Th17 and Tregs at Early HIV Infection Influence Disease Progression and HIV Specific CD8⁺ Responses

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ABSTRACT

Th17 and Treg subsets have been related to HIV/SIV disease progression. Their role and direct relation with specific HIV-adaptive T-cell responses during primary HIV infection (PHI) are unexplored topics. The aim of this work is to analyze the frequency and balance of Th17 and Treg subsets and the correlation with clinical parameters, immune activation and HIV-specific responses during early infection. For this PBMCs were obtained from 6 healthy donors (HDs) and 28 HIV infected subjects: 6 elite controllers (ECs), 5 chronics (Chs), and 17 PHIs (within 6 months post-infection (mpi)). Th17 and Treg cells (baseline and 8 to 14 mpi samples) were identified as $CD3^+/CD4^+$ IL-17^+ or $CD25^+/FoxP3^+$ (respectively). Data was compared inter/intragroups and correlated to clinical parameters (viral load (VL), CD4 counts), $CD8^+$ $CD38^+/HLA\text{-}DR^+$ activated T-cells and specific anti-HIV $CD8^+$ responses (specifically a viral inhibitory activity (VIA) assay and intracellular cytokine staining (ICS) for IFN-γ production and $CD107_{a/b}$ cytotoxicity markers) measured at baseline and 8 to 14 mpi, using parametric and nonparametric statistics. We observed that chronic patients have lower % of Th17 cells compared to HDs and ECs (p=0.004 and 0.042 respectively), among which frequencies were similar (median 0.14 vs 0.11 respectively). In PHI group, CD4 counts at 8 to 14 mpi directly correlated with baseline Th17 counts (p=0.048, r=0.5). Moreover, VL at 8 to 14 mpi inversely correlated with baseline % of Th17 (p=0.041, r=-0.596). Th17/Tregs ratio was higher for HDs compared to any HIV^+ group (p<0.01), and within PHIs Th17/Tregs was directly associated with CD4 counts at 8 to 14 mpi (p=0.017, r=0.793). Significantly, Th17 and quality of HIV-specific $CD8^+$ responses, previously observed to be protective, were directly associated: higher basal % of Th17 correlated with higher $CD8^+$-mediated VIA at late times post-infection (p=0.004, r=0.829) and Th17 counts at 8 to 14 mpi also related with % of HIV $CD8^+/CD107_{a/b}{}^+/IFN\text{-}\gamma^+$ cells (p=0.024, r=0.847). Regarding immune activation, as expected HDs had the lowest and Chs the highest % of activated T cells compared to the other groups (p<0,05). Of note, in PHIs activation occurs rapidly after infection and diminishes at late times post-infection (p=0,004). Importantly, Th17/Tregs ratio of groups altogether, negatively correlated with activated $CD8^+$ T-cells (p<0.05), suggesting a proper balance is associated with a lower level of immune activation. **Our preliminary results indicate that higher counts and frequency of Th17 and higher Th17/Tregs ratio at early stages of HIV infection associate with slower disease progression in terms of clinical parameters and immune activation. Importantly, preservation of Th17 subset associates with the capacity to exert protective HIV-specific $CD8^+$ responses at later times post-infection.**

AIM

To analyze the frequency and balance of Th17 and Treg subsets and their correlation with clinical parameters, immune activation and HIV-specific responses during early HIV infection.

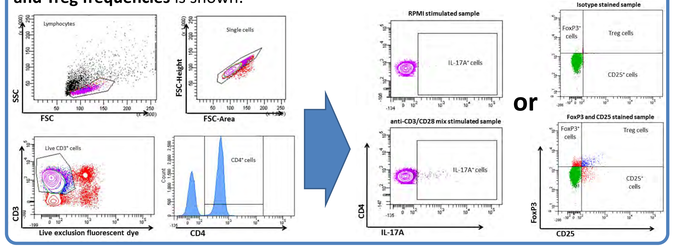
METHODS

Table 1 describes the characteristics of the groups of subjects included in the present study.

Table 1. Groups of individuals: characteristics and inclusion criteria

EXPERIMENTAL GROUPS		
Group	N	Characteristics
Primary HIV infection (PHI)	17	Acute/early HIV infected patients, defined by one of the following diagnosis criteria: i) negative HIV serology followed by a positive one and confirmed by western blott (WB) whithin 6 months; ii) positive HIV serology, indeterminate WB and detectable plasma viral RNA; iii) acute infection simptoms with negative or indeterminate HIV serology and latter infection confirmation. Samples were taken at 1, 2, 3, 6, 9 y 12 months post-infection (mpi). For some analysis this group was sub-divided in PHI<350 and PHI>350 according to if CD4 counts dropped below 350 cells/µl within 12 mpi or not (respectibely).
Elite controllers (ECs)	6	Patients with established HIV infection capable of controlling viremia in the absence of therapeutic intervention. Criteria: Viral load (VL) <50 copies RNA/plasma ml (≥ 8 years, with a minimum of 3 determinations of VL/year), antiretroviral treatment naive, CD4 counts ≥ 500 cells/µl and without history of opportunistic infections.
CONTROL GROUPS		
Chronics	5	Patients HIV infected for more than 1 year, antiretroviral therapy naive and with a VL >4 log_{10} copies RNA/plasma ml.
Healthy donors (HDs)	6	Individuals with negative HIV serology and absence of infection signs.

