 T cells (Vδ2). The ectonucleotidases CD39 and CD73 are part of the purinergic pathway which can regulate inflammation by degradation of pro-inflammatory ATP to Adenosine. Human γδ T cells may exhibit immunosuppressive functions and a potential role of $CD39^+$ γδT with a regulatory function in mice has previously been discussed. However, the expression of the ectoenzymes CD73 and CD39 in human γδT and their role in HIV remains unclear. Therefore, the main aim of this dissertation was to study the expression patterns of CD39 and CD73 on γδ T cells in people living with HIV and to identify a potentially suppressive activity of ($CD39^+$) γδ T cells through adenine signaling. Additionally, a distinct group of patients, elite controllers who control their HIV infection without any medication, was evaluated.

Methods: PBMCs of 86 people living with HIV (36 viremic patients; 32 antiretroviral therapy (ART) –treated; 8 elite controllers; 10 long-term non-progressors) and 26 HIV-negative individuals using a multiparametric flow cytometry panel (16-colored) were studied by determining the surface expression of CD39 and CD73 on Vδ1 and Vδ2 in association with markers of differentiation (CD45RA, CD28, CD27), activation (CD38 and HLA-DR) and exhaustion (TIGIT and PD-1), and evaluating the intracellular production of pro- and anti-inflammatory cytokines (IL-2, TGF-ß, TNF-a, Granzyme B, IL-10, IFN-g) after *in vitro* stimulation with PMA/Ionomycin.

Results: A significant increase in the frequency of $CD39^+$ γδ T cells in samples of viremic HIV-infected individuals in comparison to uninfected controls was observed, while the frequency of $CD73^+$ γδ T cells decreased. CD39, but not CD73, expression on γδ T cells of ART-treated individuals returned to levels comparable with those of uninfected individuals.

The frequency of $CD39^+$ γδ T cells correlated with disease progression (higher viral load, lower $CD4^+$ T-cell count) and immune activation ($HLA\text{-}DR^+CD38^+$ expression of γδ T cells as well as $CD8^+$ T cells). Inversely, the frequency of $CD73^+$ γδ T cells correlated negatively with disease progression.

The expression of CD39 on the Vδ2 T-cell subset was significantly lower than on the Vδ1 γδ T-cell subset in all study groups except in elite controllers.

The expression of CD39 on activated, HLA-DR^{+}CD38^{+} γδ T cells was found to be higher than on HLA-DR^{-}CD38^{-} γδ T cells while the activation level of CD39^{+} Vδ1 and CD39^{+} Vδ2 T cells was similar. Moreover, there were significantly more exhausted (PD-1^{+}TIGIT^{+}) and terminally differentiated (CD27^{-}CD28^{-}) CD39^{+} Vδ1 compared to CD39^{+} Vδ2.

A higher production of IL-10 in CD39^{+} γδ T cells vs CD39^{-} γδ T cells regardless of disease status was noticed and the highest IL-10 production level of γδ T cells was seen in viremic HIV patients. The expression of CD39^{+}CD73^{+} on γδ T cells remained low (< 1%) regardless of disease status, but nevertheless, showed the highest release of IL-10. Also, CD39^{+} Vδ2 T cells produced IL-10 more frequently than their CD39^{+} Vδ1 counterparts in all studied individuals. Analysis of other cytokines revealed that Vδ2 lost their polyfunctionality in parts and produced fewer anti-inflammatory cytokines in HIV infection.

Elite controllers appear to assume an intermediate position between HIV-uninfected and -infected individuals regarding CD39 and CD73 expression and cytokine profile on γδ T cells.

Conclusions: Our results suggest a potential immunomodulatory role of CD39^{+} and CD73^{+} γδ T cells in the pathogenesis of chronic HIV infection. To identify novel therapeutic approaches for HIV infections, suppression assays, immunotherapeutic approaches with Vδ2 T cells, and blocking monoclonal antibodies should be considered.

