Immune activation, apoptosis, and Treg activity are associated with persistently reduced CD4$^+$ T-cell counts during antiretroviral therapy

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Background: Persistently reduced CD4$^+$ T-lymphocyte counts in the face of undetectable HIV viremia are seen in a sizable percentage of HIV-infected patients undergoing antiretroviral therapy (ART). We analyzed the immune correlates of this phenomenon.

Materials and methods: Sixty-seven HIV-infected patients with undetectable viremia (<50 copies/μl) after more than 7 years of ART were enrolled in the study and divided into two groups (CD4 cell counts >500 cells/μl or <500 cells/μl). Duration of HIV infection (>16 years) was comparable. Peripheral blood mononuclear cell were stimulated with gag+env or with cytomegalovirus peptides. Activated T cells (Ki67$^+$), Treg lymphocytes (CD4$^+$/CD25high/Foxp3+), divided into naive and activated cells based on PD1 expression, interleukin (IL)-10 and transforming growth factor (TGF)-β production, annexin V, activation of caspases 8 and 9, Toll-like receptor (TLR)2 and TLR4 expression on immune cells, and plasma lipopolysaccharide (LPS) concentration were analyzed.

Results: CD4$^+$/Ki67$^+$ T cells; plasma LPS; total, naive, and activated Treg; TLR2-expressing and TLR4-expressing Treg; IL-10 production; and early and late apoptotic CD4 T cells, were significantly increased in patients with undetectable viremia and CD4 cell counts less than 500 cells/μl after more than 7 years of ART. As previously shown, CD4 nadir were also lower in these individuals. Immune activation, LPS concentration, Treg, and degree of apoptosis were negatively correlated with CD4 cell counts.

Conclusion: Lack of CD4 recovery in individuals in whom ART suppresses HIV replication is associated with complex immune alterations. Immune activation, likely driven by altered gut permeability and resulting in augmented Treg activity could play a pivotal role in this process. © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins

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Introduction

Antiretroviral therapy (ART) changed the natural history of HIV infection. Notably, though, despite optimal suppression of HIV replication, restoration of CD4$^+$ T-cell counts is not always achieved in ART-treated individuals. Several factors seem to be involved in the lack of CD4$^+$ T-cell recovery which is observed in 20–

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30% of ART-treated patients. Thus, residual viral replication [1], altered thymic function [2], older age [3], immuneactivation [4,5], apoptosis, and viral coinfections [6,7] were all proposed to play a significant role in this phenomenon. On the contrary, basal viral load [8,9], genetic factors [10], younger age [11,12], and the percentage of naive cells [13] were reported to have a positive influence on the restoration of $CD4^+$ T-cell levels.

The importance of proper immune reconstitution is underlined by data obtained in a French cohort of HIV-infected patients showing that, once therapy is initiated, mortality becomes comparable with that of HIV-seronegative individuals only if $CD4^+$ T cells reach physiological values [14]. In the Athena cohort, $CD4^+$ T-cell normalization (800 cells/μl) was achievable only after several years of ART and was not obtained in HIV-infected individuals who started therapy with CD4 cell count less than 350 cells/μl. Moreover, HIV-infected ART-treated individuals older than 50 years at initiation of ART, and in whom viremic spikes (>1000 copies/μl) were observed, showed a more limited increase in $CD4^+$ T cells and reached an early plateau at CD4 levels lower than normal values [15]. According to these results, $CD4^+$ T cells nadir influences not only the possibility to normalize cell counts in response to therapy but also affects the time needed to obtain this goal. Similar conclusions were reported by another study performed in patients stratified on the basis of their $CD4^+$ T-cell counts before initiation of therapy [16]. Results showed that the time between ART initiation and achievement of a $CD4^+$ T-cell count more than 500 cells/μl was significantly longer in individuals with lower baseline lymphocyte counts. Finally, other data indicate that $CD4^+$ T-cell nadir correlates with, and is predictive for, responses to vaccines, suggesting that this parameter directly influences the functionality of the immune system [17].

Several studies indicate that immune activation plays a key role in the pathogenesis of HIV. Notably, recent results showed that a degree of $CD4^+$ and $CD8^+$ T-cell immune activation persists over time during ART even in the presence of fully satisfactory suppression of HIV replication [18]. A definite consensus on which are the clinical consequences, or the causes, of immune activation in HIV infection is nevertheless still lacking. The most recent pathogenic hypothesis postulates that the main cause of immune activation is the destruction of $CD4^+$ T cells in the gastrointestinal mucosa observed in the earliest phases of the disease [19]. Several evidence indicates that the resulting mucosal alterations allow gut bacterial translocation into the peripheral blood, resulting in Toll-like receptor (TLR)-mediated immune-activation. T-lymphocyte homeostasis and the activation of the immune system are controlled by several mechanisms; among them regulatory T cells play a crucial role as they produce immunosuppressive cytokines [interleukin (IL)-10, transforming growth factor (TGF-β)] and induce apoptosis of antigen-specific cells. All these mechanisms, proteins, and cell populations could play a role in impeding $CD4^+$ T-cell recovery in ART-treated HIV infected individuals with suppressed HIV viremia [20–23].

