

Memory and naive-like regulatory CD4⁺ T cells expand during HIV-2 infection in direct association with CD4⁺ T-cell depletion irrespectively of viremia

Russell B. Foxall^a, Adriana S. Albuquerque^a, Rui S. Soares^a,
António P. Baptista^{a,*}, Rita Cavaleiro^a, Rita Tendeiro^a,
Perpétua Gomes^b, Rui M.M. Victorino^{a,c} and Ana E. Sousa^a

Objective: The dynamics of CD4⁺ regulatory T cells (Treg) during HIV-1 infection remains unclear. To further investigate Treg in this context, we characterized, for the first time, this population in HIV-2-positive individuals. Although both HIV infections are associated with hyperimmune activation and CD4⁺ T-cell lymphopenia, most HIV-2-positive individuals display slower disease progression and low-to-undetectable viremia.

Design/methods: Samples were obtained from cohorts of untreated HIV-2-positive and HIV-1-positive, treated HIV-1-positive and seronegative individuals. The proportion of CD4⁺ T cells bearing a Treg phenotype, defined in terms of high-level CD25 or Foxp3 expression, was assessed by flow cytometry and correlated with markers of disease progression. The proportions of naive and memory-like subsets as well as cycling cells were determined.

Results: We observed an increased proportion of Treg, associated with disease progression, as well as increased proportions of cycling (Ki67⁺) memory Treg, in untreated HIV-2-positive and HIV-1-positive individuals. We also noted an expansion of Treg that persisted over time in treated, immunologically discordant HIV-1-positive individuals, who, similarly to HIV-2-positive patients, present undetectable viremia and low CD4 T-cell count.

Conclusion: Overall, we demonstrated that Treg frequency was increased in all lymphopenic HIV-2-positive and HIV-1-positive individuals irrespectively of the presence or absence of viremia or antiretroviral treatment. This, in turn, suggests that the observed alterations in Treg frequency in HIV/AIDS are more directly related to the degree of CD4 depletion than to viremia.

© 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins

AIDS 2011, **25**:1961–1970

Keywords: HIV/AIDS, HIV-2, immune activation, regulatory T cells

Introduction

CD4⁺ regulatory T cells (Treg) play vital roles in T-cell homeostasis [1] and the control of immuno-

pathology associated with persistent pathogens [2]. In HIV/AIDS, they may contribute to viral-persistence through the suppression of specific T-cell responses [3] while simultaneously limiting damage

^aInstituto de Medicina Molecular, Faculdade de Medicina, Universidade de Lisboa, ^bLaboratório de Biologia Molecular, Serviço de Medicina Transfusional, Hospital Egas Moniz Lisboa, and ^cClínica Universitária de Medicina 2, Hospital de Santa Maria, Faculdade de Medicina, Universidade de Lisboa, Lisbon, Portugal.

Correspondence to Ana E. Sousa, MD, PhD, Unidade de Imunologia Clínica, Instituto de Medicina Molecular, Faculdade de Medicina de Lisboa, Av. Prof. Egas Moniz, 1649-028 Lisbon, Portugal.

Tel: +351 21 7999525; fax: +351 21 7999527; e-mail: asousa@fm.ul.pt

* Current address: Department of Molecular Cell Biology and Immunology, Vrije University Medical Center, Amsterdam, The Netherlands.

Received: 5 May 2011; revised: 1 July 2011; accepted: 22 July 2011.

DOI:10.1097/QAD.0b013e32834b3554

resulting from continuous immune stimulation [4].

Chronic HIV-1 infection is associated with persistent hyperimmune activation that is thought to be a main determinant of the progressive CD4⁺ T-cell depletion [5–7]. Although, Treg, with a demonstrable capacity to suppress HIV-1-specific T-cell responses [8–11], have been consistently shown to be present in significant numbers in lymph nodes of HIV-1-positive individuals [12,13], even in advanced stages [14,15], data on the frequency of circulating Treg during HIV-1 infection are much more conflicting [16–18].

