CE: ; QAD/AIDS-D-24-00020; Total nos of Pages: 6;
AIDS-D-24-00020

CONCISE COMMUNICATION

Distinct features of immune activation and exhaustion markers in people with perinatally acquired HIV

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Objective: The aim of this study was to characterize T-cell activation, exhaustion, maturation and Treg frequencies in individuals who acquire perinatal HIV (PHIV), in individuals who acquired HIV as adult (AHIV), and in healthy controls.

Design: This cross-sectional study included people with HIV at least 14 and younger than 40 years, HIV-RNA less than 50 copies/ml on antiretroviral therapy for at least 6 months, and HC.

Methods: We assessed the expression of PD-1, TIM-3, EOMES, $CD38^+$ DR+, maturation status by $CD4^+$ and $CD8^+$ T cells and the frequency of $CD4^+$ and $CD8^+$ Treg cells. Principal component analysis (PCA) and k-means cluster analysis investigated which combination of immunological parameters better associated with each group.

Results: Twenty-six PHIV and 18 AHIV with median ages of 26 (8.0) and 28 (6.8) years were consecutively enrolled. PHIV showed significant higher frequency of naive and lower frequency of terminal effector memory $CD4^+$ and $CD8^+$ T cells than AHIV. AHIV exhibited higher expression of exhaustion and activation markers. The statistical analysis returned two clusters with 94% of specificity and 88% of sensitivity identifying PHIV vs. AHIV. The nine healthy controls had a lower expression of exhaustion markers on both $CD4^+$ and $CD8^+$ T lymphocytes than PHIV and AHIV.

Conclusion: These data may exclude major alterations of lymphopoiesis in PHIV, with even lower state of immune-activation and exhaustion compared with AHIV. This suggests that recent lack of virological control, may affect immune activation and exhaustion of $CD4^+$ and $CD8^+$ T cells.

AIDS 2024, **38**:000–000

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Received: 10 January 2024; revised: 11 August 2024; accepted: 18 August 2024.

DOI:10.1097/QAD.0000000000004001

Keywords: activation, correlates and predictors, exhaustion, HIV, perinatally acquired HIV

Introduction

Although antiretroviral therapy (ART) is highly effective in inhibiting HIV viral replication, it does not eradicate the infection [1], making it a chronic condition characterized by immune activation and premature senescence [2]. Individuals who acquire perinatal HIV (PHIV) receive ART from a young age and continue it for life, potentially leading to long-term therapy consequences and chronic inflammation [3,4]. Previous studies have shown that untreated PHIV, in the first year of life exhibit significantly altered proportions of $CD8^+$ T-cell maturation stages [5]. Additionally, older children and adolescents with PHIV have higher levels of inflammatory markers compared with age-matched healthy controls [6–11]. Whether this results in a different cellular pattern in adulthood compared with those who acquire HIV when already adult (AHIV) remains under investigation.

The primary objective of this study was to compare immune activation and cellular exhaustion in both PHIV and AHIV. This was assessed by examining the expression of activation markers ($CD38^+$ and HLA-DR+) in $CD4^+$ and $CD8^+$ T lymphocytes; the proportions of naive, central memory, effector memory, terminal effector memory (TEM) in $CD4^+$ and $CD8^+$ T-cell subpopulations; the frequency of $CD4^+$ and $CD8^+$ T-regulatory (Treg) cells; and immune exhaustion markers (PD-1, TIM-3, EOMES) in both total $CD4^+$ and $CD8^+$ T-cell subsets and in $CD4^+$ and $CD8^+$ Treg.

Secondary objectives included identifying which cellular subsets best differentiate the two study populations (PHIV and AHIV) and evaluating differences in the expression of immune exhaustion and activation markers among PHIV, AHIV, and heathy controls.

Materials and methods

Study population

This monocentric cross-sectional study, conducted from 1 January 2018, included consecutively people with HIV (PWH) at least 14 and younger than 40 years if they were on ART and had HIV-RNA less than 50 copies/ml for at least 6 months.

Exclusion criteria included active concurrent infections, previous AIDS-defining conditions, opportunistic diseases or malignancies, lack of virological control (HIV-RNA $\geq$50 copies/ml) in the last 6 months, age below 14 years, or lack of consent.

PHIV were followed since birth and had documented mother-to-child transmission of HIV. All were on ART since childhood and treated with various combination regimens.

AHIV had documented HIV diagnosis in adulthood. Their inclusion was irrespective of the time elapsed between the time of HIV acquisition and the date of the diagnosis.