In an attempt to shed light on the relationship between immune activation and lack of $CD4^+$ T-cell-recovery during virologically successful ART we performed extensive immunological analyses in a population of HIV-infected, ART-treated individuals in whom therapy did or did not result in immune recovery. Results indicated that a complex pattern of immune alterations is present in ART-treated patients with persistently reduced $CD4^+$ T-lymphocyte counts.

Materials and methods

Study population

Sixty-seven HIV-infected patients with suppressed viremia (<50 copies/μl) (50 men and 17 women; mean age = 49.21 years, range = 33–71 years) were enrolled in the study. All patients were treated with combined ART according to currently accepted guidelines (NRTI + PI or NRTI + NNRTI). HIV plasma viremia had been below the threshold of detection for at least 7 years in all patients. Patients who in the past had undergone changes in their therapy because of simplification or toxicity were included in the study. Patients were subdivided into two groups on the basis of the CD4 cell counts (>500 $CD4^+$ T cells/μl, $n = 32$, and <500 $CD4^+$ T cells/μl, $n = 35$). Duration of HIV infection (>16 years) and of ART (>7 years) was comparable between the two groups. All individuals were enrolled by the Departments of Infectious Diseases of the Luigi Sacco Hospital in Milano and of the San Gerardo Hospital in Monza; written informed consent was obtained before enrolment.

Blood sample collection and peripheral blood mononuclear cell separation

Whole blood was collected by venipuncture in Vacutainer tubes containing EDTA (Becton Dickinson, Rutherford, New Jersey, USA). Plasma was stored and peripheral blood mononuclear cells (PBMC) were separated on lymphocyte separation medium (Cedarlane Laboratories Limited, Hornby, Ontario, Canada) and washed twice in phosphate-buffered saline (PBS) (PBI, Milan, Italy). The number of viable leukocytes was determined by trypan blue exclusion test.

Stimulation of peripheral blood mononuclear cell

PBMC were incubated for 18 h in the presence/absence of a pool of gag+env peptides (HIV) [24] or cytomegalovirus (CMV) protein (Microbics Biosystems inc., Toronto, Ontario, Canada). For cytokine analyses, 10 μg/ml Brefeldin A (Sigma-Aldrich, St. Louis, Missouri, USA)

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was added to cell cultures during the last 6 h. In some experiments PD1 or PD-L1 neutralizing antibodies (eBiosciences, San Diego, California, USA) were added to the cultures to evaluate the PD1–PD-L1 pathway activity.

Ki67 evaluation

PBMC washed in PBS and stained for CD4PE mAb (Beckman-Coulter, Fullerton, California, USA) for 15 min at room temperature in the dark were then fixed in 1% paraformaldehyde (PFA; Sigma-Aldrich) for 15 min at 4°C. After washing, cells were resuspended in 0.5% saponin (Sigma-Aldrich) and stained for Ki67 or mouse fluorescein isohiocyanate (FITC)-coupled IgG1 isotype control (BD Biosciences, San Diego, California, USA). Cells were finally incubated for 45 min at 4°C in the dark, washed, and fixed in 1% PFA.

Plasma lipopolysaccharide concentration

Lipopolysaccharide (LPS) concentration was measured on plasma samples with the LAL Chromogenic Endopoint Assay (Hycult Biotechnology, Uden, the Netherlands). Samples, prepared according to manufacture's instructions, were plated in a 96-well plate, followed by LAL reagent. After 45-min incubation at room temperature, absorbance was measured at 405 nm with a spectrophotometer. LPS concentration was expressed in EU/ml and calculated relatively to standard curve.

Identification of T-regulatory lymphocytes

PBMC were incubated with anti-CD4, anti-CD25, and anti-PD-1 for 15 min at room temperature. The intracellular detection of PD-1 and FoxP3 was performed following the manufacture's protocol (eBioscience). Intracellular or surface costaining of PD-1 and intracellular FoxP3 was performed by flow cytometry on $CD4^{+}CD25^{bright}$ gated T-cell $CD4^{+}$ T cells/μl.

Evaluation of Toll-like receptor expression

PBMC resuspended in fresh medium were stained for CD14, CD4, CD25, Foxp3, TLR2, and TLR4 monoclonal antibodies. After a 15-min incubation at room temperature in the dark, cells were washed and fixed in 1% PFA.

Intracellular cytokine concentration

Antigen-stimulated PBMC were stained for CD4 expression. After a 15-min incubation at room temperature in the dark, cells were fixed in 1% PFA, incubated for 15-min at 4°C in the dark, and permeabilized with 0.5% saponin (Sigma-Aldrich). TGF-β and IL-10 monoclonal antibodies were then added. After a 30-min incubation at 4°C in the dark, cells were washed and fixed 1% PFA in PBS.

Identification of early apoptotic, late apoptotic, and necrotic cells

Stimulated PBMC resuspended in D-PBS (Euroclone, Siziano, Pavia, Italy) were stained with CD4, annexin V, and 7AAD monoclonal antibodies (Beckman-Coulter). After 20-min incubation at room temperature, cells were washed in cold D-PBS and resuspended in D-PBS.

