

T Cell Activation but Not Polyfunctionality after Primary HIV Infection Predicts Control of Viral Load and Length of the Time without Therapy

Andrea Cossarizza^{1*}, Linda Bertonecelli¹, Elisa Nemes¹^{‡a}, Enrico Lugli¹^{‡b}, Marcello Pinti², Milena Nasi¹, Sara De Biasi¹, Lara Gibellini¹, Jonas P. Montagna¹, Marco Vecchia¹, Lisa Manzini¹, Marianna Meschiari¹, Vanni Borghi³, Giovanni Guaraldi^{3,4}, Cristina Mussini^{1,3}

1 Department of Surgery, Medicine, Dentistry and Morphological Sciences, University of Modena and Reggio Emilia, Modena, Italy, **2** Department of Life Sciences, University of Modena and Reggio Emilia, Modena, Italy, **3** Infectious Diseases Clinics, Azienda Ospedaliero-Universitaria Policlinico di Modena, Modena, Italy, **4** Department of Medical and Surgical Sciences of Mother, Child and Adult, University of Modena and Reggio Emilia, Modena, Italy

Abstract

Objective: Immune changes occurring after primary HIV infection (PHI) have a pivotal relevance. Our objective was to characterize the polyfunctionality of immune response triggered by PHI, and to characterize immune activation and regulatory T cells, correlating such features to disease progression.

Patients and Methods: We followed 11 patients experiencing PHI for 4 years. By polychromatic flow cytometry, we studied every month, for the first 6 months, T lymphocyte polyfunctionality after cell stimulation with peptides derived from HIV-1 gag and nef. Tregs were identified by flow cytometry, and T cell activation studied by CD38 and HLA-DR expression.

Results: An increase of anti-gag and anti-nef CD8+ specific T cells was observed 3 months after PHI; however, truly polyfunctional T cells, also able to produce IL-2, were never found. No gross changes in Tregs were present. T lymphocyte activation was maximal 1 and 2 months after PHI, and significantly decreased in the following period. The level of activation two months after PHI was strictly correlated to the plasma viral load 1 year after infection, and significantly influenced the length of period without therapy. Indeed, 80% of patients with less than the median value of activated CD8+ (15.5%) or CD4+ (0.9%) T cells remained free of therapy for >46 months, while all patients over the median value had to start treatment within 26 months.

Conclusions: T cell activation after PHI, more than T cell polyfunctionality or Tregs, is a predictive marker for the control of viral load and for the time required to start treatment.

Citation: Cossarizza A, Bertonecelli L, Nemes E, Lugli E, Pinti M, et al. (2012) T Cell Activation but Not Polyfunctionality after Primary HIV Infection Predicts Control of Viral Load and Length of the Time without Therapy. PLoS ONE 7(12): e50728. doi:10.1371/journal.pone.0050728

Editor: Clive M. Gray, University of Cape Town, South Africa

Received: August 9, 2012; **Accepted:** October 24, 2012; **Published:** December 7, 2012

Copyright: © 2012 Cossarizza et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This study was partially supported by "Programma Nazionale di Ricerca sull'AIDS 2009–2010 – Istituto Superiore di Sanità", Rome, Italy (grant 40H71 to AC). No additional external funding received for this study. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: andrea.cossarizza@unimore.it

‡ These authors contributed equally to this work.

‡a Current address: University of Cape Town, Cape Town, South Africa

‡b Current address: National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland

Introduction

Primary infection with the human immunodeficiency virus type-1 (HIV) is a crucial moment for establishing relationships between virus and host [1,2,3]. The high plasma viral load (pVL) causes a relevant and persistent immune activation that can trigger apoptosis [6–8], and becomes chronic in the absence of a valid immune response or without efficient antiretroviral therapy. The immune activation present in this phase is recognizable by typical changes [4], such as an increase in activated/memory CD8+ T cells that express CD38, CD45RO, human leukocyte antigen-DR, and high amounts of cell adhesion molecules, and which can

represent most part of circulating lymphocytes; a decrease in CD4+ T cells is not always present. High plasma levels of proinflammatory cytokines have been described, along with changes in mitochondrial functionality, augmented tendency to apoptosis and expression of cell death markers (such as CD95) in almost all white blood cells [5,6,7]. However, no gross alterations in V β T-cell repertoire have been found, and the functionality of the T cell repertoire seems well preserved [8]. In turn, immune activation can promote viral replication, so facilitating the infection of other T cells [9,10]. Several studies, including those in animal models, where primary infection has been experimentally induced and strictly monitored, showed that a strict

correlation exists between immune activation and progression of the infection [11].

During PHI, the appearance of virus-specific cytotoxic T lymphocytes (CTL) coincides with the decay of viral replication, so that patients with a high frequency of HIV-specific CTL display a low pVL and a slow decrease in CD4+ T cell count [12,13]. A significant direct association between the frequency of CD8+ gag-specific T cells and the length of AIDS-free period has been observed during chronic infection [14]. Specific T helper cells are crucial for the anti-HIV immune response, since they provide help to B and CD8+ cells. A recent study in SIV-infected macaques has shown that depleting CD4+ during PHI worsens the infection [15].

HIV preferentially infects HIV-specific CD4+ lymphocytes [16]. The efficacy of a specific immune response is due to CD4+ and CD8+ T cell clones with multiple effectors functions, such as production of different cytokines and chemokines, activity of costimulatory molecules, capacity to perform degranulation and to express cytotoxic molecules (e.g., perforin) [17,18]. These cells, defined “polyfunctional”, are present at relatively low frequency in HIV+ patients, but at high frequency in the blood of patients who control the virus, such as long term non progressors (LTNPs) or “elite controllers”, where the presence of HIV-specific polyfunctional CD8+ lymphocytes is associated with spontaneous control of viral replication [19,20,21,22]. Very few data exist on the polyfunctionality of T cells immediately after primary infection [23], and we were interesting in investigating this aspect in a longitudinal manner.

Regulatory T cells (Tregs) have a crucial importance, being a viral reservoir, as shown by the presence of HIV-DNA in resting CD4+ Tregs from patients assuming HAART [24]. However, their role during the infection remains unclear. CD4+ Tregs might be important for the reduction of immune activation after PHI or even in chronic infection [25]. During chronic infection they could cause the deregulation of HIV-specific response [26], so favoring the progression of the infection, and a decrease of such cells has been associated to an increase in CD4+ and CD8+ specific responses to the virus. In chronically infected HIV+ patients, increased proportions, but reduced absolute numbers of circulating Tregs were found, and Treg frequency was largely normalized by HAART [27].

Thus, in order to identify some crucial immunological events that occur during PHI, we analyzed specific response to viral antigens such as gag and nef, regulatory CD4+ T cells, and T cell activation in a group of patients who experienced a well documented PHI, and have been followed for more than 4 years. Our main finding is that T cell activation after PHI, more than T cell polyfunctionality or the presence of Tregs, could be considered as a predictive marker for the viral setpoint and time required to treatment.

Materials and Methods

Patients

This longitudinal study enrolled 11 patients (9 males) experiencing PHI, who have been followed by the Infectious Diseases Clinics, University Hospital, Modena (Northern Italy). Median age of patients at enrolment was 37 years (range: 20–56); 7 acquired the infection through homosexual intercourses, 4 were heterosexual. All patients had acute PHI documented by positive ELISA and undefined Western Blot, and were in Fiebig stage III [28]. The date of infection was estimated as about 1 month before undetermined Western Blot or 2 weeks before symptoms onset.

In these patients, clinical events who took patients to the clinical observation were: syphilis (1 case), gonorrhoea (1), diarrhoea (1), candidiasis (1). Furthermore, one had gallbladder stones, another

psoriasis; such pathologies were not considered related to HIV infection. All patients came to the medical observation and HIV testing because they realized to have had a risk because of unprotected sexual intercourses, that occurred few weeks before their first visit.

At enrolment, median plasma viral load (VL) was 305,943 copies/mL, median CD4+ T cell count was 816 cells/ μ L. Viro-immunological parameters (standard CD4+ T cell count and quantification of VL) were performed in untreated patients up to 48 months from PHI (specifically at 12, 24, 36, 48 months) or up to the start of therapy. Chiron branched-DNA was used for plasma HIV RNA, and a value below 50 copies/mL was considered undetectable. Immunological analyses were performed in the first (M1), second (M2), third (M3), fourth (M4) and sixth (M6) month after infection. The different length of the observation period, during which no patients took antiretroviral therapy, was due to different time of enrollment; the longer period of observation in the survival analysis is due to the fact that during the time required to perform the analyses here described patients continued to be followed.

The study has been conducted according to Declaration of Helsinki principles, and approved by the Modena University Review Board. All patients gave written informed consent for the studies here described, according to the Italian laws.

Samples

Peripheral blood mononuclear cells (PBMC) were purified from EDTA-treated whole blood using Ficoll gradient [29], and cryopreserved according to standard procedures [30]. Thawed PBMC were immediately divided in two aliquots: the first part was stained for phenotype analysis; cells in the second part were rested at least 4 hours at 37°C, in a 5% CO₂ incubator, in complete RPMI medium [RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), and 1% of each L-glutamine, sodium pyruvate, non-essential amino acids and antibiotics; all obtained from Invitrogen, Carlsbad, CA] before stimulation.