3.2 Zusammenfassung in deutscher Sprache

Hintergrund: Eine HIV-Infektion führt beim Menschen zu einer chronischen Aktivierung und Dysfunktion des Immunsystems. γδ-T-Zellen sind unkonventionelle T-Zellen, die nachweislich eine wichtige Rolle bei der Pathogenese und der Immunreaktion auf Virusinfektionen im Allgemeinen und insbesondere bei der HIV-Infektion spielen. Zusätzlich zu den konventionellen T-Zellen beeinflusst das HI-Virus auch γδ-T-Zellen und kehrt das Verhältnis ihrer Subgruppen Vδ1-T-Zellen und Vδ2-T-Zellen um. Die Ektonukleotidasen CD39 und CD73 sind Teil des purinergen Signalwegs, der Entzündungen durch den Abbau von entzündungsförderndem ATP zu Adenosin regulieren kann. Menschliche γδ-T-Zellen können immunsuppressive Funktionen aufweisen. Eine mögliche Rolle von $CD39^+$ γδT mit einer regulatorischen Funktion bei Mäusen wurde bereits diskutiert. Die Expression der Ektoenzyme CD73 und CD39 auf menschlichen γδ-T-Zellen und ihre Rolle in der HIV-Infektion sind jedoch weitgehend unklar. Daher bestand das Hauptziel dieser Arbeit darin, die Expressionsmuster von CD39 und CD73 auf γδ-T-Zellen von Menschen, die mit HIV leben, zu untersuchen und eine potenziell suppressive Aktivität von ($CD39^+$) γδ-T-Zellen durch Adenin-Signalisierung zu identifizieren. Darüber hinaus wurde eine bestimmte Gruppe von Patient:innen, die sogenannten Elite-Controller, die in der Lage sind, ihre HIV-Infektion ohne Medikamente zu kontrollieren, untersucht.

Methoden: PBMCs von 86 Menschen, die mit HIV leben (36 virämische, 32 mit antiretroviraler Therapie (ART) behandelte, 8 Elite-Controller, 10 Long-term non-progressors), und 26 nicht HIV-infizierte Personen wurden untersucht. Dafür wurde ein multiparametrisches Durchflusszytometrie-Panel (16-farbig) zur Bestimmung der Oberflächenexpression von CD39 und CD73 auf Vδ1- und Vδ2-T-Zellen verwendet. Besonders Augenmerk wurde auf Marker dieser Zellen gelegt, die mit Differenzierung (CD45RA, CD28, CD27), Aktivierung (CD38 und HLA-DR) und Erschöpfung (TIGIT und PD-1) assoziiert sind. Anschließend wurde die intrazelluläre Produktion von entzündungsfördernden und entzündungshemmenden Zytokinen (IL-2, TGF-ß, TNF-a, Granzyme B, IL-10, IFN-g) nach *In-vitro*-Stimulation mit PMA/Ionomycin bestimmt.

Ergebnisse: Es wurde ein signifikanter Anstieg des Anteils von $CD39^+$ γδ-T-Zellen in Proben von virämischen HIV-Infizierten im Vergleich zu gesunden Kontrollen beobachtet, während der Anteil von $CD73^+$ γδ-T-Zellen abnahm. Die Expression von CD39 auf γδ-T-Zellen von mit ART behandelten Personen kehrte auf ein Niveau zurück, das mit dem von gesunden Personen vergleichbar war. Bei der Expression von CD73 war dies nicht der Fall.

Die Frequenz von $CD39^+$ γδ-T-Zellen korrelierte mit dem Fortschreiten der Krankheit (höhere Viruslast, niedrigere $CD4^+$-T-Zellzahl) und der Immunaktivierung ($HLA\text{-}DR^+CD38^+$ Expression auf γδ-T-Zellen sowie $CD8^+$ T-Zellen). Umgekehrt korrelierte die Frequenz von $CD73^+$ γδ-T-Zellen negativ mit dem Fortschreiten der Krankheit.

Die Expression von CD39 auf der Vδ2-T-Zell-Untergruppe war in allen Studiengruppen mit Ausnahme der Elite-Controller signifikant niedriger als auf der Vδ1-γδ-T-Zell-Untergruppe.

Die Expression von CD39 auf aktivierten $HLA\text{-}DR^+CD38^+$ γδ-T-Zellen war höher als auf $HLA\text{-}DR^-CD38^-$ γδ-T-Zellen, während das Aktivierungsniveau von $CD39^+$ Vδ1 und $CD39^+$ Vδ2-T-Zellen ähnlich war. Außerdem gab es deutlich mehr erschöpfte ($PD\text{-}1^+TIGIT^+$) und terminal differenzierte ($CD27^-CD28^-$) $CD39^+$ Vδ1 im Vergleich zu $CD39^+$ Vδ2-γδ-T-Zellen.