Detection of activated caspases 8 and 9

The FLICA Apoptosis detection kit (Immunochemistry Technologies, Bloomington, Minnesota, USA) was used to analyze caspases. FLICA reagents were prepared according to manufacture's instructions and added to the resuspended cells; the mix was then incubated for 1 h at 37°C under 5%CO_2. After incubation cells were washed twice with buffer. The cell pellet was resuspended in wash buffer and stained with CD4 and CD8 monoclonal antibodies for 30 min in ice. Finally, cells were stained with propidium iodide and analyzed by flow cytometry.

Monoclonal antibodies (mAbs)

The following mAbs were used: anti-CD4 (mouse IgG1 isotype) Phycoerythrin-Cy7 (PECy7), anti-CD14 (mouse IgG2a isotype), all coupled to R-Phycoerythrin-Cyanine 5 (PECy5), anti-CD4 (mouse IgG1 isotype) R-Phycoerythrin (PE), anti-CD14 (mouse IgG2a isotype), all coupled to FITC, anti-CD25 (mouse IgG2a) coupled to phycoerythrin-Texas red (ECD) (Beckman-Coulter); anti-CD178 (mouse IgG1 isotype) PE (BioLegend, San Diego, California, USA), anti-B7H1 PE (mouse IgG1 isotype), anti-TLR4 PE (mouse IgG2a isotype), anti-PD1 PE (mouse IgG1 isotype), anti-TLR2 FITC (mouse IgG2a isotype) (eBioscience); recombinant protein annexin V PE (Bender MedSystem, Burlingame, California, USA). The intracellular staining detection mAb used were anti-Foxp3 (rat IgG2a isotype) PECy5, anti-PD1 FITC (mouse IgG1 isotype) (eBioscience); anti human TGF-β PE (mouse IgG1 isotype) (BioLegend, San Diego, California, USA); anti human IL-10 FITC (mouse IgG2b isotype) (R&D System); anti-human Ki67 FITC (mouse IgG1 isotype) (BD Biosciences).

Cytometric analysis

Cytometry was performed using a FC500 flow cytometer (Beckman-Coulter) equipped with a double 15-mW argon ion laser operating at 456 and 488 nm interfaced with Intercorp computer. For each analysis 20 000 events were acquired and gated on CD4 and CD14 expression and SSC (side scatter) properties. Green fluorescence from FITC (FL1) was collected through a 525-nm band-pass filter, orange-red fluorescence from R-PE (FL2) was collected through a 575-nm band-pass filter, Texas red fluorescence from ECD (FL3) was collected through a 613-nm band-pass filter, red fluorescence from PECy5 and APC (FL4) were collected through a 670-nm band-pass filter, far red fluorescence from PECy7 (FL5) was collected through a 770-nm band-pass filter. Data were collected using linear amplifiers for forward and SSC and logarithmic amplifiers for FL1, FL2, FL3, FL4, and FL5.

Statistical analysis

Data were analyzed according to standard statistical tests; *t* tests were performed to compare groups. Procedures

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Table 1. Demographic and clinical characteristics of the HIV-infected antiretroviral therapy-treated patients enrolled in the study.

Parameter	CD4 cell count >500 cells/μl	CD4 cell count <500 cells/μl	*P*
No.	32	35	
Sex (M/F)	25M; 7F	25M; 10F	
Age (years)	48.13 ± 1.12	52.11 ± 1.39	0.03
Years of ART	10.34 ± 0.46	11.51 ± 0.52	NS
CD4 nadir (cells/μl)	195.84 ± 24.35	119.37 ± 15.70	0.01
CD4 cell count (cells/μl)	705.52 ± 25.87	245.83 ± 24.31	<0.001
CD4 (%)	20.96 ± 1.49	12.93 ± 1.34	0.002

ART, antiretroviral therapy. Mean values, SE, and *P* values are shown.

were based on parametric analyses. The rank-transformed variables were analyzed if distributions were not normal. To account for different patient's characteristics between groups, an analysis of variance was run through a general linear model, including sex and age as independent variables. Possible relationships were evaluated using Pearson's correlation test.

Results

Study population

Sixty-seven HIV-infected, ART-treated individuals with undetectable viremia (<50 copies/μl) were enrolled in the study. Patients were divided in two groups on the basis of the CD4 cell counts reached after at least 7 years of ART. HIV-infected individuals in whom ART was not associated with $CD4^+$ T-cell recovery were characterized by a lower CD4 nadir ($P = 0.01$). As per inclusion criteria, lower absolute numbers ($P < 0.001$) and percentages ($P = 0.002$) of $CD4^+$ T cells were seen in patients with less than 500 $CD4^+$ T cells/μl. The epidemiologic characterization of the two groups of individuals is presented in Table 1.

$CD4^+$ T lymphocytes activation

T lymphocytes activation was evaluated by Ki67 expression. The percentage of $CD4^+/Ki67^+$ T cells was significantly augmented in individuals with $CD4^+$ cell counts less than 500 cells/μl compared with individuals with more than 500 $CD4^+$ T cells/μl. This difference was detected both in unstimulated cells and upon stimulation with HIV (basal: $P = 0.032$; HIV: $P = 0.027$) (Fig. 1a).

Plasma lipopolysaccharide concentration

Plasma LPS concentration is an index of microbial translocation; augmented LPS concentration is associated with alterations of the gut permeability. LPS plasma concentration was significantly higher in individuals with $CD4^+$ cell counts less than 500 cells/μl compared with individuals with more than 500 $CD4^+$ T cells/μl ($P < 0.001$) (Fig. 1b).

Regulatory T cells

Total ($CD4^+CD25^{bright}Foxp3+$), naive ($CD4^+CD25^{bright}Foxp3+$ intracellular PD1+), and activated ($CD4^+CD25^{bright}Foxp3+$ extracellular PD1+) regulatory T cells were analyzed on whole blood samples. All the populations examined were increased in patients with $CD4^+$ cell counts

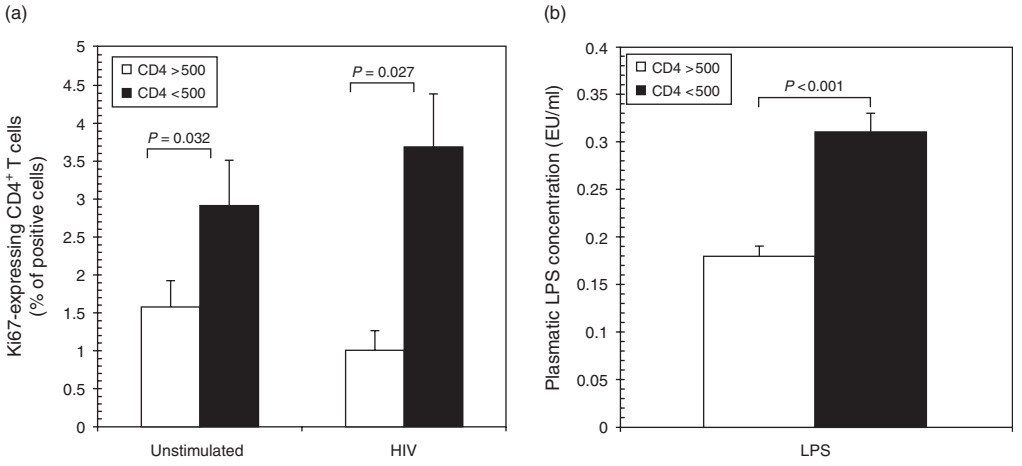

Fig. 1. Activated T cells and plasma LPS concentration. (a) Percentage of $Ki67^+$ $CD4^+$ T lymphocytes in patients with CD4 cell counts >500 cells/μl (□) and in patients with CD4 cell counts <500 cells/μl (■) in basal condition and upon HIV-specific stimulation. (b) Plasma lipopolysaccharide concentration in patients with CD4 cell counts >500 cells/μl (□) and in patients with CD4 cell counts <500 cells/μl (■). Mean values, SE, and *P* values are shown.

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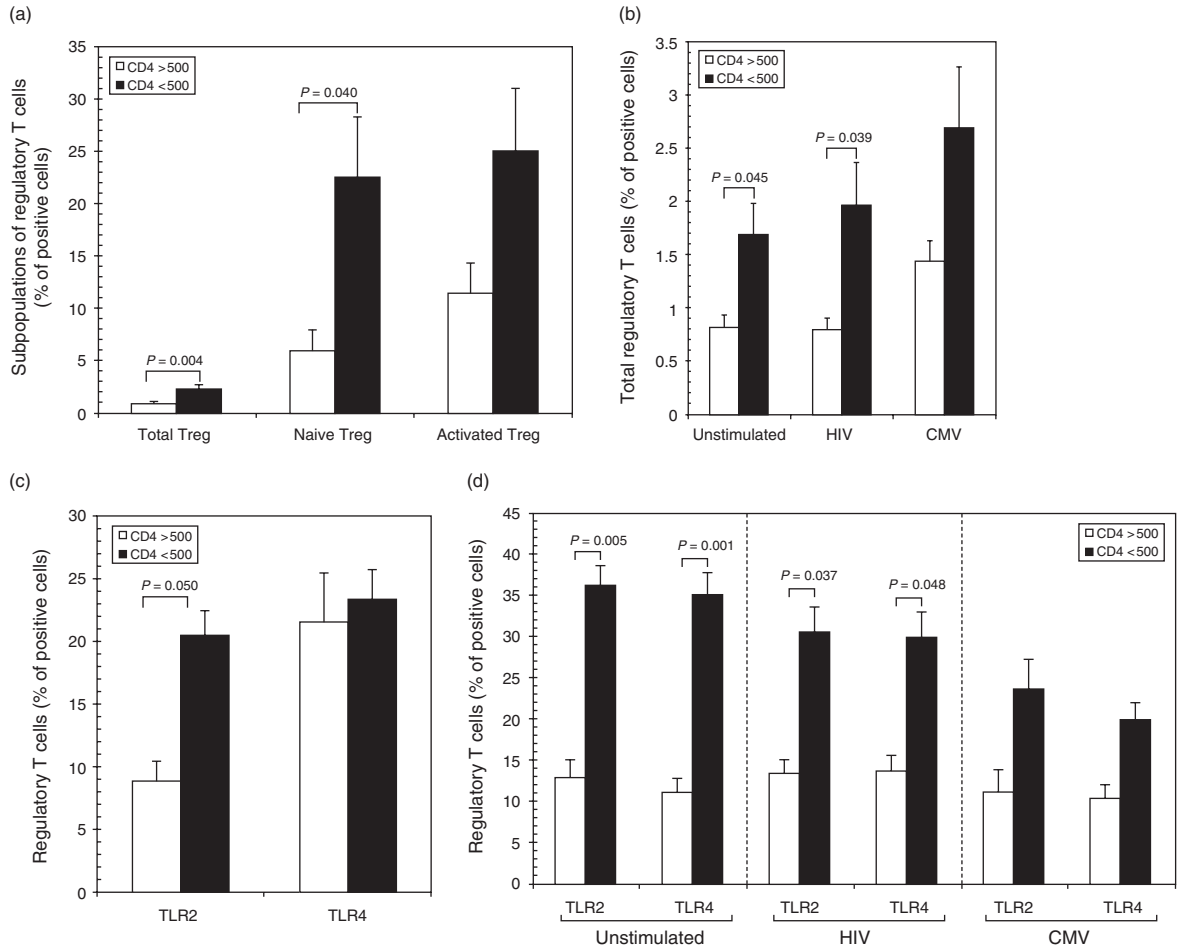

Fig. 2. Regulatory T cells. (a) Percentage of total, naive and activated regulatory T lymphocytes in patients with CD4 cell counts >500 cells/μl (□) and in patients with CD4 cell counts <500 cells/μl (■) in whole blood. (b) Percentage of total Treg in basal condition and upon HIV- or CMV-specific stimulation in patients with CD4 cell counts >500 cells/μl (□) and in patients with CD4 cell counts <500 cells/μl (■). (c) Percentage of Toll-like receptor (TLR)2-expressing and TLR4-expressing regulatory T lymphocytes in patients with CD4 cell counts >500 cells/μl (□) and in patients with CD4 cell counts <500 cells/μl (■) in whole blood. (d) Percentage of TLR2-expressing and TLR4-expressing Treg in basal condition and upon HIV- or CMV-specific stimulation in patients with CD4 cell counts >500 cells/μl (□) and in patients with CD4 cell counts <500 cells/μl (■). Mean values, SE, and *P* values are shown.

less than 500 cells/μl compared with individuals with more than 500 $CD4^+$ T cells/μl. These differences reached statistical significance in the case of total ($P = 0.004$) and naive ($P = 0.040$) Treg cells (Fig. 2a).

The three different populations of Treg cells were subsequently analyzed after stimulation of PBMC with HIV or CMV. HIV-stimulated, but not CMV-stimulated total Treg cells were significantly increased in patients with $CD4^+$ cell counts less than 500 cells/μl compared to patients with more than 500 $CD4^+$ T cells/μl ($P = 0.039$) (Fig. 2b).

Toll-like receptor2-expressing and Toll-like receptor4-expressing Treg

TLR2 and TLR4 expression was analyzed on Treg cells, as TLR-mediated activation plays a pivotal role in regulating the immunosuppressive functions of these cells. Notably, TLR4 is the ligand for LPS. Results showed that TLR2-expressing as well as TLR4-expressing and HIV-stimulated Treg were significantly increased in patients with $CD4^+$ cell counts less than 500 cells/μl compared with patients with more than 500 $CD4^+$ T cells/μl (HIV-stimulated:TLR2: $P = 0.037$; TLR4: $P = 0.048$) (Fig. 2c, d).

Immunosuppressive cytokines

IL-10 and TGF-β production by $CD4^+$ T lymphocytes was evaluated upon stimulation of PBMC with HIV and CMV. HIV-specific IL-10+/$CD4^+$ and TGF-β+/$CD4^+$ T cells were augmented in patients with $CD4^+$ cell counts less than 500 cells/μl in comparison to patients with CD4 cell counts more than 500 cells/μl (IL-10: $P = 0.037$) (Fig. 3). No differences were observed in CMV-stimulated PMBC.

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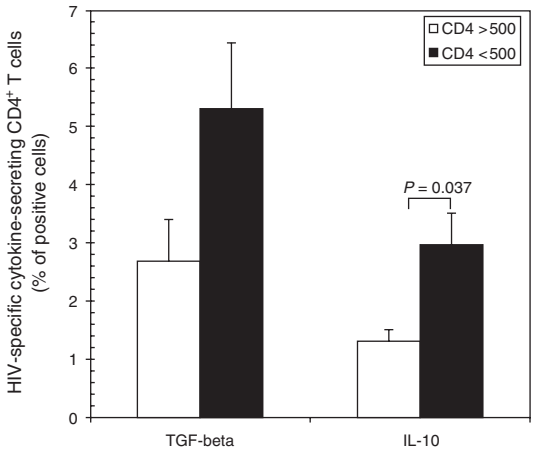

Fig. 3. Percentage of HIV-specific transforming growth factor (TGF)-β- or IL-10-secreting CD4$^+$ T cells in patients with CD4 cell counts >500 cells/μl (□) and in patients with CD4 cell counts <500 cells/μl (■). Mean values, SE, and *P* values are shown.

Viable, early apoptotic, and late apoptotic T cells

Viable, early and late apoptotic CD4$^+$ T cells were evaluated in basal condition and after stimulation with HIV and CMV. Viable cells were identified as CD4$^+$/annexin V^{neg}/7AAD^{neg} T cells; cells in early step of apoptosis as CD4$^+$/annexin V+/7AAD^{neg} T cells; and cells in late apoptosis as CD4$^+$/annexin V+/7AAD+ T cells. Both in basal conditions and upon HIV stimulation viable CD4$^+$ T cells were diminished, whereas early apoptotic and late apoptotic CD4$^+$ T cells were augmented in individuals with CD4$^+$ T-cell counts less than 500 cells/μl (Fig. 4).