PBMC stimulation

After resting and washing, 2×10^6 cryopreserved PBMC were incubated overnight in presence of a pool of 15-mer peptides overlapping by 11 amino acids (obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH; final concentration was 2 μ g/mL/peptide) spanning the sequence of HIV-1 gag (123 peptides) and nef (49 peptides), consensus sequence B. For each sample 0.5×10^6 cells were left unstimulated as negative control and for each experiment another 0.5×10^6 cells were stimulated with 1 μ g/mL *Staphylococcus aureus* enterotoxin B (SEB, Sigma-Aldrich, St. Louis, MO) as positive control. All samples were incubated in presence of the secretion inhibitors monensin (2.5 μ g/mL; Sigma-Aldrich) and brefeldin A (5 μ g/mL; Sigma-Aldrich), the costimulatory monoclonal antibodies (mAb) anti-CD28 (1 μ g/mL, R&D Systems, Minneapolis, MN) and anti-CD49d (1 μ g/mL, Serotec, Oxford, UK); anti-CD107a mAb conjugated with PE-Cy5 (BD Biosciences, San José, CA) was simultaneously added to detect degranulation [21].

Flow cytometry analysis

Different mAb directly conjugated with different fluorochromes, obtained from eBioscience (San Diego, CA) (anti-CD154-FITC, anti-IL-2-PE, anti-IFN- γ -PE-Cy7, anti-CD4-APC-Alexa 750, anti-HLA-DR-PE-Cy7, anti-CD38-PE), R&D Systems (anti-CD8-APC) and Serotec (anti-CD3-Alexa 405) were pre-titrated with the appropriate buffer before use to identify the optimal combinations and concentrations [31].

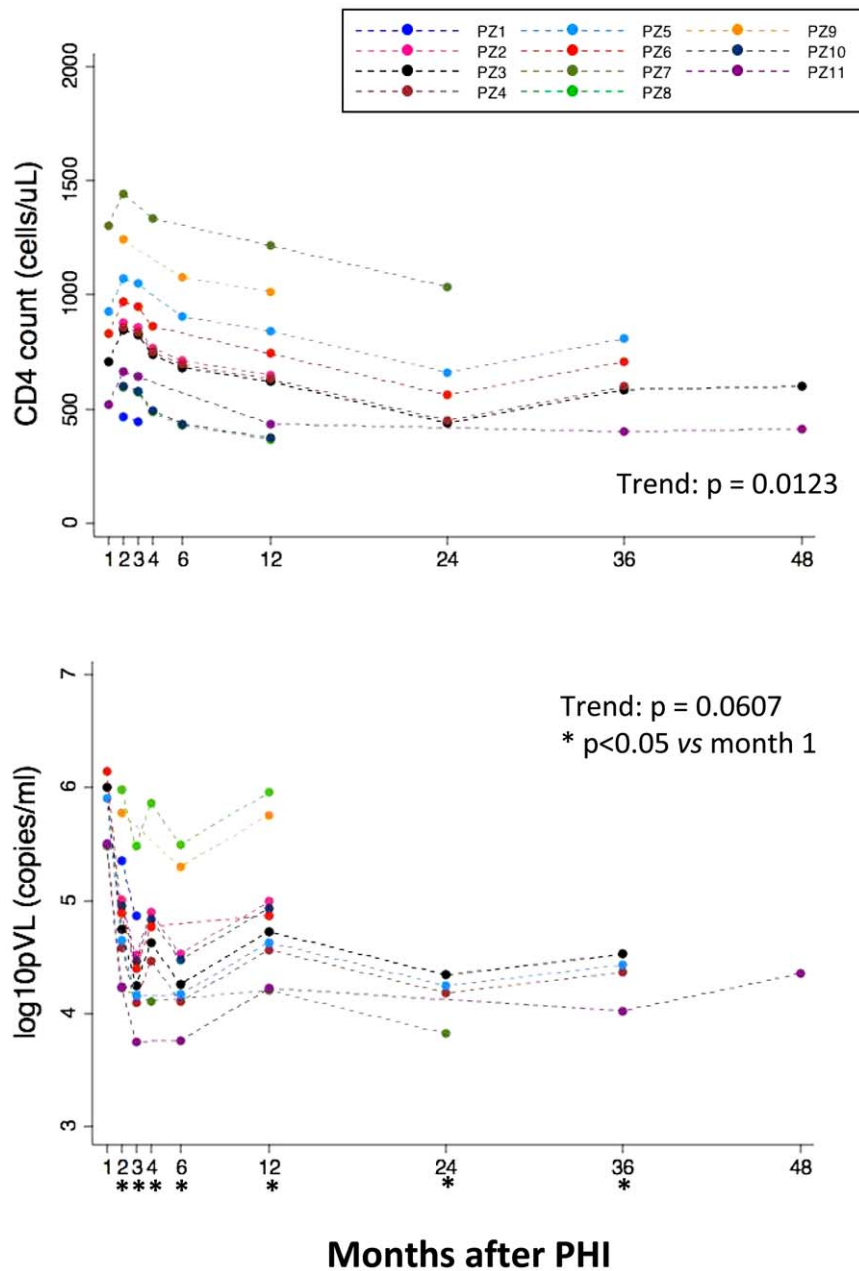


Figure 1. Kinetics of changes in CD4+ T cell count (cell/ μ L blood, upper panel) and plasma viral load (pVL, number of copies/mL blood, lower panel) after primary HIV infection. Each patients is represented by a different colour.
doi:10.1371/journal.pone.0050728.g001

Cells were stained with the LIVE/DEAD Red Stain Kit (Molecular Probes, Eugene, OR) and with different mAb for surface antigens, incubated for 20 minutes at room temperature and washed with PBS containing 5% FBS and 5 mM EDTA. Cells were fixed and permeabilized with the “Cytofix/Cytoperm buffer set” from Becton Dickinson for intracellular cytokine detection or with the “Foxp3 Fixation/Permeabilization” kit from eBioscience prior to quantify the intracellular FoxP3 detection. Samples were finally fixed in PBS+1% paraformaldehyde, kept at 4°C.

A multilaser CyFlow ML flow cytometer (Partec GmbH, Münster, Germany) was used to acquire the samples, and the data were analyzed using FloMax (Partec) and FlowJo v8.8.6

(Tree Star Inc., Ashland, OR, USA) softwares. Single staining and “Fluorescence Minus One” (FMO) controls were performed, and gates defining the positive and negative expression of cell surface antigens were combined by boolean gating strategy, as described [32]. Simplified Presentation of Incredibly Complex Evaluations (SPICE) software (kindly provided by Dr. Mario Roederer, Vaccine Research Center, NIAID, NIH) was used to graphically depict polychromatic flow cytometry data [33]. For T cell function analysis, we put a threshold of 0.02% on the basis of the distribution of negative values generated after background subtraction, with a minimum of 10 events [34].