To better describe immunological features, we also conducted immunological and statistical analyses on healthy controls (voluntary donors).

All clinical and routinely collected laboratory data were retrieved via the MedInfo online platform (www.reteligureHIV.it), an anonymous and automatic database [12]. Immunological markers were tested by flow cytometry. The authors did not have access to information that could identify individual participants during or after data collection.

Ethics

All study participants signed a written informed consent form agreeing to the use of their clinical data, in anonymous form, for scientific purposes. The use of the Ligurian HIV Network database for scientific purposes and all flow cytometry analyses was approved by the Ligurian Ethics Committee (28 August 2013).

Immunological analysis

Cryopreserved peripheral blood mononuclear cells (PBMCs) were thawed, washed, and resuspended at 10^7/ml in RPMI 1640 complete medium supplemented with 10% FBS and 1×10^6 cells/100 μl were seeded per well in a 96-round bottom plate for immunofluorescence analyses. PBMC were incubated with specific fluorochrome-conjugated monoclonal antibodies (mAbs) listed in Supplementary Tables 1–3, http://links.lww.com/QAD/D301. Samples were acquired and analysed using a BD Fortessa X20 flow cytometer (BD Biosciences, San Jose, CA, USA) and BD FACS Diva software version 8.0 (BD Biosciences, San Jose, CA, USA).

Supplementary Table 1, http://links.lww.com/QAD/D301 lists reagents selected to evaluate both $CD4^+$ and $CD8^+$ T-regulatory (Treg) lymphocyte frequencies [13] and markers of exhaustion: Programmed Cell Death protein 1 (PD-1), transmembrane immunoglobulin and mucin domain 3 (TIM-3), and Eomesodermin (Eomes). Supplementary Table 2, http://links.lww.com/QAD/D301 lists reagents for analysing the maturation status of $CD4^+$ and $CD8^+$ T cells by examining the expression of CD45RA and CCR7. Supplementary

Table 3, http://links.lww.com/QAD/D301 lists reagents for studying T-cell activation status based on HLA-DR and CD38 markers. Cytometric data are expressed as percentages relative to the parental population.

Gating strategies for evaluating Treg subsets and T-lymphocyte subpopulations expressing exhaustion or activation markers are shown in Supplementary Figure 1, http://links.lww.com/QAD/D301 (panels a, b and c, respectively).

Statistical analysis

Data were described using mean and standard deviation (SD) for normally distributed continuous variables, median and interquartile range (IQR) for nonnormally distributed continuous variables, and frequency (%) for categorical and ordinal variables. Cellular markers were compared using *t* test or Mann–Whitney *U* test. To identify cellular subsets that best differentiate the study populations, we discarded individuals with missing markers and markers characterized by high variability (coefficient of variation $\geq$1.5) within the subpopulations (AHIV, PHIV, healthy controls). The remaining data were age-corrected before analysis. We performed a feature reduction procedure using a Mann–Whitney *U* test to identify features with the highest intergroup differences (AHIV vs. PHIV) and the lowest intragroup variability. Bonferroni correction was applied. This step led to the selection of significant markers used in subsequent clustering procedures. We performed a k-means cluster analysis [14] on the selected variables to investigate the distribution of PHIV and AHIV individuals and the positioning of healthy controls in an unsupervised manner. The number of ideal clusters was obtained via silhouette analysis with Dice distance. To visualize the clustering result, we performed a principal component analysis (PCA) on the considered variables, a dimensionality-reduction method [15] that uses a combination of the considered features to find a smaller set of privileged directions preserving the maximum amount of information from the initial set of features. This approach facilitates straightforward data visualization and interpretability.

Results

We consecutively enrolled 26 PHIV (13 women and 13 men) and 18 AHIV (7 women and 11 men) with median ages (IQR) of 26 (23.25–30.5) and 28 (26.0–33.0) years ($P = 0.080$), respectively and history of 20 (15.75–23.25) and 2.5 (2.0–4.25) years of ART, respectively. Further characterization of the two study populations is provided in Supplementary Tables 4 and 5, http://links.lww.com/QAD/D301.

Figure 1 shows the distributions of the frequencies of different T-cell subsets based on their activation status, regulatory function, exhaustion condition, and maturation stage in both PHIV and AHIV.