Because activated Treg express PD1, and the PD1/PDL1 pathway is of fundamental importance in inducing apoptosis of CD4 T cells, we analyzed this pathway adding to the cultures antibodies able to block this interaction. PD1 blocking resulted in an increase of viable and a decrease of early and late apoptotic cells in individuals with CD4$^+$ T-cell counts less than 500 cells/μl, whereas no effects were observed in patients with more than 500 CD4$^+$ T cells/μl (data not shown).

Caspases 8 and 9 activation in CD4$^+$ T cells

Activation of caspases 8 and 9 is associated with apoptosis. The activation of these caspases was evaluated in CD4$^+$ T cells in basal condition and after HIV and CMV stimulation. As shown in Table 2, expression of caspases 8 and 9 was augmented in cells of patients with CD4$^+$ T-cell counts less than 500 cells/μl. These differences reached statistical significance for HIV-specific and CMV-specific CD4$^+$/caspase 9+ cells (HIV: $P = 0.044$; CMV: $P = 0.026$).

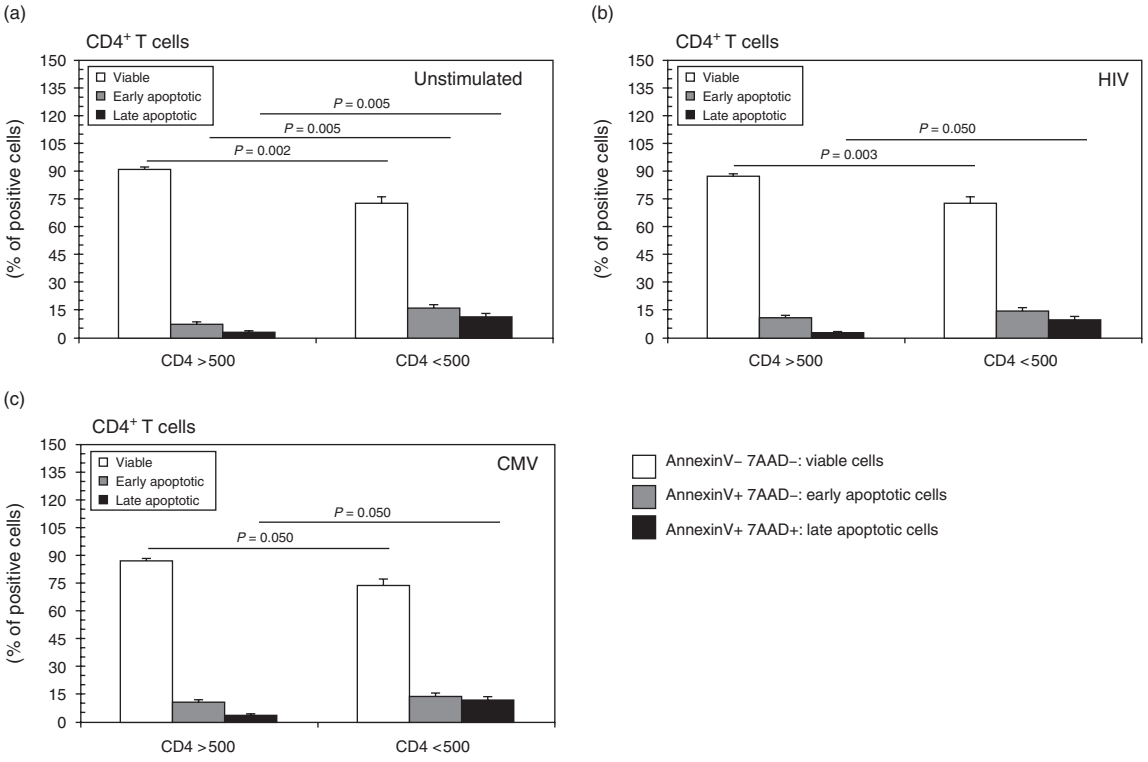

Fig. 4. Percentage of viable (AnnexinV− 7AAD−)(□), early apoptotic (AnnexinV+ 7AAD−) (■) and late apoptotic (AnnexinV+ 7AAD+) (■) CD4$^+$ T cells in patients with CD4 cell counts >500 cells/μl and in patients with CD4 cell counts <500 cells/μl, in basal condition and upon HIV-specific or cytomegalovirus-specific stimulation. Mean values, SE, and *P* values are shown.

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Table 2. Percentage of caspase 8+/CD4$^+$ and caspase 9+/CD4$^+$ T cells in unstimulated condition and upon HIV-specific or cytomegalovirus-specific stimulation in HIV-infected antiretroviral therapy-treated patients enrolled in the study.

	CD4 cell count >500 cells/μl	CD4 cell count < 500 cells/μl	*P*
Unstimulated			
Caspase 8/CD4	2.51 ± 0.29	7.87 ± 2.42	NS
Caspase 9/CD4	2.18 ± 0.15	8.56 ± 2.45	NS
HIV			
Caspase 8/CD4	3.60 ± 0.47	9.11 ± 2.39	NS
Caspase 9/CD4	3.43 ± 0.36	9.38 ± 2.12	0.044
CMV			
Caspase 8/CD4	3.38 ± 0.45	10.39 ± 2.71	NS
Caspase 9/CD4	3.09 ± 0.21	10.39 ± 2.17	0.026

CMV, cytomegalovirus. Mean values, SE, and *P* values are shown.

Correlations

Correlations between CD4$^+$ T cells, lymphocyte activation, LPS concentration, Treg and apoptosis were analyzed. Significant negative correlations were observed between CD4$^+$ T-cell counts and HIV-specific CD4$^+$/Ki67$^+$ cells (Pearson correlation: $P = 0.01$); plasma LPS concentration (Pearson correlation: $P = 0.01$); total, naive, and activated Treg (total and naive Treg: Pearson correlation: $P = 0.01$; activated Treg: Pearson correlation: $P = 0.04$); and unstimulated and HIV-specific late apoptotic CD4$^+$ T cells (Pearson correlation: $P = 0.01$) (Fig. 5).

Discussion

Normalization of CD4$^+$ T cells is a pivotal target in the management of HIV infection, as the likelihood of developing non-AIDS comorbidities is associated with the duration of the period during which CD4$^+$ T cells are lower than 500 cells/μl (DAD) [25,26]. The FIRST study also showed that lower CD4$^+$ T cell counts on treatment are associated with a higher immediate risk of cardiovascular and renal diseases and cancer [27–29]. The implication of this study, which was confirmed by later observations, is that even patients with suppressed viremia are at risk for significant morbidity and mortality if their CD4$^+$ T cells are reduced [30].

Our aim was to analyze possible relationships between CD4 nadir and the lack of CD4 recovery in HIV-infected patients in whom ART did not result in normalization of CD4$^+$ T lymphocytes despite suppressing HIV replication, and to identify immune mechanisms involved in these interactions. Data herein indicate that defective CD4 cell counts recovery in ART-treated patients with suppressed viremia is associated with lower CD4 nadir, gut microbial translocation and immune activation, augmented percentage and activity of Treg lymphocytes,

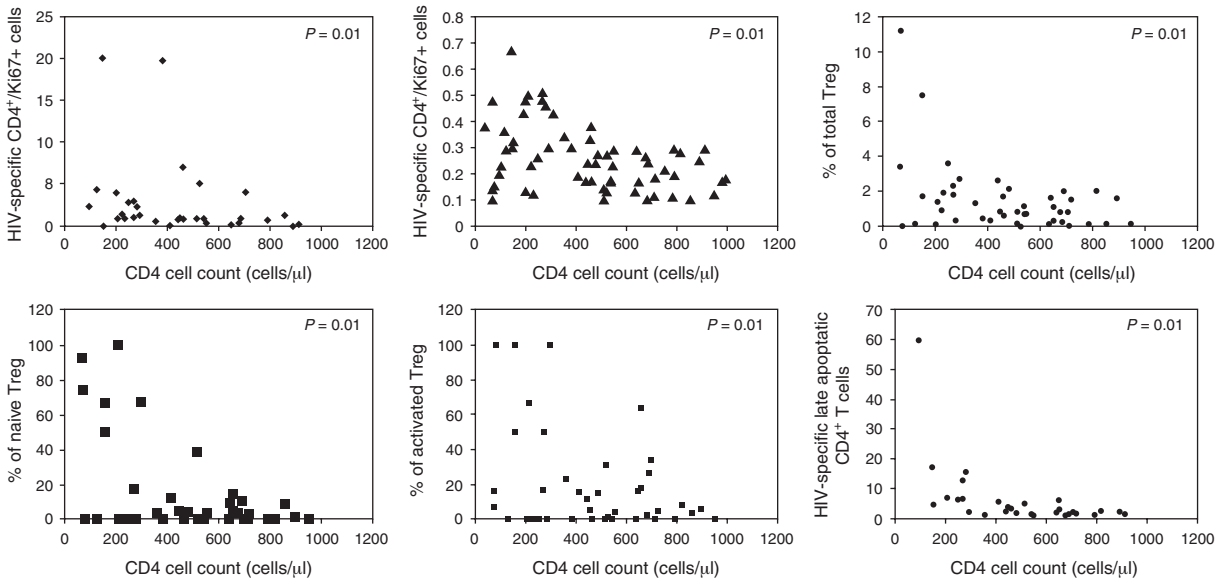

Fig. 5. Correlations between percentage of HIV-specific Ki67$^+$CD4$^+$ cells, plasma lipopolysaccharide concentration, percentage of total Treg, percentage of naive Treg, percentage of activated and percentage of HIV-specific late apoptotic CD4$^+$ T cells and CD4 T-cell counts in HIV-infected antiretroviral therapy-treated individuals. *P* values are shown.

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and higher susceptibility to apoptosis. Our results are in agreement with earlier studies reporting a correlation between CD4 nadir and increases in $CD4^+$ T cells during ART, and identify novel mechanisms possibly responsible for lack of $CD4^+$ T cells during ART. Our working hypothesis is that the complex pattern of immune dysfunctions observed in patients with incomplete immune reconstitution causes low CD4 cell counts; this argument could nevertheless be turned upside down: lack of CD4 recovery could be the cause, and not the consequence of immune impairment. This problem does not offer any easy solution; immunological analyses performed during the first year of therapy in a large group of patients, some of whom will not show significant increases in CD4 cell counts, will be needed to settle this question.