There are no data regarding Treg in HIV-2-positive individuals. HIV-2, in contrast to HIV-1 infection, is associated with low-to-undetectable levels of circulating virus [19,20] and a very slow rate of CD4⁺ T-cell decline [19,20], leading to a reduced impact on the survival of the majority of infected adults [21,22]. Nevertheless, comparable degrees of hyperimmune activation and naive-memory CD4⁺ T-cell imbalances have been observed in both infections, when infected individuals were matched for a given level of CD4 depletion [23,24]. These findings further support a closer link between CD4 depletion and immune activation than viral load in HIV/AIDS. It is plausible that Treg play a role in the much slower rate of disease progression associated with HIV-2 infection.

To address this possibility, we investigated, for the first time, the circulating Treg pool in HIV-2-positive patients. In addition to untreated HIV-2 and HIV-1 infections, we studied antiretroviral therapy (ART) treated HIV-1-positive individuals that, despite suppression of viremia, exhibited variable degrees of CD4⁺ T-cell recovery, that may in part be due to reduced thymic activity [25,26]. The study of these cohorts, characterized by distinct degrees of viremia, CD4⁺ T-cell depletion, immune activation and putative thymic impairment allowed us to address potential factors contributing to the maintenance of circulating Treg.

Participants and methods

Studied cohorts

Thirty-one HIV-2-positive, and 26 HIV-1-positive ART-naïve individuals without clinical signs of opportunistic infections or tumors at the time of study were assessed together with 20 seronegative controls (Table 1). Both HIV cohorts featured individuals with a similar range of CD4⁺ T-cell depletion. As expected, the majority of HIV-2-positive individuals were aviremic, whereas a wide range of viremia was observed in the HIV-1 cohort [27]. A previously described cohort of ART-treated HIV-1-positive individuals with variable degrees of CD4⁺ T-cell recovery despite the suppression of viremia was also assessed in this study [28]. All participants gave informed consent to blood sampling and processing, and the study was approved by the Ethical Board of the Faculty of Medicine of Lisbon.

Cell preparation and flow cytometric studies

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood by Ficoll-Hypaque (Gibco-Invitrogen, Carlsbad, California, USA) gradient centrifugation, immediately after venipuncture and stained with combinations of the following monoclonal antibodies (mAbs): FITC-conjugated CD45RA, HLA-DR and Ki67; PE-conjugated CCR7, CD38, CD45RO and CD62L; PerCP-conjugated CD3, CD4 and CD8; PE-Cy7-conjugated CD25; allophycocyanin (APC)-conjugated CD25 and CD45RA; and APC-Cy7-conjugated CD4; purchased from either BD Biosciences (San Jose, California, USA), eBiosciences (San Diego, California, USA) or R&D Systems (Minneapolis, Minnesota, USA). Intracellular staining for Foxp3 was performed according to manufacturer's instructions (FoxP3 staining set, clone PCH101; eBiosciences), and the same protocol was used for Ki67 intracellular staining. The CD4⁺CD25⁺ population was separated into dim and bright, according to criteria that we have previously detailed [29]. The lower limit of the CD25^{bright} region was set to include only the discrete CD4⁺ T-cell population expressing CD25 at the highest levels. Freshly

Table 1. Characteristics of the cohorts.

	Seronegative	Untreated HIV-2	Untreated HIV-1
Number (males/females)	20 (6/14)	26 (20/6)	31 (8/23)
Age (years)	42 ± 3 (22–58)	49 ± 3 (19–78)	39 ± 2 ^a (23–61)
% CD4 ⁺ T cells	43.6 ± 1.8 (31.1–61.1)	28.4 ± 2.4 ^b (7.1–54.1)	23.3 ± 3 ^b (1.3–47.2)
CD4 cell count (cells/μl)	942 ± 54 (518–1332)	658 ± 74 ^c (52–1511)	571 ± 93 ^c (18–1848)
Viremia (RNA copies/ml)	NA	2966 ± 1049 ^d (200–2.63 × 10 ⁵)	5.75 × 10 ⁵ ± 2.63 × 10 ^{5a,e} (40–4.47 × 10 ⁶)

Data are mean ± SEM with limits in brackets. NA, not applicable.