Cluster analysis in individuals who acquired HIV as adult, individuals who acquire perinatal HIV, and healthy controls

By discarding individuals without markers' expression, we obtained data from 25 PHIV, 16 AHIV, and 9 healthy controls (median age 46 years, IQR 35–64). By excluding markers with high coefficients of variation ($\geq$1.5) within subgroups, we identified 33 feasible markers (Supplementary Table 6, http://links.lww.com/QAD/D301). A Mann–Whitney *U* test (Bonferroni-corrected, $P < 10^{-2}$) led to the identification of seven significant markers, listed in Supplementary Table 7, http://links.lww.com/QAD/D301, whose distribution showed at the same time the highest inter-group difference (PHIV and AHIV) and the lowest intra-group variability, after age correction. These variables were used in the subsequent analyses.

In the spider chart in Fig. 2, panel a shows the distribution of the normalized mean values of each variable across the groups (PHIV, AHIV, and heathy controls). A first descriptive analysis indicated that PHIV and healthy controls are indistinguishable from each other by means of the selected variables ($P > 0.1$ for all variables, Bonferroni-corrected).

We then performed a k-means cluster analysis to investigate the distribution of individuals in a data-driven unsupervised manner, using the seven most significant markers differentiating PHIV and AHIV. Silhouette analysis returned $k = 2$ as the ideal number of clusters for the given data distribution. The analysis resulted in two clusters (19 vs. 31 people), with PHIV, AHIV, and healthy controls distributed as shown in the confusion matrix in Supplementary Table 8, http://links.lww.com/QAD/D301.

Figure 2, panel b provides a graphical representation of the clustering result, where individuals are plotted along the two principal dimensions derived from PCA, accounting for 50.8 and 15.3% of data variability, respectively.

Analysing the composition of the two clusters, we observed that the cluster 1 (dots in Fig. 2, panel b, comprising 19 participants) primarily includes AHIV. Conversely, the cluster 2 (triangles in Fig. 2, panel b, comprising 31 participants) mostly contains PHIV and all healthy controls. The analysis showed a specificity of 94% and a sensitivity of 88%.

The spider chart in Fig. 2, panel c, depicts the distribution of the normalized mean values of the considered variables across the groups given by the two clusters (cluster 1 and cluster 2).

Discussion

This study utilized clustering analysis (unsupervised learning for clustering results, and PCA for visualization)