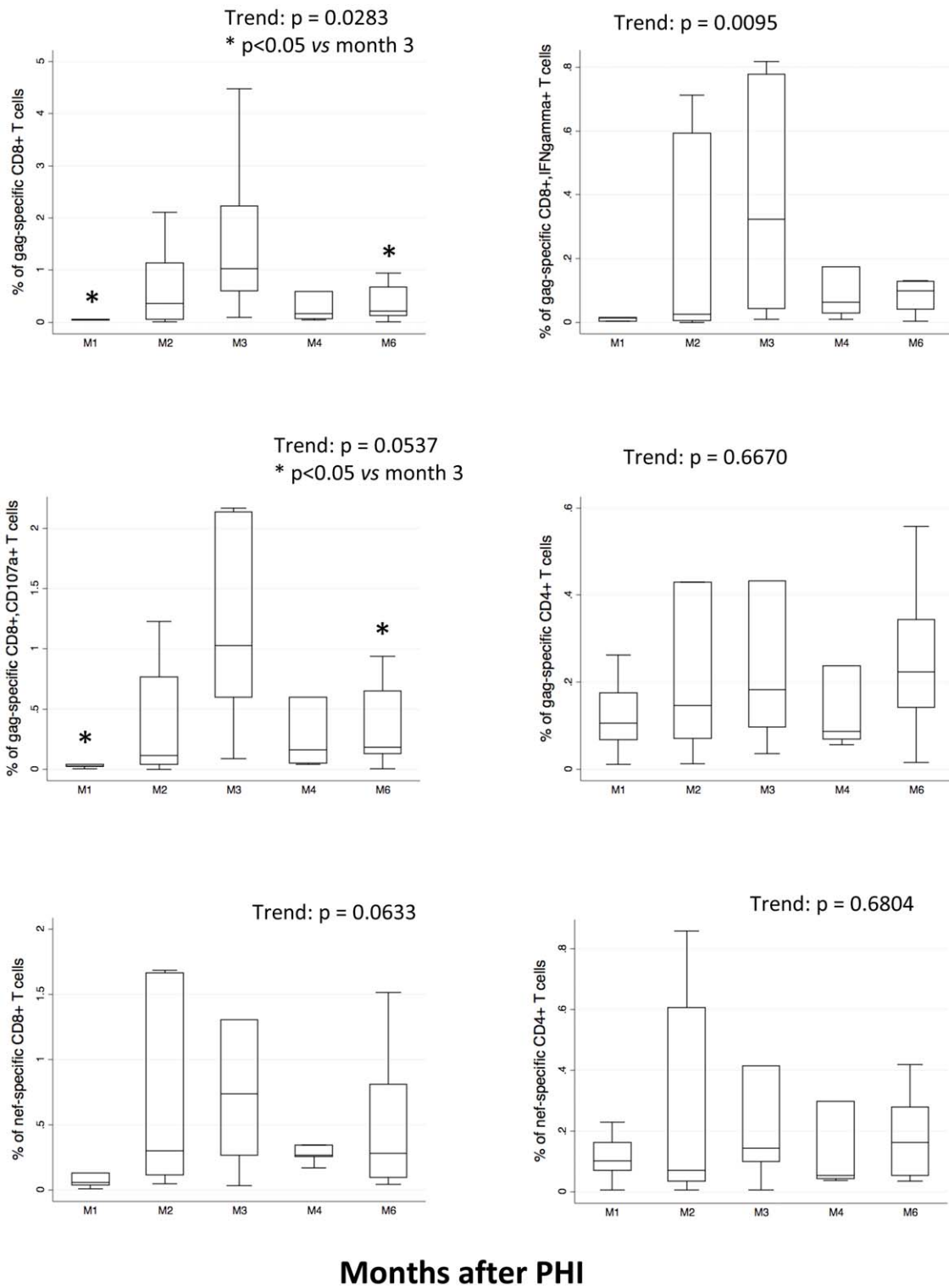


Figure 2. Trends of CD8+ T and CD4+ T cell response to gag- and nef-derived peptides. Boxes indicate median values with 25th and 75th percentiles, whiskers show minimum and maximum. Figure shows the total response to viral peptides, *i.e.*, the sum of all cells positive for at least one of the markers studied (IL-2, IFN- γ , CD154 and CD107a). Patients were studied at month 1 (M1), month 2 (M2), month 3 (M3), month 4 (M4), and month 6 (M6) after PHI. The values of nonparametric analysis of variance (Skillings-Mack, and p values) are reported in figures. Stars in graphs indicate the significant differences of pairwise comparisons between the indicated months, performed by Tukey-Kramer test.
doi:10.1371/journal.pone.0050728.g002

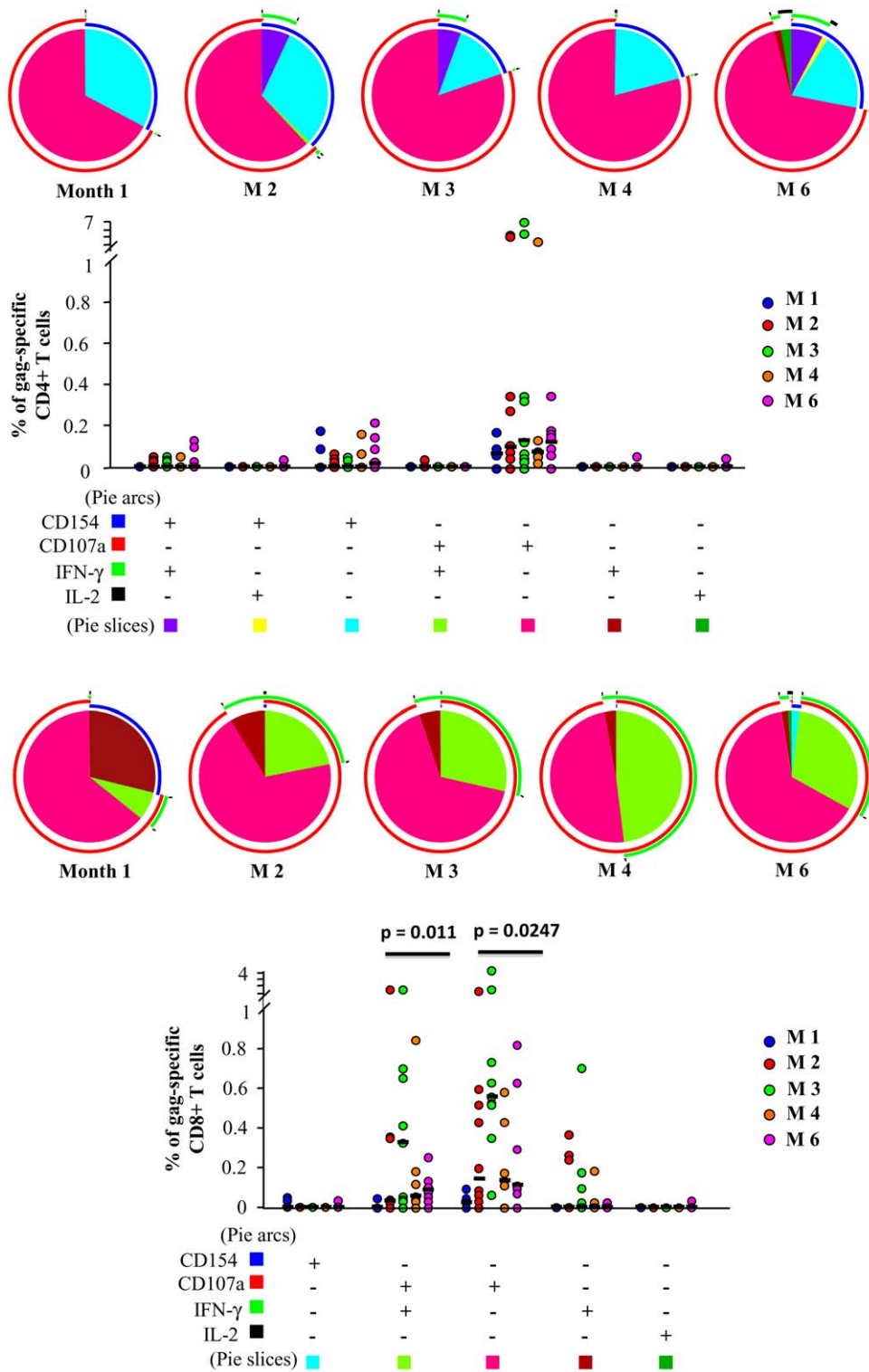


Figure 3. Characterization of the T cell response to gag-derived peptides. Pie charts show the qualitative composition of total gag-specific CD4+ or CD8+ T cell response; each pie slice represents the mean proportion of the total CD4+ T cell response contributed by a single functional pattern, as indicated in the bottom legend. Arcs designed outside the pies represent the fraction of total cells expressing a particular marker, irrespectively of the positive or negative expression of other markers (blue: CD154; red: CD107a; green: IFN- γ ; black: IL-2).
doi:10.1371/journal.pone.0050728.g003

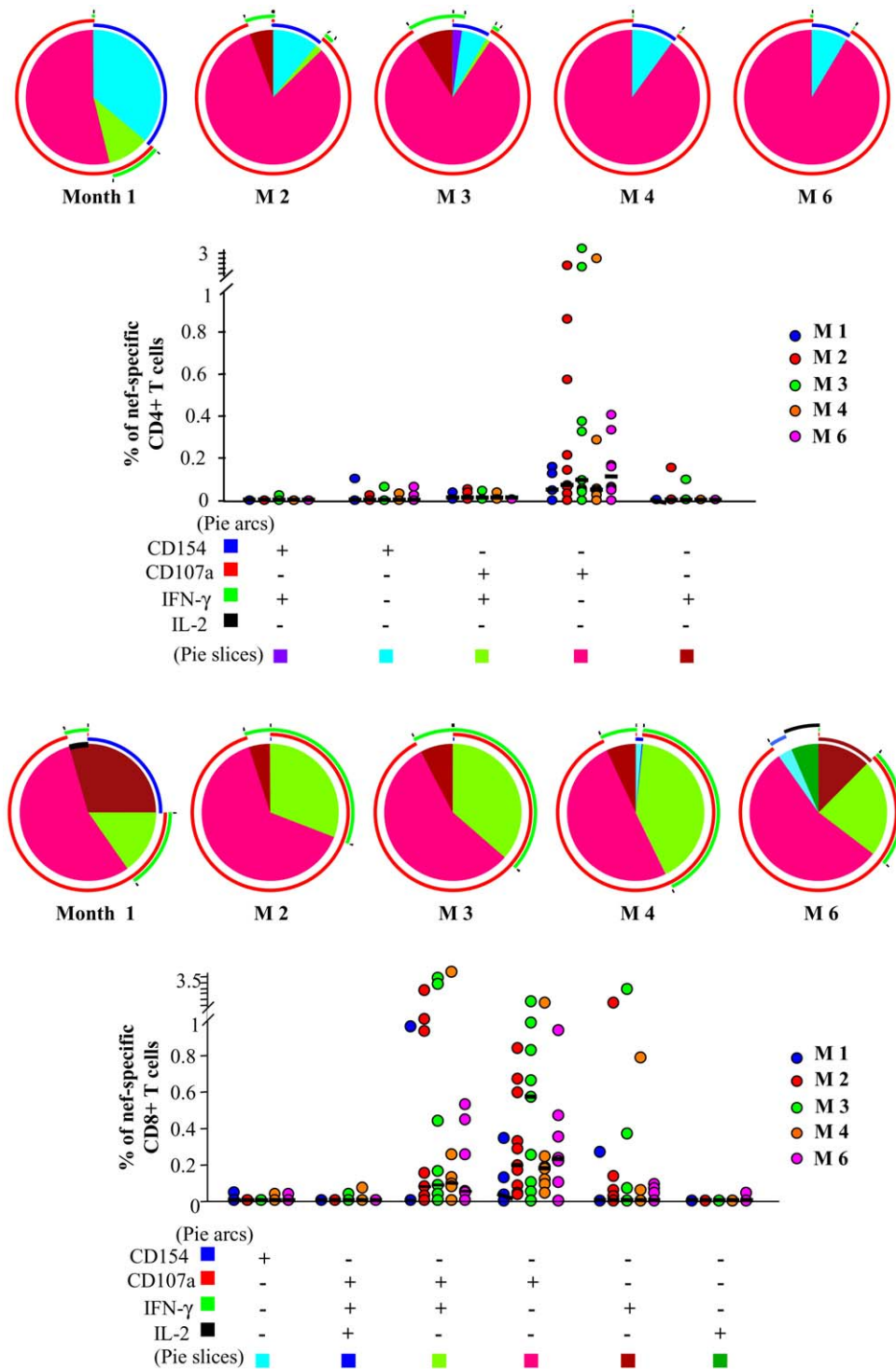


Figure 4. Characterization of the T cell response to nef-derived peptides. Pie charts show the qualitative composition of total gag-specific CD4+ or CD8+ T cell response; each pie slice represents the mean proportion of the total CD4+ T cell response contributed by a single functional pattern, as indicated in the bottom legend. Arcs designed outside the pies represent the fraction of total cells expressing a particular marker, irrespectively of the positive or negative expression of other markers (blue: CD154; red: CD107a; green: IFN- γ ; black: IL-2). doi:10.1371/journal.pone.0050728.g004

Statistical analyses

The time-dependent behavior of T lymphocyte activation, Tregs and gag- or nef-specific responses were analyzed by the nonparametric analysis of variance using the Skillings-Mack test to address the presence of missing data. Tukey-Kramer test

was used for pairwise comparisons between the months. Differences were considered statistically significant when $p < 0.05$. Linear regressions were performed to investigate the associations between viro-immunological parameters (CD4+ T cell count and pVL), time without therapy and CyFlow data.

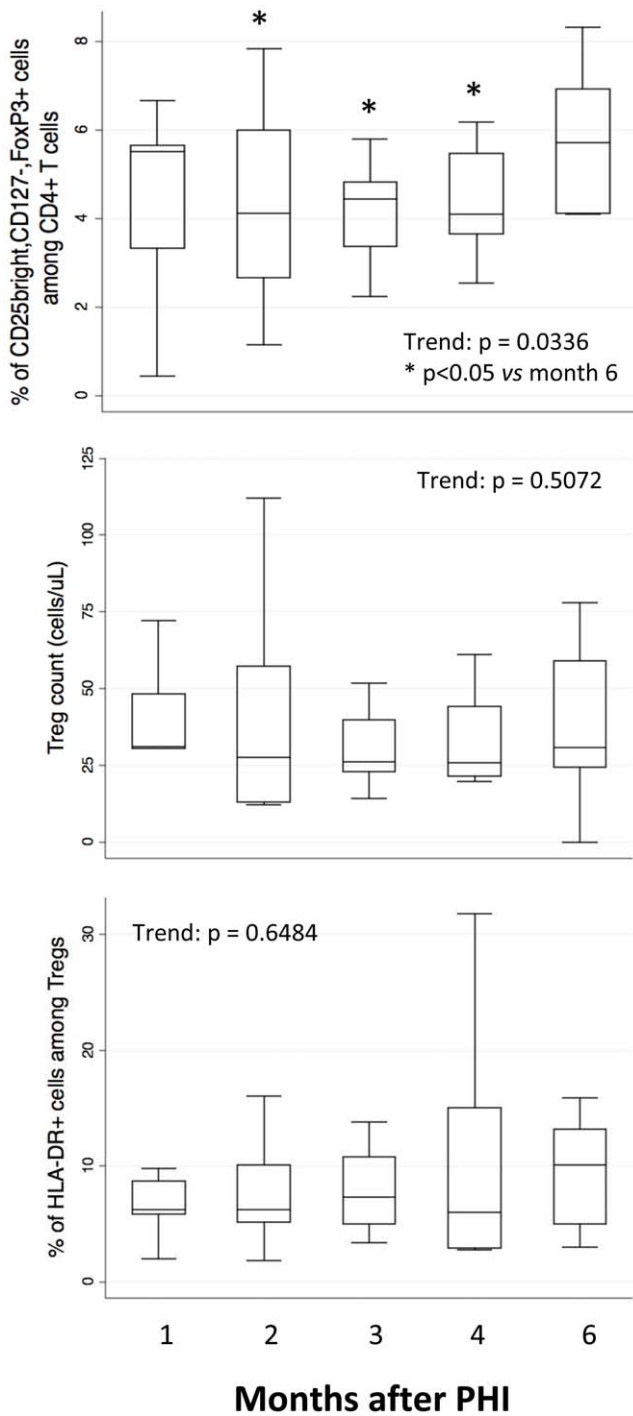


Figure 5. Trends of the Treg frequency (upper panel), absolute number (middle panel) and activated Treg (lower panel), in the patients under investigation. Tregs were considered as those cells that were CD3+,CD4+, positive to FoxP3, highly positive (*i.e.*, bright) to CD25 and negative to CD127. Boxes indicate median values with 25th and 75th percentiles, whiskers show minimum and maximum. doi:10.1371/journal.pone.0050728.g005

Survival analysis and Cox proportional hazards model were also performed. STATA 11 for Mac (College Station, TX) was used for performing statistical analyses and obtaining part of the graphics.

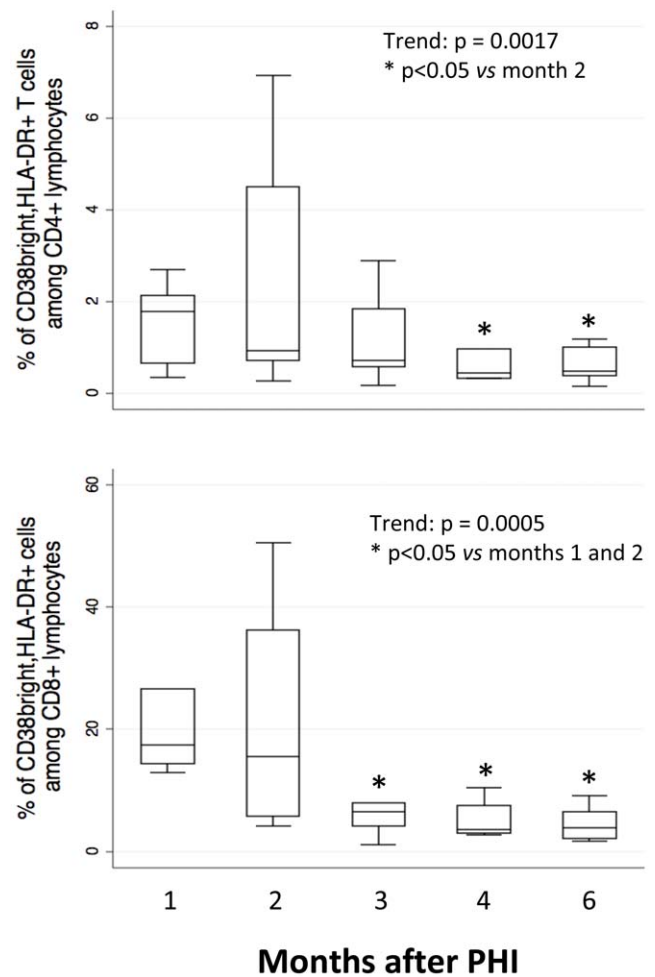


Figure 6. Percentages of activated CD4+ (upper panel) and CD8+ (lower panel) T cells. Activated cells were considered those expressing high levels of CD38 (CD38 bright) and MHC class II (HLA-DR) molecules. Boxes indicate median values with 25th and 75th percentiles, whiskers show minimum and maximum. doi:10.1371/journal.pone.0050728.g006

Results

PHI cohort and viro-immunological parameters

CD4+ T cell count and pVL were monitored in PHI patients from the first month up to 4 years after infection. It has to be underlined that the number of missing data (in terms of analysis not performed or data not reliable for technical problems), even if carefully considered by the statistical methods we used, was negligible in all cases, *i.e.* <2%. Figure 1, upper panel, shows that CD4+ T cell count increased during the first three months, with a gradual decline in following months ($p = 0.0123$). In parallel, we observed a decrease in plasma viral load ($p = 0.0607$) until the onset of a stabilization of plasma viral load by the second month of infection (Figure 1, lower panel). Indeed, at months 2, 3, 4, 6, 12, 24 and 36, viral load was significantly reduced in comparison to M1.

CD107a expression dominates CD4 gag- and nef- specific response

We studied CD4+ and CD8+ T lymphocyte specific polyfunctional response to gag and nef peptides, considering the production or expression of molecules such as IFN- γ , CD107a, CD154 and IL-2. We identified both the “total” response, *i.e.* the sum of all cells

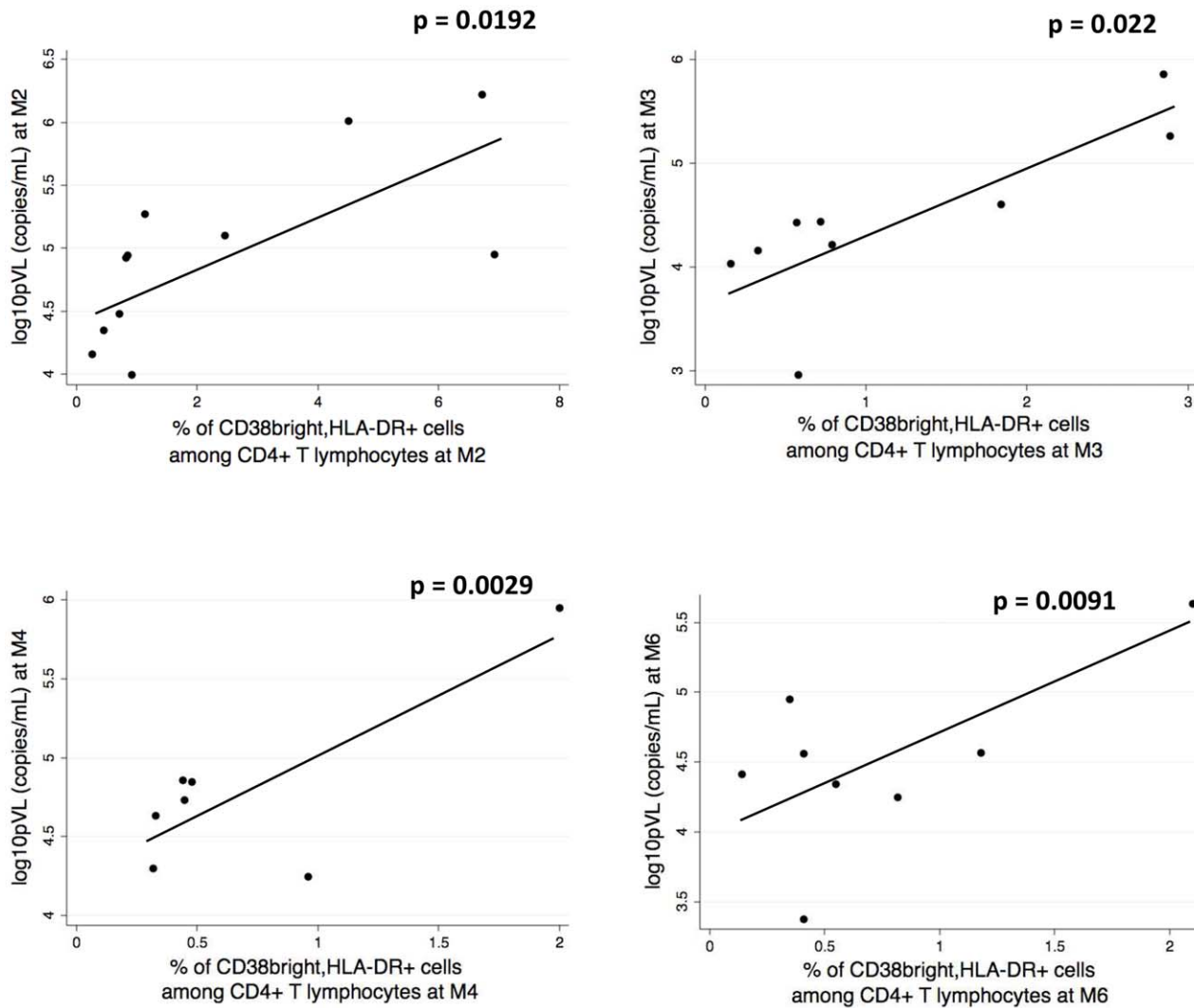


Figure 7. Correlation between the level of CD4⁺ T cell activation status and pVL levels, at all months analyzed. Activated cells were identified as described in the legend to Figure 6.
doi:10.1371/journal.pone.0050728.g007

positive for at least one marker (that provides the overall “frequency” of responding cells among T lymphocytes), and the “qualitative” response, which describes the contribution of each functional pattern to the total specific response. Figure 2 shows a significant change over time in the percentage of total CD8⁺ gag-specific cells, and indicates that CD8 response was higher at M3 than at M1 or M6. The same trend was found considering those gag-specific CD8⁺ T lymphocytes that produced IFN- γ , or those that expressed CD107a. We could not detect any significant variation in the percentage of gag-specific CD4⁺ T cells, or in the percentages of both nef-specific CD4⁺ and CD8⁺ T cells over time.

Regarding the quality of T cell specific response to gag (Figure 3, upper part), we found that at all the time points >50% of gag-specific CD4⁺ T lymphocytes were CD107a⁺; only a low percentage was CD154⁺ or CD154⁺,IFN- γ ⁺. IL-2 production was detectable only at M6, and in a negligible amount of cells. Figure 3, lower part, shows that gag-specific CD8⁺ T lymphocytes were predominantly CD107a⁺, and many of them also produced IFN- γ at all time points. IL-2 production was almost never detected. A significant trend over time was observed in both CD107a⁺,IFN- γ ⁺ ($p = 0.0011$) and CD107a single positive

($p = 0.0247$) CD8⁺ T cells, with an increase at M2 and M3 and a reduction in the following months.

Figure 4 (upper part) shows that also nef-specific CD4⁺ T cell response was characterized a relevant expression of CD107a; only a small amount of cells were able to express CD154 or to produce IFN- γ ; IL-2 production was almost never detected. The nef-specific CD8⁺ T cell response (Figure 4, lower part) was similar to that observed for gag: a large proportion of cells were CD107a⁺ and/or IFN- γ ⁺, while a negligible amount of cells expressed CD154 or produced IL-2.

Treg frequency returns to baseline level 6 months after HIV infection

We analyzed the frequency and absolute number of Tregs, defined as CD3⁺,CD4⁺,CD25⁺,CD127⁻,FoxP3⁺ cells. As shown in Figure 5, the frequency of CD4⁺ T cell with regulatory phenotype increased over time (upper panel). However, the absolute number of Treg did not change significantly (middle panel), as well as the amount of Treg showing an activated phenotype (*i.e.*, those expressing HLA-DR, lower panel).

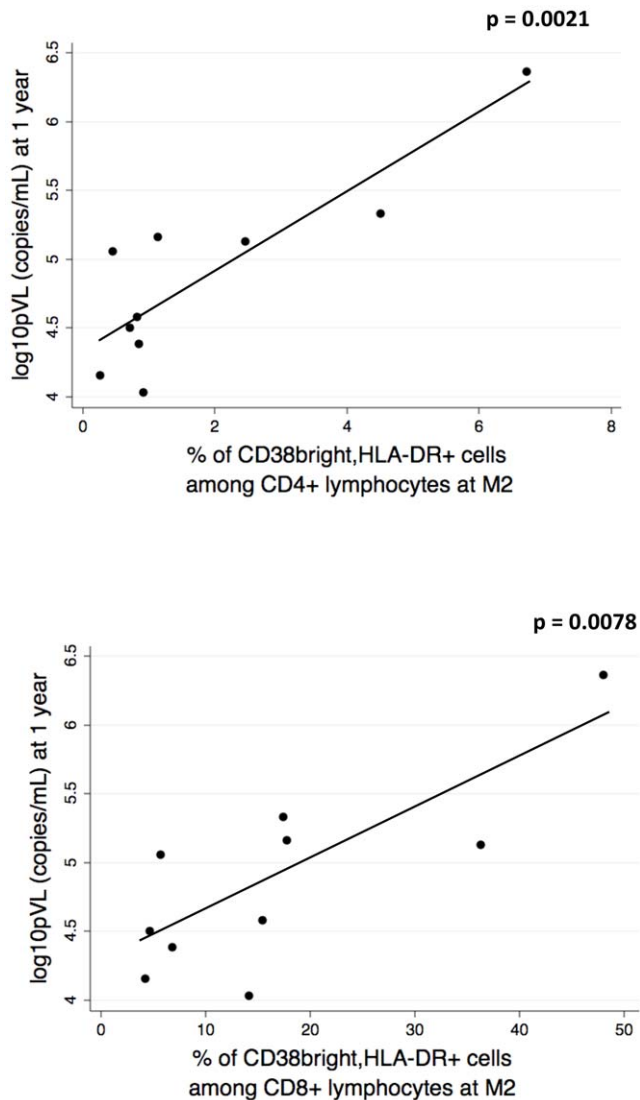


Figure 8. Association between the frequency of activated T cells (either CD4⁺ or CD8⁺) measured at M2, and plasma viral load, analyzed 1 year after PHI. Activated cells were identified as described in the legend to Figure 6. doi:10.1371/journal.pone.0050728.g008

Trend of T cell activation

The activation of CD4⁺ and CD8⁺ T cells was studied taking into account the co-expression of HLA-DR and of high levels of CD38 (CD38^{bright}), as described [7]. Figure 6 (upper panel, referred to CD4⁺, and lower panel, referred to CD8⁺ T cells) shows a high level of activation at M1 and M2, and a significant decrease in the following period.

T cell activation after PHI is a predictive marker for viral setpoint and length of the period without therapy

Figure 7 shows that a direct correlation was present between the level of CD4⁺ T cell activation status and pVL levels, at all months analyzed. As reported in Figure 8, a direct association was then found between the frequency of activated T cells (either CD4⁺ or CD8⁺) measured at M2, and plasma viral load, analyzed 1 year after PHI.

Finally, an inverse association between CD4⁺ or CD8⁺ T cell activation at M2 and M3 and the length of the period free of therapy was also found (Figure 9).

Activation of CD8⁺ T cells predicts the length of the period without therapy

We analyzed the role of CD8⁺ T cell activation in predicting the length of the period without treatment, and performed a “drug free” survival analysis in which we considered the importance of T cell activation in influencing the length of the period that did not require any treatment, i.e. from PHI to the failure of virological control (the time of starting HAART).

Figure 10 (upper part) shows time free of therapy of all our patients, performed by Kaplan Meyer analysis: 25% of patients failed (i.e., had to start therapy) within 18 months of HIV infection, and 50% within 26 months. Five out of 11 patients were still out of therapy 48 months after PHI.

Two months after PHI, the median percentage of activated CD8⁺ T cells was 15.5%, that of CD4⁺ T cells was 0.9%. By Cox analysis, we found that activation of CD8⁺ T cells had a significant impact on the risk of starting therapy (Hazard ratio = 1.124 $p > |z| = 0.013$; 95% Conf. Interval: 1.030–1.232); the increase in one unit of CD8 activation leads to an increase in the instantaneous risk of 2.5% to 23%. As shown in Figure 10 (lower part), we found that all patients with values of activated CD8⁺ T cells above the median (5 out of 11) had to start therapy within 26 months from PHI. Five out of 6 whose values of CD8⁺ T cell activation were below the median were still out of therapy for more than 48 months. It is to note that identical results were obtained considering activated CD4⁺ T cells: 80% of patients with less than the median value remained free of therapy for >48 months, while all patients above the median had to start treatment within 26 months (not shown).

Discussion

Acute, primary HIV infection has a crucial importance in determining the entire course of the disease, since in this phase the immune activation set point predicts the loss of CD4⁺ T lymphocytes [2]. Indeed, it has been shown that the level of CD4⁺ and CD8⁺ T cell activation during acute infection is able to influence the level of HIV viremia, that the level of CD8⁺ T cells that express the activation marker CD38 predicts the loss of CD4⁺ T cells [35], and that not only the reduction of viremia by antiretroviral treatment reduces immune activation and inflammatory phenomena, but also that the reduction of T cell activation by anti-inflammatory drugs can reduce viremia [36]. A strong immune activation, that includes the production of proinflammatory cytokines and rapid destruction of CD4⁺ T cells that reside in the gut-associated lymphoid tissue, favors massive replication of the virus and its dissemination in lymphoid tissues. In turn, the impaired local cellular immunity favors damages to the gastrointestinal mucosa, allowing the translocation of microbial products, including LPS, which contribute to persistent inflammation through the activation of Toll like receptors.

Furthermore, self-molecules containing the so-called “damage-associated molecular pattern”, or alarmins, that include mitochondrial proteins and mitochondrial DNA [37], can further activate inflammatory pathways and increase the damages [38,39]. Gaining information on the events that occur during primary infection is thus crucial to find strategies that can either arrest the initial virus spread, or rescue host cells. To better understand the importance of immune activation in determining the course of

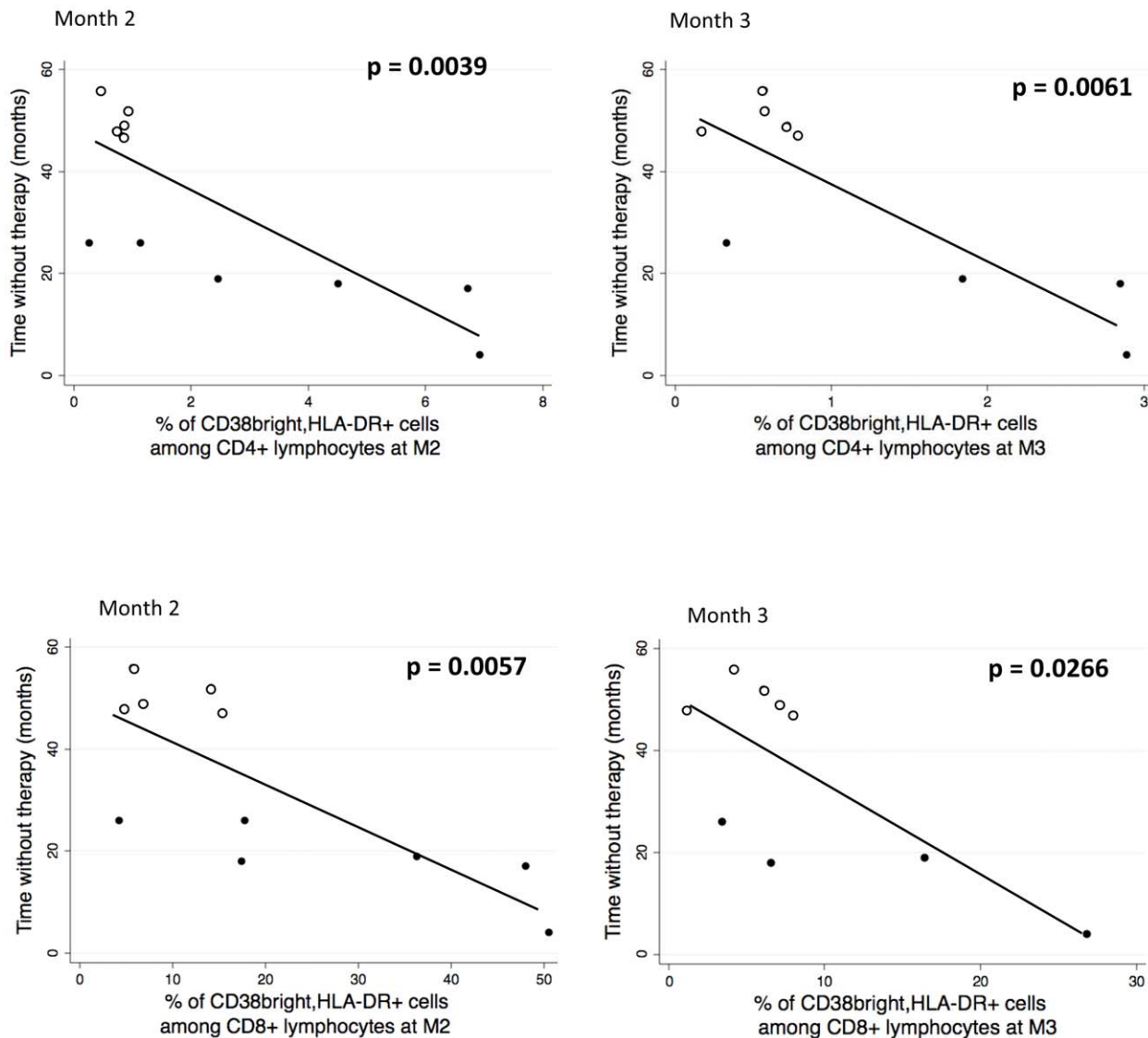


Figure 9. Association between CD4+ or CD8+ T cell activation at M2 and M3 and the length of the period free of therapy. Activated cells were identified as described in the legend to Figure 6. doi:10.1371/journal.pone.0050728.g009

HIV infection, we have performed a longitudinal study in a group of patients in whom several immune parameters were studied for six months after primary infection, and who were then followed for up to 5 years.

We analyzed both the subtype and magnitude of specific T lymphocytes that respond to the viral proteins gag and nef, and observed an increased level of activity of CD8+ T cells 3 months after infection. CD4+ T cell specific response against HIV peptides did not significantly change during the first 6 months and, as observed in our previous studies on treatment interruption (which could be considered a sort of secondary acute infection) or by others (during acute primary infection) was mainly characterized by cytotoxic features, including the expression of CD107a [21,40]. The efficacy of anti-HIV specific response has been linked to the “polyfunctionality” of specific clones, *i.e.* to those cells able to exert simultaneously multiple effectors functions. However, in the first months after PHI, we could not find truly polyfunctional CD4+ and CD8+ T cells, *i.e.* those performing 4 functions: indeed, we could not detect significant amounts of cells able to produce IL-2.

The influence and role of Treg during HIV infection remain unclear. Discrepant results have been reported, likely depending on the patient populations, the type and length of treatment, patient’s age, the time points analyzed, and the way Tregs were characterized [41,42,43]. Most studies showed that during HIV infection Treg increase in frequency but decrease in absolute number, either in blood or in other compartments, likely because of an increased generation, survival or proliferation in the periphery, or because of a different redistribution among tissues [reviewed in 44]. The role played by Tregs during HIV infection is still poorly understood, as two opposing hypotheses have been proposed. A detrimental role of Tregs during HIV infection was suggested based on the evidence that Tregs suppress virus-specific immune responses. Conversely, Tregs could be beneficial by limiting immune activation, thus controlling the availability of HIV targets as well as preventing immune-based pathologies. Recently, it has been shown that untreated, chronically infected patients can display a 2-fold increase in the frequency of Tregs [26]. On the contrary, HIV+ patients defined “elite long-term

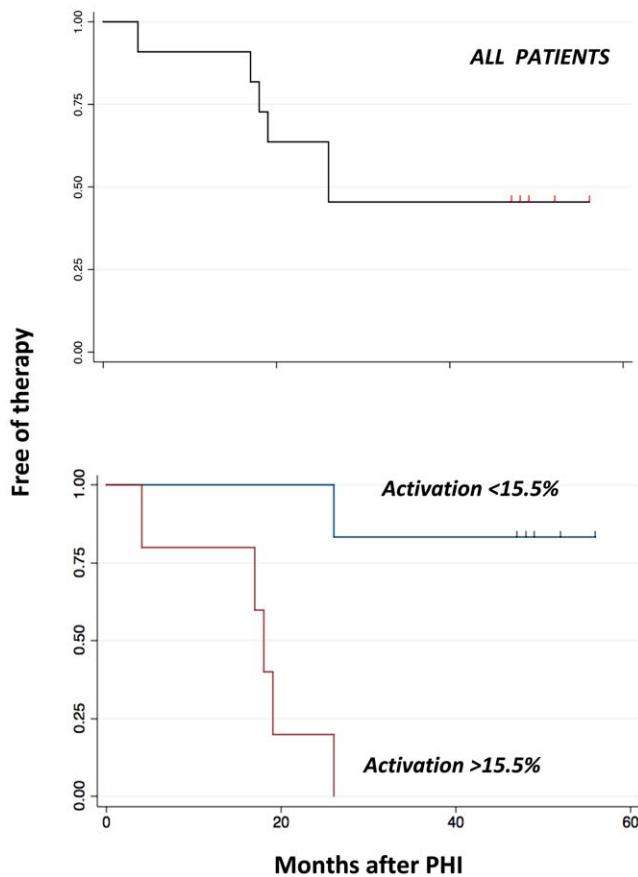


Figure 10. Kaplan-Meier survival curves related to the free-of-therapy period. The survival analysis was performed considering the time elapsed from PHI to the occurrence of failure, defined as the start of antiretroviral therapy. Upper pane, all patients; lower panels, patients with different degree of CD8+ T cell activation.
doi:10.1371/journal.pone.0050728.g010

nonprogressors³³, with a documented history of at least 12 years of infection and an undetectable viremia showed had fewer activated Treg [45].

Very few data are available on these cells in the first stages of HIV infection. Tregs could be involved in the regulation of the hyper-activation that occur during PHI, but in our study the

References

- Al-Harhi L, MaWhinney S, Connick E, Schooley RT, Forster JE, et al. (2007) Immunophenotypic alterations in acute and early HIV infection. *Clin Immunol* 125: 299–308.
- Deeks SG, Kitchen CM, Liu L, Guo H, Gascon R, et al. (2004) Immune activation set point during early HIV infection predicts subsequent CD4+ T-cell changes independent of viral load. *Blood* 104: 942–947.
- Maenette P, Riou C, Casazza JP, Ambrozak D, Hill B, et al. (2010) A steady state of CD4+ T cell memory maturation and activation is established during primary subtype C HIV-1 infection. *J Immunol* 184: 4926–4935.
- Cossarizza A, Ortolani C, Mussini C, Borghi V, Guaraldi G, et al. (1995) Massive activation of immune cells with an intact T cell repertoire in acute human immunodeficiency virus syndrome. *J Infect Dis*, 1995; 172: 105–112.
- Cossarizza A, Mussini C, Mongiardo N, Borghi V, Sabbatini A, et al. (1997) Mitochondria alterations and dramatic tendency to undergo apoptosis in peripheral blood lymphocytes during acute HIV syndrome. *AIDS* 11: 19–26.
- Cossarizza A, Mussini C, Borghi V, Mongiardo N, Nuzzo C, et al. (1999) Apoptotic features of peripheral blood granulocytes and monocytes during primary, acute HIV infection. *Exp Cell Res* 247: 304–311.
- Cossarizza A, Stent G, Mussini C, Paganelli R, Borghi V, et al. (2000) Deregulation of the CD95/CD95L system in lymphocytes from patients with primary acute HIV infection. *AIDS* 14: 345–355.
- Cossarizza A, Ortolani C, Mussini C, Guaraldi G, Mongiardo N, et al. (1995) Lack of selective V beta deletion in CD4+ or CD8+ T lymphocytes and functional integrity of T-cell repertoire during acute HIV syndrome. *AIDS* 9: 547–553.
- Hunt PW (2007) Role of immune activation in HIV pathogenesis. *Curr HIV/AIDS Rep* 4: 42–47.
- Giorgi JV, Hultin LE, McKeating JA, Johnson TD, Owens B, et al. (1999) Shorter survival in advanced human immunodeficiency virus type 1 infection is more closely associated with T lymphocyte activation than with plasma virus burden or virus chemokine coreceptor usage. *J Infect Dis* 179: 859–870.
- Bosinger SE, Sodora DL, Silvestri G (2011) Generalized immune activation and innate immune responses in simian immunodeficiency virus infection. *Curr Opin HIV AIDS* 6: 411–418.
- Koup RA, Safrit JT, Cao Y, Andrews CA, McLeod G, et al. (1994) Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. *J Virol* 68: 4650–4655.
- Borrow P, Lewicki H, Hahn BH, Shaw GM, Oldstone MB (1994) Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. *J Virol* 68: 6103–6110.
- Ogg GS, Jin X, Bonhoeffer S, Dunbar PR, Nowak MA, et al. (1998) Quantitation of HIV-1-specific cytotoxic T lymphocytes and plasma load of viral RNA. *Science* 279: 2103–2106.

frequency and number of Tregs were not correlated to the control of the immune activation, to the disease progression, nor to viro-immunological parameters. Thus, it might be hypothesized that the time required by the virus (or by the inadequate response to the virus) to provoke functional or phenotypic Treg alterations is longer than that we have considered in our study.

Finally, we found high levels of activation in the first two months after primary infection, that decreased over time. Immune activation is a well known important and predictive marker in patients with chronic infection [46], being related to several phenomena, including loss of CD4+ T cells in the gastrointestinal tract and the consequent microbial translocation [47,48].

We are aware that our study has some limitations, the first of which is the limited number of patients followed for a relatively short time. However, it has to be noted that the enrolment of patients with acute HIV infection is quite difficult, and we could only observe a few cases per year. A second limitation of the study is that the percentage of peripheral blood lymphocytes responding to specific HIV peptides were quite low, and that in some cases the individual variability was quite high. These phenomena, along with the number of patients, were likely responsible for the lack of any statistical significance of parameters related to T cell polyfunctionality.

However, here we show that patients who had a lower frequency of activated CD4+ and CD8+ T lymphocytes in the first 2 months after primary infection could remain much longer without antiretroviral therapy, and confirm the importance of the immune activation set point after primary infection [2]. Since patients with low immune activation could remain out of therapy – and thus were able to maintain a relatively high CD4+ T cell count – for a relatively long period, the identification of this immune parameter has to be considered when clinicians visit patients with primary, acute HIV infection. Finally, it is our opinion that the use of relatively simple flow cytometry methods, based on the simultaneous detection of no more than 3 or 4 parameters at the single cell level, could be more than sufficient to identify this biomarker, whose importance is actually not adequately taken into consideration.

Author Contributions

Conceived and designed the experiments: AC LB EN GG CM. Performed the experiments: LB EN EL MP MN SDB LG JPM. Analyzed the data: AC LB EN MP MN CM. Wrote the paper: AC LB EN MP CM. Followed the HIV+ patients: MV LM MM VB GG CM.