^aStatistical differences between HIV-1-positive and HIV-2-positive individuals: $P < 0.01$.

^bStatistical differences between a given HIV-positive cohort and the seronegative controls: $P < 0.05$.

^cStatistical differences between a given HIV-positive cohort and the seronegative controls: $P < 0.0001$.

^dHIV-2 viremia was quantified in 30 individuals and was below 200 RNA copies/ml (cut-off value of the assay) in 21/30. For the sake of comparison, aviremic individuals were assigned the cut-off value of the assay (200 copies/ml).

^eHIV-1 viremia was quantified in 25 individuals and was below 40 RNA copies/ml (cut-off value of the assay) in three of 25. For the sake of comparison, aviremic individuals were assigned the cut-off value of the assay (40 copies/ml).

isolated PBMCs were used except where otherwise specified. In these cases, PBMCs were frozen immediately after isolation and stored in liquid nitrogen until thawing. Cells were acquired using either FACSCalibur or FACSCanto flow cytometers (BD Biosciences). Analysis was performed using Cellquest software (BD Biosciences) and FlowJo (Tree Star Inc., Ashland, Oregon, USA). The absolute numbers of lymphocyte subsets were calculated by multiplying their representative frequency by the absolute lymphocyte count obtained at the clinical laboratory.

Plasma viral load assessment

Viremia was quantified using RT-PCR-based assays with a detection threshold of 40 RNA copies/ml for HIV-1 (Roche, Basel, Switzerland) and 200 RNA copies/ml for HIV-2 [30]. The cut-off values of the tests were considered for the purpose of the analysis in the cases where detection was below this level.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.00 (GraphPad Software, San Diego, California, USA). The data are presented as box and whisker plots, with the upper and lower whiskers set at the 90th and 10th percentile, respectively, or as mean \pm SEM, and were compared using Mann–Whitney test and Wilcoxon-matched pairs test, as appropriate. Spearman's correlation coefficient was used to assess the correlation between two variables. Multivariate analysis was performed using stats package of R statistical environment (www.R-project.org). *P* values less than 0.05 were considered significant.

Results

The frequency of regulatory T cells increases with the progression of HIV-2 infection in direct correlation with CD4⁺ T-cell depletion

There are currently no markers absolutely specific for the Treg subset. Human Treg are apparently restricted to CD4⁺ T cells expressing the IL-2R α chain (CD25) at very high intensity [31,32], based on their suppressive capacity and high-level expression of other putative Treg markers, particularly the forkhead-box transcription factor P3 (Foxp3) [31,33]. Although initially described as a highly specific Treg lineage marker, Foxp3 has increasingly been shown to be transiently upregulated upon T-cell receptor stimulation of human CD4⁺ T cells [34,35]. Low-level expression of IL-7R α (CD127) is also an additional Treg marker, but its use in HIV/AIDS is debatable given the acknowledge disturbances of the IL-7/CD127 network [36,37].

We found that the percentage of CD25^{bright} within CD4⁺ T cells was similar in both the untreated HIV-2 and HIV-1 cohorts (2.15 ± 0.22 and 2.21 ± 0.28 ,

respectively), despite the distinct viral load (Table 1). However, the negative correlation between this population and the absolute number of CD4⁺ T cells we observed was more marked in the HIV-2 cohort ($r = -0.656$, $P < 0.0001$, $n = 31$ for HIV-2; $r = -0.438$, $P = 0.0252$, $n = 26$ for HIV-1). In agreement with this observation, HIV-2-positive patients exhibited a reduced percentage of Treg in early-stage disease (>350 CD4⁺ T cells/ μ l), which reached significance with respect to late-stage infection (<350 CD4⁺ T cells/ μ l) and seronegative individuals (Fig. 1a). As noted above, a significant number of HIV-2-positive individuals had undetectable viremia (Table 1). Nevertheless, the assessment of the relationship between the percentage of CD25^{bright} CD4⁺ T cells and viremia revealed no significant correlation, in contrast to HIV-1-positive individuals ($r = 0.216$, $P = 0.253$, $n = 30$ for HIV-2; $r = 0.625$, $P = 0.0008$, $n = 25$ for HIV-1).