Es wurde eine höhere Ausschüttung von IL-10 in $CD39^+$ γδ-T-Zellen im Vergleich zu $CD39^-$ γδ-T-Zellen unabhängig vom Krankheitsstatus festgestellt und die höchste IL-10-Produktion von γδ-T-Zellen bei virämischen HIV-Patienten gesehen. Die Expression von $CD39^+CD73^+$ auf γδ-T-Zellen blieb unabhängig vom Krankheitsstatus niedrig (< 1 %), zeigte nichtsdestotrotz dazu die höchste Freisetzung von IL-10. Außerdem produzierten $CD39^+$ Vδ2-T-Zellen bei allen untersuchten Personen häufiger IL-10 als ihre $CD39^+$ Vδ1 Gegenparts. Die Analyse anderer Zytokine ergab, dass Vδ2-γδ-T-Zellen ihre Polyfunktionalität teilweise verloren hatten und bei einer HIV-Infektion weniger entzündungshemmende Zytokine produzierten.

Elite-Controller scheinen hinsichtlich der CD39- und CD73-Expression und des Zytokinprofils auf γδ-T-Zellen eine Zwischenstellung zwischen gesunden und HIV-infizierten Personen einzunehmen.

Schlussfolgerungen: Die Ergebnisse deuten auf eine mögliche immunmodulatorische Rolle von $CD39^+$ und $CD73^+$ γδ-T-Zellen bei der Pathogenese der chronischen HIV-Infektion hin. Um neue therapeutische Ansätze für HIV-Infektionen zu identifizieren, sollten Suppressionstests, immuntherapeutische Ansätze mit Vδ2-T-Zellen und blockierende monoklonale Antikörper in Betracht gezogen werden.

4 Declaration Regarding My Own Contribution

Katharina Kolbe and Dr. rer. nat. Melanie Wittner have contributed equally to the publication and share the first authorship.

The topic as well as the initial study design were developed by Prof. Dr. med. Julian Schulze zur Wiesch and Katharina Kolbe.

Methods and experimental set-up were selected, established, and discussed by Prof. Dr. med. Julian Schulze zur Wiesch and Katharina Kolbe with the assistance of Dr. rer. nat. Melanie Wittner and Dr. rer. nat. Philip Hartjen.

The recruitment of patients and the collection of samples and patient data were carried out by Prof. Dr. med. Julian Schulze zur Wiesch, Dr. med. Anja-Dorothee Hüfner, Dr. med. Olaf Degen, Prof. Dr. med. Hans-Jürgen Stellbrink and Dr. rer. nat. Christin Ackermann.

The equipment and premises were provided by Prof. Dr. med. Ansgar Lohse and Prof. Dr. med. Julian Schulze zur Wiesch. Prof. Dr. med. Julian Schulze zur Wiesch solicited and provided the funding.

The surface and intracellular stainings and subsequent FACS analyses were carried out significantly by Katharina Kolbe and Dr. rer. nat. Melanie Wittner with the help of Robin Woost and Silke Kummer.

The evaluation of the flow cytometry experiments and the preparation of the figures was done by Katharina Kolbe with assistance from Dr. rer. nat. Melanie Wittner. Dr. rer. nat. Melanie Wittner conducted SPICE analysis and preparation of the respective figures. Prof. Dr. med. Julian Schulze zur Wiesch, Dr. rer. nat. Philip Hartjen and Prof. Dr. med. Friedrich Haag supervised this process.

The discussion and interpretation of the results was done by Katharina Kolbe, Dr. rer. nat. Melanie Wittner, Prof. Dr. med. Julian Schulze zur Wiesch, Dr. rer. nat. Philip Hartjen, Prof. Dr. med. Friedrich Haag, Jana Libera, Dr. rer. nat. Johanna Eberhard, Dr. rer. nat. Christin Ackermann, Parimah Ahmadi, and Leon Cords.

Prof. Dr. med. Julian Schulze zur Wiesch and Dr. rer. nat. Melanie Wittner wrote the manuscript with the assistance of Katharina Kolbe.

The writing of this dissertation was done by Katharina Kolbe.

Prof. Dr. med. Julian Schulze zur Wiesch supervised the whole process and was the charge of the overall research project.

5 Acknowledgements

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6 Curriculum Vitae

Lebenslauf entfällt aus datenschutzrechtlichen Gründen

Lebenslauf entfällt aus datenschutzrechtlichen Gründen

7 Eidesstattliche Versicherung

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.

Ferner versichere ich, dass ich die Dissertation bisher nicht einem Fachvertreter an einer anderen Hochschule zur Überprüfung vorgelegt oder mich anderweitig um Zulassung zur Promotion beworben habe.

Ich erkläre mich einverstanden, dass meine Dissertation vom Dekanat der Medizinischen Fakultät mit einer gängigen Software zur Erkennung von Plagiaten überprüft werden kann.

Unterschrift: