T-CELLS HOMEOSTASIS DISTURBANCES IN A COHORT OF LONG-TERM ELITE CONTROLLERS

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I. Background

• Elite controllers (EC) are an exceptional group of people living with HIV (PLWH) able to control HIV replication without antiretroviral therapy and have been proposed as a model of functional HIV cure.

• However, several evidences suggest that this spontaneous control of HIV has a cost in terms of systemic inflammation and immune activation.

• Herein, we have performed a deep phenotypic study to get insight into the T-cell homeostasis disturbances in EC subjects maintaining long-term virologic and immunologic control of HIV (long-term elite controllers; LTEC).

II. Methods

• Three PLWH groups were included: PLWH-LTEC group (long-term elite controllers), PLWH-onART group (non-controllers under successful cART), and PLWH-offART group (non-controllers cART-naïve with replicating HIV) (**Table 1**). A UC group (HIV-seronegative participants) was included as reference of uninfected controls.

• T-cells homeostasis was analysed by spectral flow cytometry using a panel of 22 different T-cells markers. A minimum of 25,000 CD4 and CD8 T cells were acquired.

• Data were analyzed using an omic approach in R software. The analysis included batch correction, tSNE (T-distributed stochastic neighbor embedding) dimensionality reduction and Louvain clustering. Abundance of CD4 and CD8 T-cell clusters was compared between groups by Mann-Whitney test and an adjusted p-value<0.05 was considered as significant.

• Using the levels of the different T-cell clusters, a random forest and a hierarchical clustering analysis were performed as classification methods in order to ascertain the T-cell clusters involved in discriminating between pairs of study groups.

Table 1. Characteristics of patients included in the study

Characteristic	PLWH-LTEC (n=20)	PLWH-onART (n=14)	PLWH-offART (n=9)	p-value
Age (years)	44 [35 – 49]	44 [42 – 49]	43 [36 – 49]	0.266
Sex (% of males)	59	80	100	**0.025**
Years since HIV diagnosis	15 [6 – 20]	12 [5 – 15]	5 [3 – 9]	**0.027**
Years as EC	13 [7 – 16]	NA	NA	NA
Years on ART	NA	6 [3 – 9]	NA	NA
Plasma HIV load (copies/mL)	50	50	57937 [28817 - 80676]	NA
CD4 count (cells/µL)	837 [603 – 1210]	820 [599 – 1127]	625 [518 – 920]	0.200

Data are given as median [Q1-Q3], except sex, expressed as %; statistical differences between the groups were tested by Kruskall-Wallis test and by Chi-square test for sex. Statistical significance was considered for p-values below 0.05 (in bold in the table). NA: not apply.

III. Results

• Dimensionality reduction using tSNE followed by clustering analysis using the Louvain method yielded 57 different cell clusters in the CD4 T cells and 54 different cell clusters in CD8 T cells (**Figure 1**).

• The proportion of each cluster over the total population of CD4 or CD8 T cells was calculated for each study volunteer and a differential abundance analysis was performed, using the UC group as reference. This analysis showed several CD4 and CD8 clusters to be differentially expressed in the PLWH groups compared to UC group (**Figure 2**).

• The PLWH group with the highest number of CD4 and CD8 clusters differentially expressed with respect to UC groups was PLWH-offART (18 CD4 and 14 CD8 clusters). In contrast, PLWH-onART group showed an almost normalized landscape of CD4 and CD8 clusters (only 1 CD4 and 2 CD8 clusters) (**Figure 2**).

• Of note, despite controlled viral replication, PLWH-LTEC group showed several CD4 and CD8 clusters differentially expressed with respect to UC group (15 CD4 and 13 CD8 clusters). Interestingly the profile of clusters alterations in PLWH-LTEC group was quite like that found in PLWH-offART group, with a high level of overlapping between these groups in the clusters differentially expressed with respect to UC group (**Figure 2**). There were, however, some differences in the profile of cluster alterations between PLWH-LTEC and PLWH-offART groups, suggesting the existence of a specific profile associated with the PLWH-LTEC phenotype (**Figure 2**).

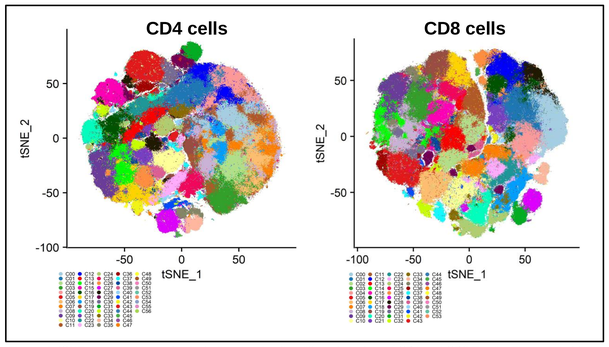

Figure 1. tSNE maps and Louvain clustering showing the different clusters of CD4 and CD8 T cells. The panel at the bottom of each map indicates the color-coding of each cluster.

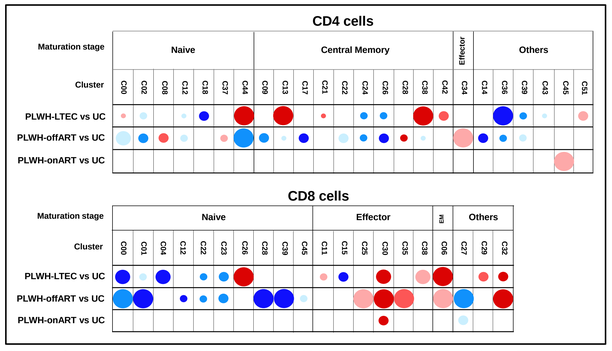

Figure 2. Schematic representation (dot-plot tables) of CD4 and CD8 T cells clusters with significant differences between the PLWH groups and the UC group. Clusters are grouped according to the maturation stage of cells. Each dot in the table represents a significant difference with respect to UC group. The size of the dot indicates the degree of difference in fold-change: <2, 2-3, 3-4, 4-5 and >5; from the smallest to the biggest size. Red colors indicate increase and blue colors indicate decrease with respect to UC group. Level of statistical significance (corrected p-value) is indicated by the color tone: 0.05-0.01, 0.01-0.001, and <0.001; for the light, medium and dark tone respectively. EM: effector memory

• Regarding the phenotype of CD4 clusters differentially expressed in PLWH groups with respect to UC group, increased clusters in PLWH-LTEC group were mainly of central memory phenotype (C13, C21, C38, C42) with high expression of activation and exhaustion markers; in contrast, among the decreased clusters there were clusters of naïve phenotype (C02, C12, C18) and of central memory phenotype (C24, C26) with low level of activation and exhaustion, as well as clusters of pTFH (peripheral T follicular helper) phenotype (C36, C39) and of regulatory T cells (Treg) phenotype (C43). Interestingly, some of these alterations overlapped with the alterations found in PLWH-offART group. Thus, of the 15 clusters altered in PLWH-LTEC, eight of them (53%) were also altered in PLWH-offART. These eight common clusters included naïve (C02, C12) and central memory (C24, C26) clusters with low level of activation and exhaustion, as well as pTFH (C36, C39) clusters; all of them were decreased both in PLWH-LTEC and in PLWH-offART groups. In contrast, increased clusters with respect to UC group were different in PLWH-LTEC (C00, C13, C21, C38, C42, C44, C51) and in PLWH-offART (C08, C28, C34, C37) (**Figure 2 and Figure 3**).

• Regarding the phenotype of CD8 clusters differentially expressed in PLWH groups with respect to UC group, increased clusters in PLWH-LTEC were mainly of effector (C11, C30, C38) or effector memory (C06) phenotype with high expression of exhaustion and activation markers. In contrast, decreased clusters in PLWH-LTEC were mainly of naïve phenotype (C00, C01, C04, C22, C23). As with CD4 clusters, some of the alterations observed in PLWH-LTEC group were also present in PLWH-offART group. Of the 13 clusters altered in PLWH-LTEC, seven of them (54%) were also altered in PLWH-offART group. Clusters C06, C30 and C32 were increased and clusters C00, C01, C22 and C23 were decreased in both groups with respect to UC group (**Figure 2 and Figure 3**).

• Lastly, the PLWH group with ART-mediated control of viral replication (PLWH-onART group) presented a CD4 and CD8 clusters profile very similar to the UC group, with only one CD4 cluster (C45) and two CD8 clusters (C27 and C30) significantly different when compared to UC group (**Figure 2 and Figure 3**).