Several evidence suggests that the accelerated immunological ageing and age-associated complications, including cardiovascular, renal, and liver diseases and malignancies, seen in HIV infection, could be explained by the chronic immune activation that characterizes this disease [18–20]. Notably, the non-AIDS comorbidities that are more common in patients with persistently reduced $CD4^+$ T cells were recently suggested to be mainly associated with immune activation [29–32]. The underlying causes of the immune activation seen in HIV infection are still not totally clarified. Interestingly, the importance of chronic immune activation in HIV pathogenesis was confirmed in mouse studies showing that this process results in severe immune dysfunctions and opportunistic infections, even in absence of viral replication activation [32].

One of the most validated and solid hypotheses to explain HIV-associated immune activation stems from the observation that acute HIV infection is associated with a rapid and probably irreversible destruction of the extensive $CD4^+$ T-cell population that reside in gut-associated lymphoid tissues [19–22]. Loss of mucosal integrity results in impaired local cellular immunity and translocation of microbial products, including LPS, which in turn contributes to persistent inflammation through TLR activation [19–22]. Because LPS ligates TLR4, a molecule expressed on a variety of immune cells [33,34] the LPS-TRL4 axis has been postulated as being responsible for HIV-associated immune activation [35,36]. The lamina propria macrophages can induce Treg differentiation and limit the generation of pro-inflammatory immune responses. Spontaneous IL-10 production by lamina propria macrophages controls their reactivity to various TLR ligands and promotes the generation of tolerogenic IL-10-producing T cells and, when combined with TGF-β, also of Foxp3 Treg cells. In mouse models [37] it was demonstrated that lamina propria macrophages counteracted the ability of IL-17-producing T cells. These findings emphasize the complexity of the intestinal APC network that differentially modulates mucosal and systemic immune activation [38]. Given the deleterious effects of sustained inflammatory responses, the immune system goes to great lengths to prevent such responses. The main mechanism involved in the maintenance of immune homeostasis involves Treg cells, a subset of $CD4^+$ T lymphocytes that suppress local T-cell activation via direct and indirect mechanisms [39]. There are conflicting results regarding the role of Treg in the immunopathogenesis of HIV disease. Some data indicate that these cells prevent the development of effective antiviral immune responses and are therefore harmful [40,41], whereas others have proposed that Treg play a beneficial role by preventing chronic immune activation [42–44]. Treg express TLR, and their activity, expansion, and function are likely dependent on TLR-mediated signaling [44,45]. We observed significantly increased percentages of HIV-specific TLR2- and TLR4-expressing Treg cells in patients in whom ART does not increases $CD4^+$ T lymphocytes. Notably, serum concentrations of LPS were significantly increased as well in these same patients, in whom a high degree of immune activation was detected. The presence of significant negative correlations between $CD4^+$ T lymphocytes, immune activation, microbial translocation, and CD4 cell counts indicate that these parameters likely play a pathogenic role in these patients.

Recent data indicating that Treg cells can be further subdivided into two populations based on the intra (naïve)- or extra (activated)-cellular expression of the PD1 molecule [45–50], stimulated us to examine such populations in our patients. All populations of Treg cells were indeed augmented in patients in whom ART does not increase CD4 cell counts, suggesting a full-blown effort of the immune system in the attempt to reduce immune activation. Treg cells mediate their effect via two complementary mechanisms: IL-10-mediated and TGF-β-mediated functional impairment of immune cells, and induction of apoptosis of such cells [50–54]. Our results show that both mechanisms are active in patients in whom ART does not increases CD4 cell counts. Thus, in these patients HIV-stimulated production of IL-10 and TGF-β was increased, and expression of caspases 8 and 9 (apoptotic cells), as well as early and late apoptotic $CD4^+$ T cells, were augmented. Preliminary results showing that in individuals with $CD4^+$ T cells less than 500 cells/μl the blockage of the PD1–PDL1 pathway, a mechanisms of pivotal importance in inducing apoptosis, induces an increase in viable and a decrease of early and late apoptotic cells indicate that this pathway likely plays a role in lack of CD4 recovery in ART-treated individuals.

Results herein suggest a multifactorial model explaining failed CD4 recovery in successfully ART-treated patients. Thus, altered gut permeability resulting in increased LPS serum concentrations would trigger TLR-4-mediated immune activation of multiple cell types, including Treg. Activated Treg lymphocytes could prevent full $CD4^+$ T

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cells reconstitution via both indirect (immunosuppressive cytokines) and direct (apoptosis) mechanisms. The observation that, with the exception of caspase 9-expression, all the immune parameters examined are altered upon HIV but not CMV stimulation deserves further analyses and underlines the extreme expansion of HIV-specific lymphocytes in this infection. From a clinical standpoint, the observation that lower nadir likely result in a more difficult increase of $CD4^+$ T cells heightens the suggestion that the CD4 threshold actually used to start ART could be reassessed, and reinforces the need for effective immune modulators in the therapy of HIV infection.

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