15. Ortiz AM, Klatt NR, Li B, Yi Y, Tabb B, et al. (2011) Depletion of CD4⁺ T cells abrogates post-peak decline of viremia in SIV-infected rhesus macaques. *J Clin Invest* 121: 4433–4445.
16. Douek DC, Brenchley JM, Betts MR, Ambrozak DR, Hill BJ, et al. (2002) HIV preferentially infects HIV-specific CD4⁺ T cells. *Nature* 417: 95–98.
17. Almeida JR, Price DA, Papagno L, Arkoub ZA, Sauce D, et al. (2007) Superior control of HIV-1 replication by CD8⁺ T cells is reflected by their avidity, polyfunctionality, and clonal turnover. *J Exp Med* 204: 2473–2485.
18. Daucher M, Price DA, Brenchley JM, Lamoreaux L, Metcalf JA, et al. (2008) Virological outcome after structured interruption of antiretroviral therapy for human immunodeficiency virus infection is associated with the functional profile of virus-specific CD8⁺ T cells. *J Virol* 82: 4102–4114.
19. Betts MR, Nason MC, West SM, De Rosa SC, Migueles SA, et al. (2006) HIV nonprogressors preferentially maintain highly functional HIV-specific CD8⁺ T cells. *Blood* 107: 4781–4789.
20. Rehr M, Cahenzli J, Haas A, Price DA, Gostick E, et al. (2008) Emergence of polyfunctional CD8⁺ T cells after prolonged suppression of human immunodeficiency virus replication by antiretroviral therapy. *J Virol* 82: 3391–3404.
21. Nemes E, Bertoncelli L, Lugli E, Pinti M, Nasi M, et al. (2010) Cytotoxic granule release dominates gag-specific CD4⁺ T-cell response in different phases of HIV infection. *AIDS* 24: 947–957.
22. Nemes E, Lugli E, Bertoncelli L, Nasi M, Pinti M, et al. (2011) CD4⁺ T-cell differentiation, regulatory T cells and gag-specific T lymphocytes are unaffected by CD4-guided treatment interruption and therapy resumption. *AIDS* 25: 1443–1453.
23. Turk G, Gherardi MM, Laufer N, Saracco M, Luzzi R, et al. (2008) Magnitude, breadth, and functional profile of T-cell responses during human immunodeficiency virus primary infection with B and BF viral variants. *J Virol* 82: 2853–2866.
24. Tran TA, de Goer de Herve MG, Hendel-Chavez H, Dembele B, Le Nevot E, et al. (2008) Resting regulatory CD4⁺ T cells: a site of HIV persistence in patients on long-term effective antiretroviral therapy. *PLoS One* 3: e3305.
25. Chase AJ, Yang HC, Zhang H, Blankson JN, Siliciano RF (2008) Preservation of FoxP3⁺ regulatory T cells in the peripheral blood of human immunodeficiency virus type 1-infected elite suppressors correlates with low CD4⁺ T-cell activation. *J Virol* 82: 8307–8315.
26. Aandahl EM, Michaelsson J, Moretto WJ, Hecht FM, Nixon DF (2004) Human CD4⁺CD25⁺ regulatory T cells control T-cell responses to human immunodeficiency virus and cytomegalovirus antigens. *J Virol* 78: 2454–2459.
27. Presicce P, Orsborn K, King E, Pratt J, Fichtenbaum CJ, et al. (2011) Frequency of circulating regulatory T cells increases during chronic HIV infection and is largely controlled by highly active antiretroviral therapy. *PLoS One* 6(12):e28118.
28. Fiebig EW, Wright DJ, Rawal BD, Garrett PE, Schumacher RT, et al. (2003) Dynamics of HIV viremia and antibody seroconversion in plasma donors: implications for diagnosis and staging of primary HIV infection. *AIDS* 17: 1871–1879.
29. Barbieri D, Abbraccio MP, Salvioli S, Monti D, Cossarizza A, et al. (1998) Apoptosis by 2-chloro-2'-deoxy-adenosine and 2-chloro-adenosine in human peripheral blood mononuclear cells. *Neurochem Int* 32: 493–504.
30. Cossarizza A, Poccia F, Agrati C, D'Offizi G, Bugarini R, et al. (2004) Highly active antiretroviral therapy restores CD4⁺ Vbeta T-cell repertoire in patients with primary acute HIV infection but not in treatment-naive HIV⁺ patients with severe chronic infection. *J Acquir Immune Defic Syndr* 35: 213–222.
31. Nemes E, Lugli E, Nasi M, Ferraresi R, Pinti M, et al. (2006) Immunophenotype of HIV⁺ patients during CD4 cell-monitored treatment interruption: role of the IL-7/IL-7 receptor system. *AIDS* 20: 2021–2032.
32. Lugli E, Pinti M, Nasi M, Roat E, Prada N, et al. (2007) Subject classification obtained by cluster analysis and principal component analysis applied to flow cytometric data. *Cytometry (part A)* 71: 334–344.
33. Roederer M, Nozzi JL, Nason MC (2011) SPICE: exploration and analysis of post-cytometric complex multivariate datasets. *Cytometry A* 79: 167–174.
34. Casazza JP, Betts MR, Price DA, Precopio ML, Ruff LE, et al. (2006) Acquisition of direct antiviral effector functions by CMV-specific CD4⁺ T lymphocytes with cellular maturation. *J Exp Med* 203: 2865–2877.
35. Giorgi JV, Liu Z, Hultin LE, Cumberland WG, Hennessey K, et al. (1993) Elevated levels of CD38⁺ CD8⁺ T cells in HIV infection add to the prognostic value of low CD4⁺ T cell levels: results of 6 years of follow-up. The Los Angeles Center, Multicenter AIDS Cohort Study. *J Acquir Immune Defic Syndr* 6: 904–912.
36. Rizzardì GP, Harari A, Capiluppi B, Tambussi G, Ellefsen K, et al. (2002) Treatment of primary HIV-1 infection with cyclosporin A coupled with highly active antiretroviral therapy. *J Clin Invest* 109: 681–688.
37. Zhang Q, Raouf M, Chen Y, Sumi Y, Sursal T, et al. (2010) Circulating mitochondrial DAMPs cause inflammatory responses to injury. *Nature* 464: 104–107.
38. Cossarizza A, Pinti M, Nasi M, Gibellini L, Manzini S, et al. (2011) Increased plasma levels of extracellular mitochondrial DNA during HIV infection: a new role for mitochondrial damage-associated molecular patterns during inflammation. *Mitochondrion* 11: 750–755.
39. Pinti M, Mussini C, Cossarizza A (2012) Mitochondrial DNA: a pro-inflammatory “enemy from within” during HIV infection? *Cell Death Dis* 3: 307. doi: 10.1038/cddis.2012.47.
40. Soghoian DZ, Jessen H, Flanders M, Sierra-Davidson K, Cutler S, et al. (2012) HIV-specific cytolytic CD4⁺ T cell responses during acute HIV infection predict disease outcome. *Sci Transl Med* 4(123):123ra25.
41. Oswald-Richter K, Grill SM, Shariat N, Leclawong M, Sundrud MS, et al. (2004) HIV Infection of Naturally Occurring and Genetically Reprogrammed Human Regulatory T-cells. *PLoS Biol* 2: E198.
42. Andersson J, Boasso A, Nilsson J, Zhang R, Shire NJ, et al. (2005) Cutting Edge: The Prevalence of Regulatory T Cells in Lymphoid Tissue Is Correlated with Viral Load in HIV-Infected Patients. *J Immunol* 174: 3143–3147.
43. Lim A, Tan D, Price P, Kamarulzaman A, Tan HY, et al. (2007) Proportions of circulating T cells with a regulatory cell phenotype increase with HIV-associated immune activation and remain high on antiretroviral therapy. *AIDS* 21: 1525–1534.
44. Moreno-Fernandez ME, Presicce P, Chougnat CA (2012) Homeostasis and Function of Regulatory T Cells in HIV/SIV Infection. *J Virol* 86: 10262–10269.
45. Bignami F, Pilotti E, Bertoncelli L, Ronzi P, Gulli M, et al. (2012). Stable changes in CD4⁺ T-lymphocyte microRNA expression following exposure to HIV-1. *Blood* 119: 6259–6267.
46. Hazenberg MD, Otto SA, van Benthem BHB, Roos MTL, Coutinho RA, et al. (2003) Persistent immune activation in HIV-1 infection is associated with progression to AIDS. *AIDS* 17:1881–1888.
47. Brenchley JM, Schacker TW, Ruff LE, Price DA, Taylor JH, et al. (2004) CD4⁺ T Cell Depletion during all Stages of HIV Disease Occurs Predominantly in the Gastrointestinal Tract. *J Exp Med* 200: 749–759.
48. Mehandru S, Poles MA, Tenner-Racz K, Horowitz A, Hurley A, et al. (2004) Primary HIV-1 infection is associated with preferential depletion of CD4⁺ T lymphocytes from effector sites in the gastrointestinal tract. *J Exp Med* 200: 761–770.