The **gating strategy used to obtain Th17 and Treg frequencies** is shown:



RESULTS

Importance of Th17 subset and Th17/Tregs ratio in HIV infection: early preservation of Th17 compartment and Th17/Tregs balance correlated with a better immune and virological status at later times post-infection

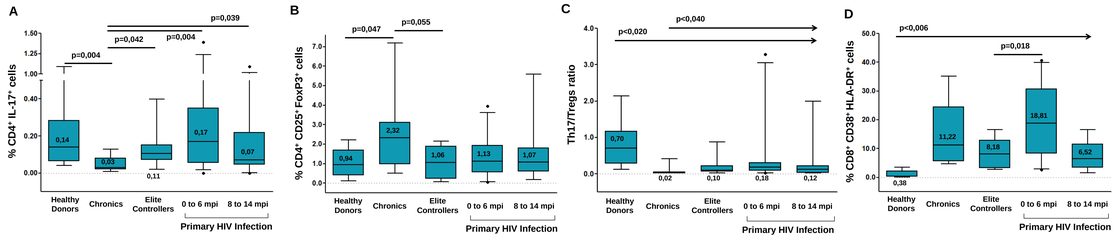


Figure 1. Th17 and Treg frequencies, Th17/Tregs balance and immune activation among HIV infected patients. Cryopreserved PBMCs were thawed and rested at 37ºC over night (ON) in RPMI 10% fetal bovine serum (FBS) to be used the next day. **A)** Intracellular staining of **IL-**17^+ producing cells within $CD3^+$ $CD4^+$ population. PBMCs were stimulated for 6 hours (hs) in the presence of monensin and anti-human CD3/anti-human CD28 mixture (10 ng/ml each) or RPMI 10% FBS (background control). Background subtracted values for each sample are shown. **B)** Intranuclear staining for detection of $FoxP3^+$ cells within $CD3^+$ $CD4^+$ $CD25^+$ population. Positive CD25 and FoxP3 cells were defined comparing each sample with the apropiate isotype controls. **C)** Using Th17 and Tregs frequencies previously obtained, Th17/Tregs ratio was calculated. **D)** Frequency of $CD3^+$ $CD8^+$ cells positive for $CD38^+$ and $HLA\text{-}DR^+$ activation markers was determined by flow cytometry. Positive staining for activation molecules was defined using matched isotype controls for each sample. Boxes indicate median values with 5 and 95 percentiles and dotted outliers. Mann-Whitney test (non parametric) was used for intergroup comparisons, the *p* values obtained are shown. Arrows indicate significant differences between a given group and all the others.

The proportion of Th17 cells in chronics is significantly diminished compared to HDs and ECs, among which frequencies are similar. PHI subjects showed a higher % of Th17 cells at baseline samples compared to chronics, but these values keep falling during the first year post-infection. Regarding the Treg compartment, chronics had higher frequencies of this subset compared to HDs and ECs.
All HIV^+ groups (even ECs) showed a diminished Th17/Treg ratio and high levels of immune activation compared to HDs. The Th17/Tregs balance is severely altered in Chs compared to other HIV^+ groups. In PHI subjects, both immune alterations occur early after HIV infection.

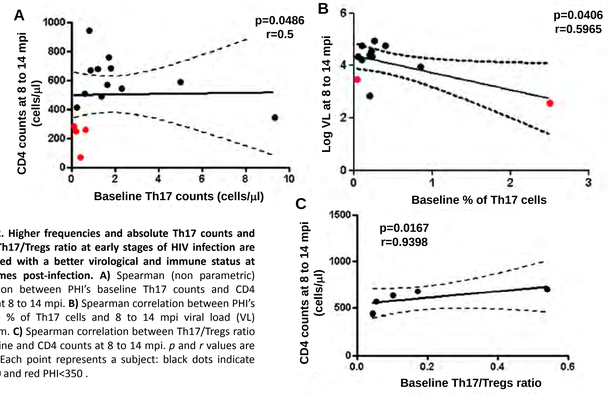


Figure 2. Higher frequencies and absolute Th17 counts and higher Th17/Tregs ratio at early stages of HIV infection are associated with a better virological and immune status at later times post-infection. A) Spearman (non parametric) correlation between PHI's baseline Th17 counts and CD4 counts at 8 to 14 mpi. **B)** Spearman correlation between PHI's baseline % of Th17 cells and 8 to 14 mpi viral load (VL) logarithm. **C)** Spearman correlation between Th17/Tregs ratio at baseline and CD4 counts at 8 to 14 mpi. *p* and *r* values are shown. Each point represents a subject: black dots indicate PHI>350 and red PHI<350 .

Among PHI subjects, higher absolute Th17 counts at early times post-infection positively correlated with higher CD4 T-cell counts at 8 to 14 mpi. In concordance, higher baseline Th17 frequencies inversely correlated with VL at 8 to 14 mpi. Importantly, Th17/Tregs ratio at baseline also positively correlated with CD4 T-cell counts at later times post-infection.