We assessed the frequency of Foxp3 expression within CD4⁺ T cells and found it was similar in representative subgroups of ART-naïve HIV-2-positive and HIV-1-positive individuals (3.86 ± 0.92 , $n = 9$; 3.82 ± 1.13 , $n = 10$, respectively) and increased with CD4⁺ T-cell depletion in both HIV cohorts (Fig. 1b). This relationship was confirmed by the significant correlations we observed between the frequency of this population and CD4 depletion in both HIV cohorts ($r = -0.8$, $P = 0.014$, for HIV-2; $r = -0.684$, $P = 0.034$, for HIV-1). Of note, when Treg were defined as the proportion of CD25^{bright}Foxp3⁺ cells, we found a similar profile to that observed for CD25^{bright} and Foxp3⁺ alone (data not shown).

The HIV-1 cohort featured an overrepresentation of individuals able to control viral replication in the absence of antiretroviral drugs, so-called 'elite controllers' [38]. We took advantage of these three individuals, who were aviremic during follow-up of 2–10 years and had CD4⁺ T-cell counts 500 cells/ μ l or higher, to further assess the relative impact of CD4⁺ T-cell depletion and viremia upon circulating Treg levels. Irrespective of viremic status and type of HIV infection, Treg frequency was reduced in individuals with CD4⁺ T-cell counts 500 cells/ μ l or higher, and increased in advanced disease, relative to that in seronegative individuals (Fig. 1c).

It is now accepted that the progressive hyperimmune activation associated with both HIV-1 and HIV-2 infections plays an important role in their immunopathogenesis. In particular, CD8⁺ T-cell activation measured as the mean fluorescence intensity (MFI) of CD38 is a known independent marker of disease progression [5,39]. Treg frequency, defined as Foxp3⁺ CD4⁺ T cells, was positively correlated with this T-cell activation parameter in our HIV cohorts ($r = 0.8$, $P = 0.0096$, $n = 31$ for HIV-2; $r = 0.73$, $P = 0.0158$, $n = 26$ for HIV-1), and similar results were found