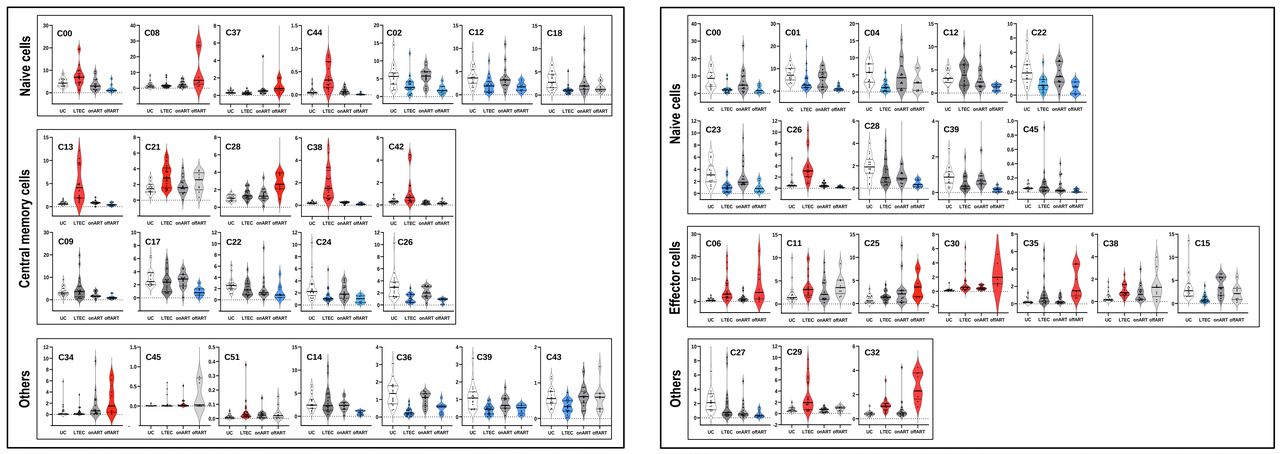

Figure 3. Violin-plots graphs showing the levels (expressed as percentage over total CD4 or CD8 T cells) of different CD4 (left) and CD8 (right) clusters (identified as C plus a number) in the four study groups. Clusters are grouped according to the maturation stage of cells. In each violin-plot graph, blue-colored violins show a statistically significant decrease and red-colored violins a statistically significant increase with respect to UC group.

• As another approach to analyze the differences in the CD4 and CD8 clusters distribution between the PLWH groups and the UC group as reference, a random forest and hierarchical clustering were carried out using the levels of the different CD4 and CD8 clusters.

• Firstly, a Boruta algorithm was applied to select the most important CD4 and CD8 clusters for classification. Using these sets of CD4 and CD8 clusters, a random forest model was generated and applied to discriminate between the different PLWH groups and the UC group (**Figure 4, left**). There was a very good discrimination between PLWH-LTEC or PLWH-offART groups and UC group; whereas discrimination between PLWH-onART group and UC group was poor (**Figure 4, left**). This poor discrimination between PLWH-onART and UC groups is in agreement with the results of the cluster distribution among PLWH groups showing a profile of clusters very similar when comparing PLWH-onART and UC groups (**Figure 3**).

• Secondly, using the sets of CD4 and CD8 clusters selected for the random forest model, a hierarchical clustering was carried out and graphically represented by heatmaps and dendrograms (**Figure 4, right**). The heatmaps showed clear differences in the profile of CD4 and CD8 clusters expression levels when comparing PLWH-LTEC or PLWH-offART groups and UC group. In contrast a much poorer discrimination in the heatmap profile was observed when comparing PLWH-onART and UC groups (**Figure 4, right**).

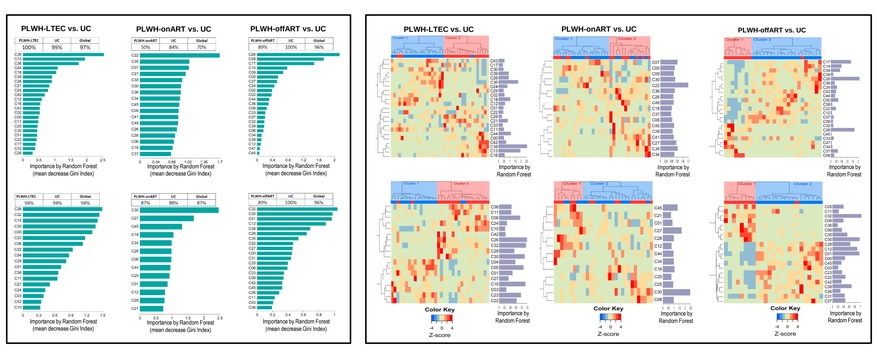

Figure 4. **Left)**: Bar graphs showing the clusters of CD4 cells (top) and of CD8 cells (bottom) selected in the random forest model to discriminate between PLWH groups and UC group. Bars show the importance of each cluster in the model (as estimated by the mean decrease in the Gini Index). Tables on top of each group of bars show the percentage of correct classification of the different study groups by the random forest model. **Right)**: Heatmaps showing the expression level (Z-score) of those CD4 (top) and CD8 (bottom) clusters that best discriminate between PLWH groups and UC group according to the random forest classification model. In the maps, each column represents a study volunteer and each row a cell cluster. Dendrograms on the top of the maps show the clustering of study volunteers with blue color representing UC volunteers and red color PLWH volunteers. Dendrograms on the left of the maps show the grouping of cell clusters. Bars on the right side of the maps show the importance of each cluster in the Random-Forest model of classification.

IV. Funding / Acknowledgements

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V. Conclusions

- Our results suggest that, compared to ART-mediated control of HIV replication, the spontaneous control of HIV is associated with several disturbances in CD4 and CD8 T-cells homeostasis.
- Interestingly, the profile of T-cells homeostasis alterations observed in long-term elite controllers of HIV, partly overlapped with that observed in non-controller patients with high levels of HIV replication, suggesting the presence of low-level HIV replication in the setting of spontaneous control of HIV.
- The impact of these alterations in both the loss of spontaneous HIV control and the state of persistent systemic inflammation need to be further analyzed.