The preservation of Th17/Tregs balance negatively correlated with levels of immune activation

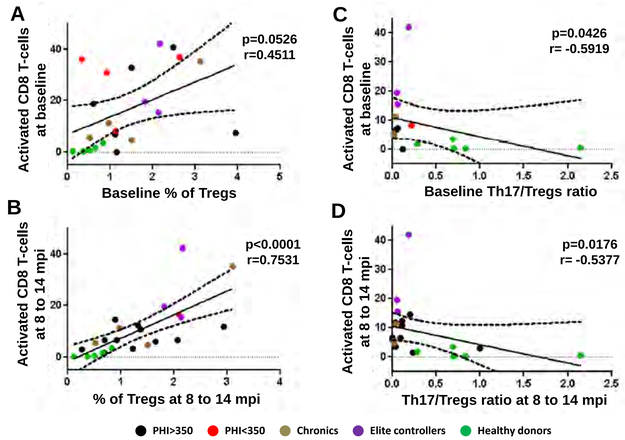


Figure 3. Th17/Tregs ratio and not Tregs frequencies, negatively correlated to levels of CD8 T-cell activation. Spearman (non parametric) correlation between A) % of Tregs and % of $CD38^+$ $HLA\text{-}DR^+$ CD8 T-cells at baseline, B) % of Tregs and % of $CD38^+$ $HLA\text{-}DR^+$ CD8 T-cells at 8 to 14 mpi, C) Th17/Tregs ratio and % of $CD38^+$ $HLA\text{-}DR^+$ CD8 T-cells at baseline, B) Th17/Tregs ratio and % of $CD38^+$ $HLA\text{-}DR^+$ CD8 T-cells at 8 to 14 mpi. *p* and *r* values are shown. Each point represents an individual.

Taking into account groups all together, Th17/Tregs ratios, and not Treg frequencies, correlated with low levels of immune activation in the CD8 T-cell compartment. The positive correlation between Tregs and activation may find its explanation in the inability of Tregs to exert suppressive activity, as reported in the literature .

Higher Th17 counts were correlated with anti-HIV specific $CD8^+$ T-cell responses of higher suppresive and cytotoxic capacities

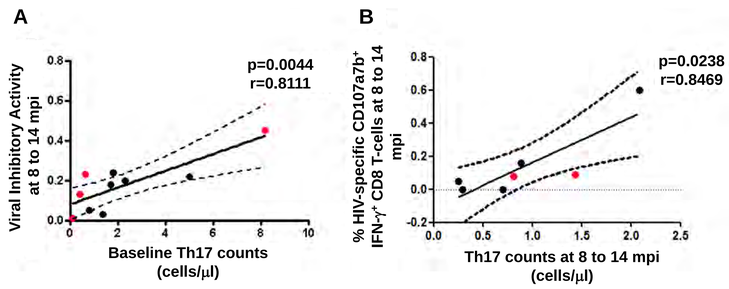


Figure 4. Higher Th17 counts were associated with a better specific celullar anti-HIV response. A) Spearman correlation between basal Th17 counts and inhibitory viral activity index at 8 to 14 mpi. This value was obtained after an *in vitro* assay which consisted in co-culture of previously activated and HIV infected $CD4^+$ T-cells with or without singeneic $CD8^+$ T-cells. After one week the supernatants of the co-cultures from each subject were obtained and p24 was measured by standard ELISA. Details of this methodology and the calculation of the inhibitory viral activity index are available at Turk et al. JVirol 2013, Vol.87 Num.13. **B)** Spearman correlation between Th17 counts at 8 to 14 mpi and % of anti-HIV specific $CD8^+$ T-cells capable of IFN-γ production and cytotoxic activity at 8 to14 mpi. The quality of the anti-HIV specific $CD8^+$ response was obtained by flow cytometry. For this, PBMCs were stimulated for 5 hours in the presence of different HIV peptide pools followed by intracelullar cytokine staining of IFN-γ and $CD107_{a/b}$ (degranulation marker). Details of this methodology can be found at Turk et al. 2013 JVirol. *p* and *r* values are shown. Each point represents a subject, black indicates PHI>350 and red PHI<350.

PHI subjects showed a positive correlation between absolute Th17 counts at baseline samples and $TCD8^+$ capacity to exert inhibitory viral activity. On the other hand, higher Th17 counts at later times post-infection correlated with a higher percentage of anti-HIV specific $CD8^+$ T-cells capable of secrete IFN-γ and degranulate ($CD107_{a/b}$, cytotoxicity marker), **both features positively correlated with better disease control during HIV infection** (Turk et al. JVirol 2013, Vol.87 Num.13).

CONCLUSIONS

Higher counts and frequency of Th17 and higher Th17/Tregs ratio at early stages of HIV infection associate with slower disease progression in terms of clinical parameters and immune activation.

Importantly, preservation of the Th17 subset associates with the capacity to exert protective HIV-specific $CD8^+$ responses at later times post-infection.

This novel results support further investigation.