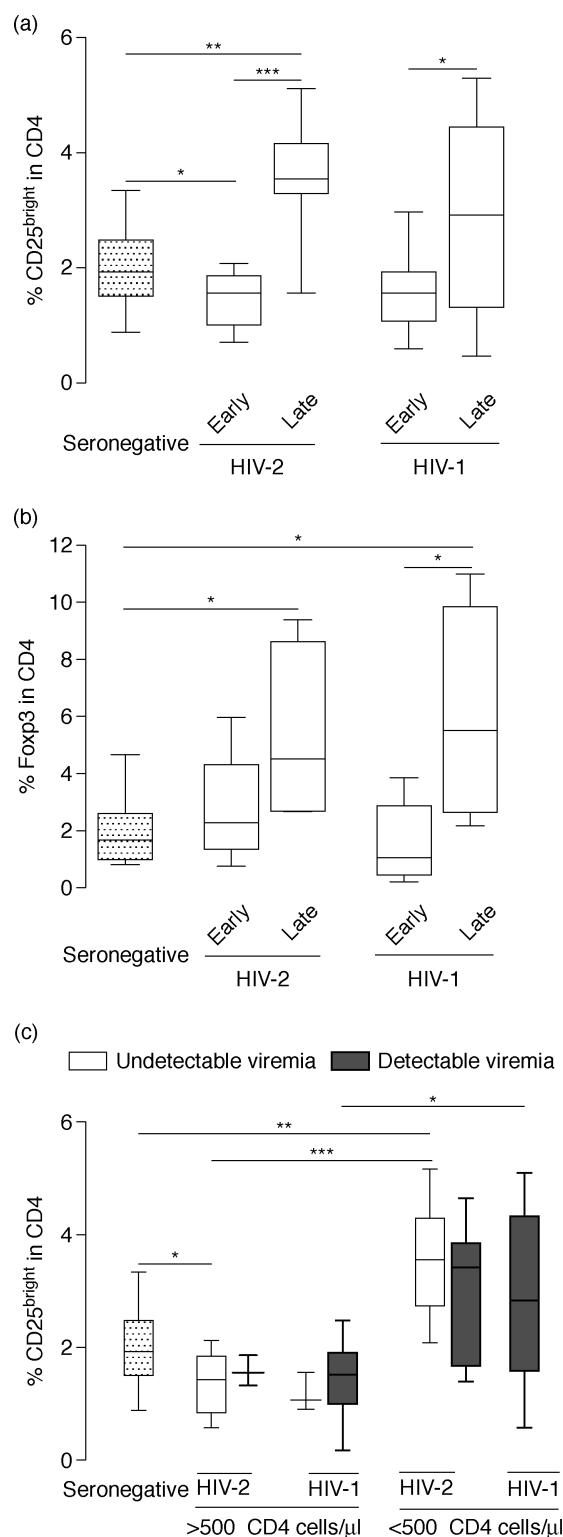


Fig. 1. Frequency of regulatory T cells within the circulating CD4⁺ T-cell subset in untreated HIV-2 and HIV-1 cohorts.

(a) Proportion of CD25^{bright} cells within CD4⁺ T cells in HIV-2-positive and HIV-1-positive cohorts and seronegative controls are shown. In order to compare these levels in early and late disease, the HIV cohorts were stratified according to CD4⁺ T-cell counts and divided into early (>350 CD4⁺

regarding CD25^{bright} CD4⁺ T cells ($r=0.415$, $P=0.0202$, $n=31$ for HIV-2; $r=0.622$, $P=0.0007$, $n=26$ for HIV-1). To further investigate the links between the expansion of the CD25^{bright} subset, immune activation and CD4⁺ T-cell depletion, a multiple regression analysis was performed (see Supplemental Digital Content 1, <http://links.lww.com/QAD/A159>, for the results of this analysis). Of note, this expansion was strongly affected by immune activation in HIV-1-positive individuals ($P<0.0001$) and, to a lesser degree, in HIV-2-positive individuals ($P=0.037$). However, a strong correlation with CD4⁺ T-cell numbers was only observed in HIV-2-positive individuals ($P<0.0001$), suggesting that the CD25^{bright} expansion in these individuals is more linked to CD4 depletion than to immune activation.

In summary, the frequency of Treg, irrespective of the markers used to define this population, progressively increased in parallel with CD4⁺ T-cell decline, reaching significantly elevated levels in advanced HIV disease. Given the reduced viral load associated with HIV-2 infection, our data suggest that Treg alterations are more directly related to CD4⁺ T-cell depletion than to viremia.

Analysis of the regulatory T-cell pool in relation to imbalances in naive and memory-effector CD4⁺ T cells during HIV-2 infection

Several reports have demonstrated that Treg can be subdivided on the basis of CD45RA expression into two distinct Treg subpopulations: CD45RA⁺ 'naive-resting' Treg and their CD45RA⁻ 'memory-activated' counterparts [32,40].