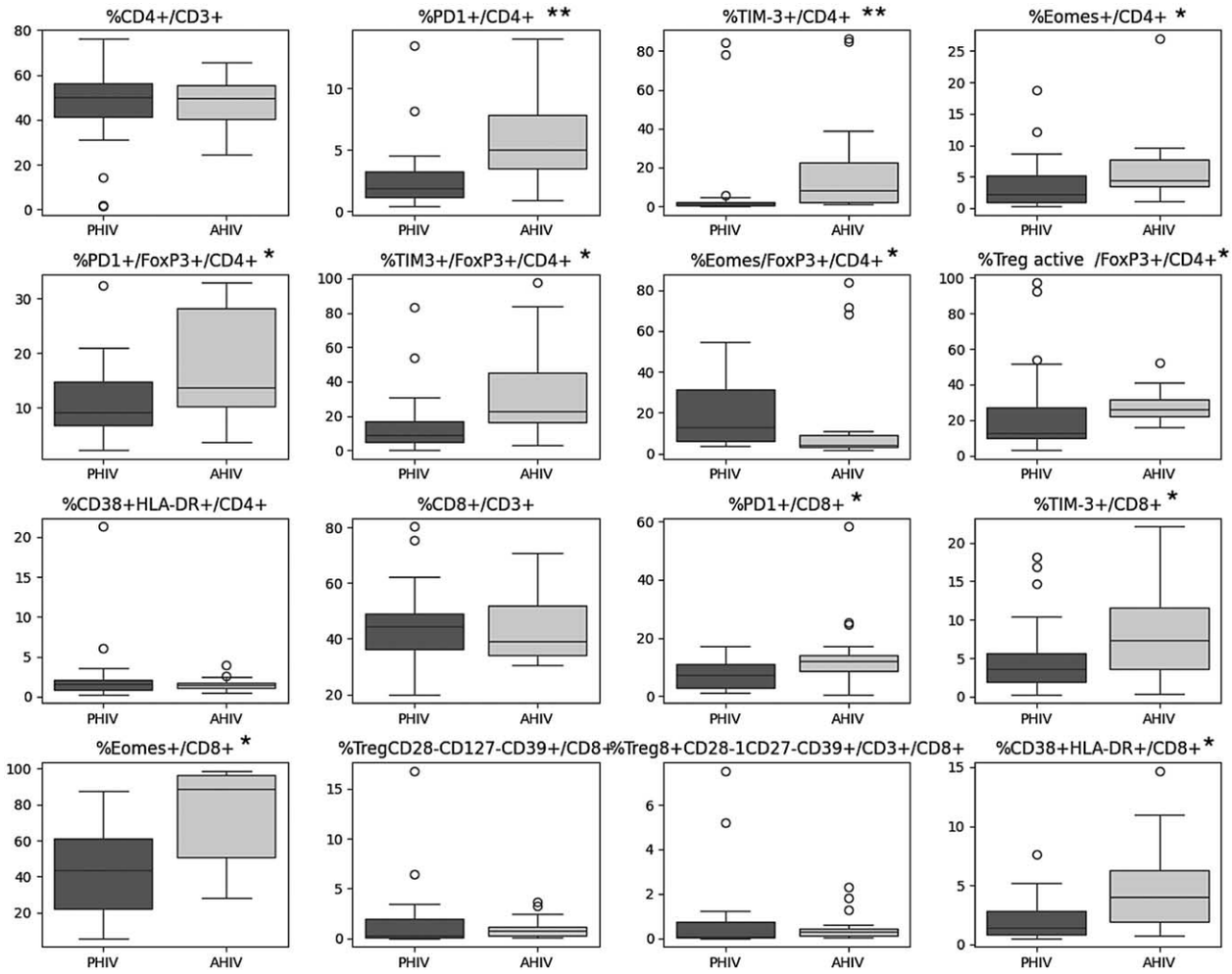


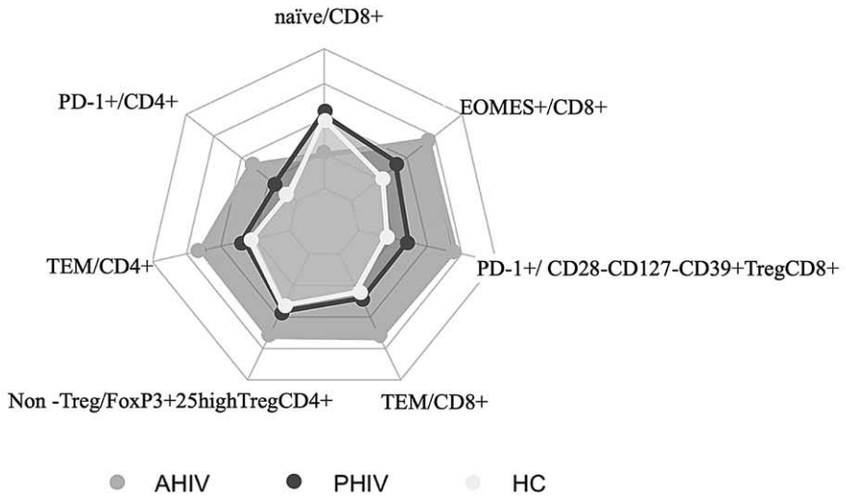
Fig. 1. Boxplots of the expression of immune exhaustion and activation markers on regulatory and total CD4$^+$ and CD8$^+$ T cells, in people who acquire perinatal HIV (dark grey) and in people who acquire HIV when already adult (light grey). The CD4$^+$ T cells are showed from first to ninth panel and the CD8$^+$ T cells from tenth to last panel (left to right, top to bottom). The CD4$^+$ and CD8$^+$ T-cell percentages have been normalized on CD3$^+$ T-cell subset.

to highlight the most relevant immune parameters differentiating these two populations of PWH. This unsupervised separation of AHIV and PHIV/healthy controls allows for the identification of outliers (i.e. misclassified participants) and provides a method to predict the clinical outcomes of future participants.

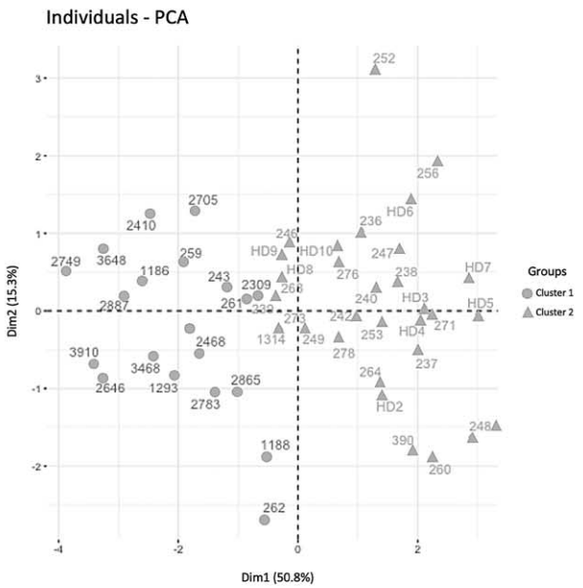
Regarding T-cell activation, marked by CD38$^+$ and HLA-DR co-expression on CD8$^+$ T cells, it serves as a prognostic indicator for disease progression at various stages of HIV [16]. In our cross-sectional study, the percentage of activated CD8$^+$CD38$^+$HLA-DR+ T cells was higher in AHIV than PHIV. Additionally, the levels of exhausted CD4$^+$ and CD8$^+$ T cells expressing PD-1, EOMES and TIM-3 were elevated.