The majority of Treg in HIV-2-positive individuals, irrespective of how they were defined, lacked CD45RA

T cells/μl, $n=21$ and 15 for the HIV-2 and HIV-1 cohorts, respectively) and late (<350 CD4⁺ T cells/μl, $n=10$ and 11 for the HIV-2 and HIV-1 cohorts, respectively) stage disease. (b) Analysis of the frequency of Foxp3⁺ cells within total CD4⁺ T cells in the HIV-2 and HIV-1 cohorts stratified as above (>350 CD4⁺ T cells/μl, $n=5$ for both the HIV-2 and HIV-1 cohorts; <350 CD4⁺ T cells/μl, $n=4$ and 5 for the HIV-2 and HIV-1 cohorts, respectively). (c) Proportion of CD25^{bright} cells within the circulating CD4⁺ T-cell subset in HIV-2 and HIV-1 cohorts subdivided according to CD4⁺ T-cell counts higher than or less than 500 CD4⁺ T cells/μl; as well as the presence (gray bars) or absence (open bars) of detectable viremia (the latter defined as viremia below the cut-off of the relevant tests; 15 out of 18 and three out of 12 for the HIV-2 and HIV-1 cohorts, respectively, in the group with more than 500 CD4⁺ T cells/μl; six out of 12 for the HIV-2 cohort in the group with less than 500 CD4⁺ T cells/μl). Of note, all 14 HIV-1-positive individuals with less than 500 CD4⁺ T cells/μl had detectable viremia. Box and whisker plots are shown, with the upper and lower whiskers set at the 90th and 10th percentile, respectively. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$.