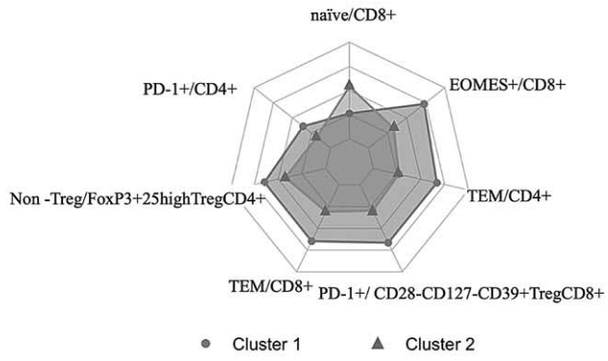
For the Treg compartment, our study focused on the nonantigen-specific CD8$^+$ Treg population, which can be considered as adaptive Treg [13,17,18]. The percentage of CD8$^+$CD28$^-$CD127$^-$CD39$^+$ Treg cells as well as CD4$^+$CD25$^+$FOXP3$^+$ was similar in PHIV and AHIV. Despite viral suppression, immune exhaustion markers on Treg CD4$^+$ and CD8$^+$, increased in individuals with HIV [19,20]. In our study, AHIV consistently exhibited higher expression of exhaustion markers on both CD4$^+$ Treg (PD-1 $P = 0.018$, TIM-3 $P = 0.009$) and CD8$^+$ Treg (PD-1 $P = 0.006$).

Regarding maturational status, our results revealed that individuals with PHIV had significantly higher rates of naive CD4$^+$ and CD8$^+$ cells ($P = 0.043$ and $P = 0.010$, respectively) and lower rates of TEM CD4$^+$ and CD8$^+$ cells ($P = 0.0002$ and $P = 0.041$, respectively) compared with AHIV, and a similar frequency of naive CD8$^+$ T cells compared with healthy controls, suggesting that controlling viral replication can revert the disadvantages observed in the first year of life for children with PHIV [21,22].



Panel A.





Panel B. Panel C.

Fig. 2. Spider chart of the normalized mean values of the seven selected variables (panel a), clustering result illustrated through principal component analysis (panel b) and spider chart of the considered variables in the two clusters (panel c). In panel a, each vertex represents the mean value of the corresponding feature for AHIV patients, in light grey, PHIV patients, in dark grey and healthy control individuals, in white. In panel b, the results of the cluster analysis are presented. Graphical representation given by the first two components resulting from the PCA. Dots represent individuals assigned to cluster 1; triangles represent individuals assigned to cluster 2. In panel c, each vertex represents the mean value of the corresponding feature computed on individuals belonging to cluster 1, dots, and to cluster 2, triangles. This spider chart showed the normalized mean values of the considered features. AHIV, individuals who acquired HIV as adult; PHIV, individuals who acquire perinatal HIV.

The main limitations of our study are its cross-sectional design, and the small number of participants.

In conclusion, these data highlight greater immune activation and the expression of certain immune exhaustion and activation markers in AHIV compared with PHIV. This suggests that recent lack of virological control, rather than the cumulative duration of living with HIV, affects immune activation and exhaustion of $CD4^+$ and $CD8^+$ T cells.

Acknowledgements

Funding: this study was supported by an unrestricted grant from Gilead (ITA Fellowship 2016). The company had no role in designing the study, collecting or analysing the data, interpreting the results or writing or approving the article.

Author contributions – conceptualization, L.T., C.D., D. F., I.C., S.G., C.C., M.P., M.B. and A.D.B. Data curation, L.T., C.D, D.F., I.C. and L.L. Formal analysis, D.F., T.A., A.P., I.C., C.C., S.G., S.M., M.G. and B.B. Methodology, T.A., C.D., I.C., M.P., S.G. and C.C. Project administration, L.T. and A.D.B. Supervision, G.F., M.B. and A.D.B. Validation, D.F., M.P., G.F. and A.D.B. Writing – original draft, L.T., C.D., D.F., I.C. and S.G.

All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

There are no conflicts of interest.

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