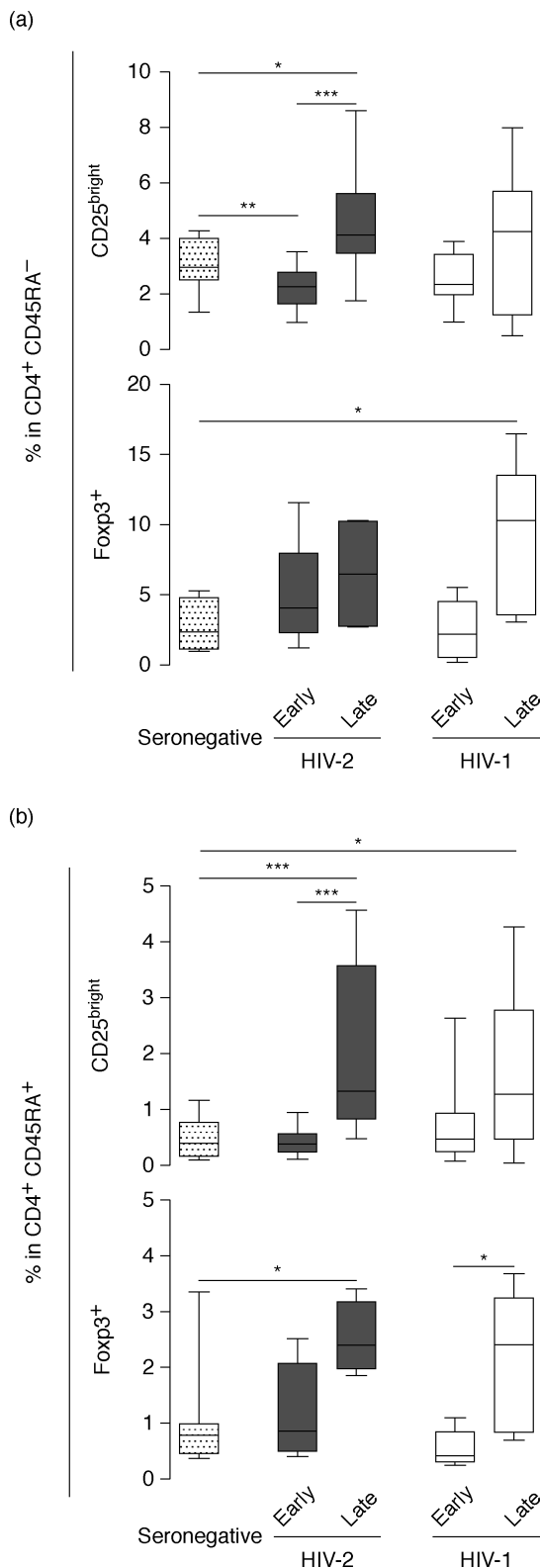


Fig. 2. Naive-memory CD4⁺ T-cell imbalances and regulatory T-cell frequency in untreated HIV-2 and HIV-1 cohorts. Proportion of CD25^{bright} cells and Foxp3⁺ cells within memory (CD45RA⁻) CD4⁺ T cells (a) and within naive (CD45RA⁺) CD4⁺ T cells (b) in seronegative individuals ($n = 20$) and in the HIV-2 and HIV-1 cohorts stratified according to their CD4⁺ T-

expression (>80%), as previously reported for HIV-1-positive and seronegative individuals [31].

Progressive HIV-2 infection is associated with a reduction in naive and a concomitant increase in memory CD4⁺ T-cell compartments [23]. Thus, it is possible that the increased frequency of Treg associated with disease progression we observed may simply reflect this phenomenon. However, we observed a significantly lower proportion of CD25^{bright} within the memory subset in early-stage HIV-2 infection and a significantly higher proportion in the late-stage infection compared with seronegative controls (Fig. 2a), resulting in a significant positive association between the proportion of CD25^{bright} within the memory subset and CD4 depletion ($r = -0.65$; $P < 0.0001$, $n = 31$). This suggests that the expansion of CD25^{bright} in CD4⁺ T cells associated with CD4 depletion observed in HIV-2-positive individuals was not solely related to the progressive naive/memory imbalance associated with disease progression. Although the same trend was observed with respect to Foxp3 expression, the differences between early-stage and late-stage HIV-2 disease were not significant (Fig. 2a).

Importantly, a distinct population of 'naive resting' CD45RA⁺ within the Treg pool was observed in the HIV-2 cohort representing $13.5 \pm 2.2\%$ of CD25^{bright} CD4 T cells and $18.1 \pm 4.2\%$ of Foxp3⁺ CD4 T cells.

Of note, the proportion of Treg within naive CD4⁺ T cells increased with disease progression in HIV-2-positive individuals, leading, in late-stage disease, to levels similar to those observed in late-stage HIV-1 infection and a significant overrepresentation of this population as compared with seronegative individuals (Fig. 2b). Moreover, a significant negative correlation was found between the proportion of CD25^{bright} cells within the naive subset and CD4 cell counts in HIV-2 infection ($r = -0.586$, $P = 0.0005$, $n = 31$).

In conclusion, these data suggest that the observed progressive increase in Treg frequency within the CD4⁺ T-cell subset in HIV-2 infection cannot be solely attributed to the relative expansion of CD4⁺ memory T cells associated with disease progression, and that a relative expansion of both resting 'naive-like' and activated Treg occurs.

cell counts into early (>350 CD4⁺ T cells/ μ L; $n = 21$ and 15 for the HIV-2 and HIV-1 cohorts, respectively) and late (<350 CD4⁺ T cells/ μ L; $n = 10$ and 11 for the HIV-2 and HIV-1 cohorts, respectively) stage disease. Box and whisker plots are shown, with the upper and lower whiskers set at the 90th and 10th percentile, respectively. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Analysis of cycling regulatory T cells during HIV-2 infection

We next assessed whether the relative Treg expansion we observed in HIV-2-positive patients was associated with increased cell turn over. Ki67 is upregulated in cycling cells and, thus, has been used as a marker to estimate proliferating cells *ex vivo* [32]. As we have previously shown [23], HIV-2, like HIV-1 infection, was associated

with increased ex-vivo cycling of CD4⁺ T cells as a whole (7.2 ± 2.53 and 10.74 ± 3.07 , respectively) as compared with seronegative individuals (1.79 ± 0.26 ; $P = 0.0101$ and $P = 0.0039$, respectively).

Regarding the Treg subset, Ki67 expression was mainly confined to 'memory' Treg in HIV-2-positive individuals, as illustrated by the representative case illustrated in

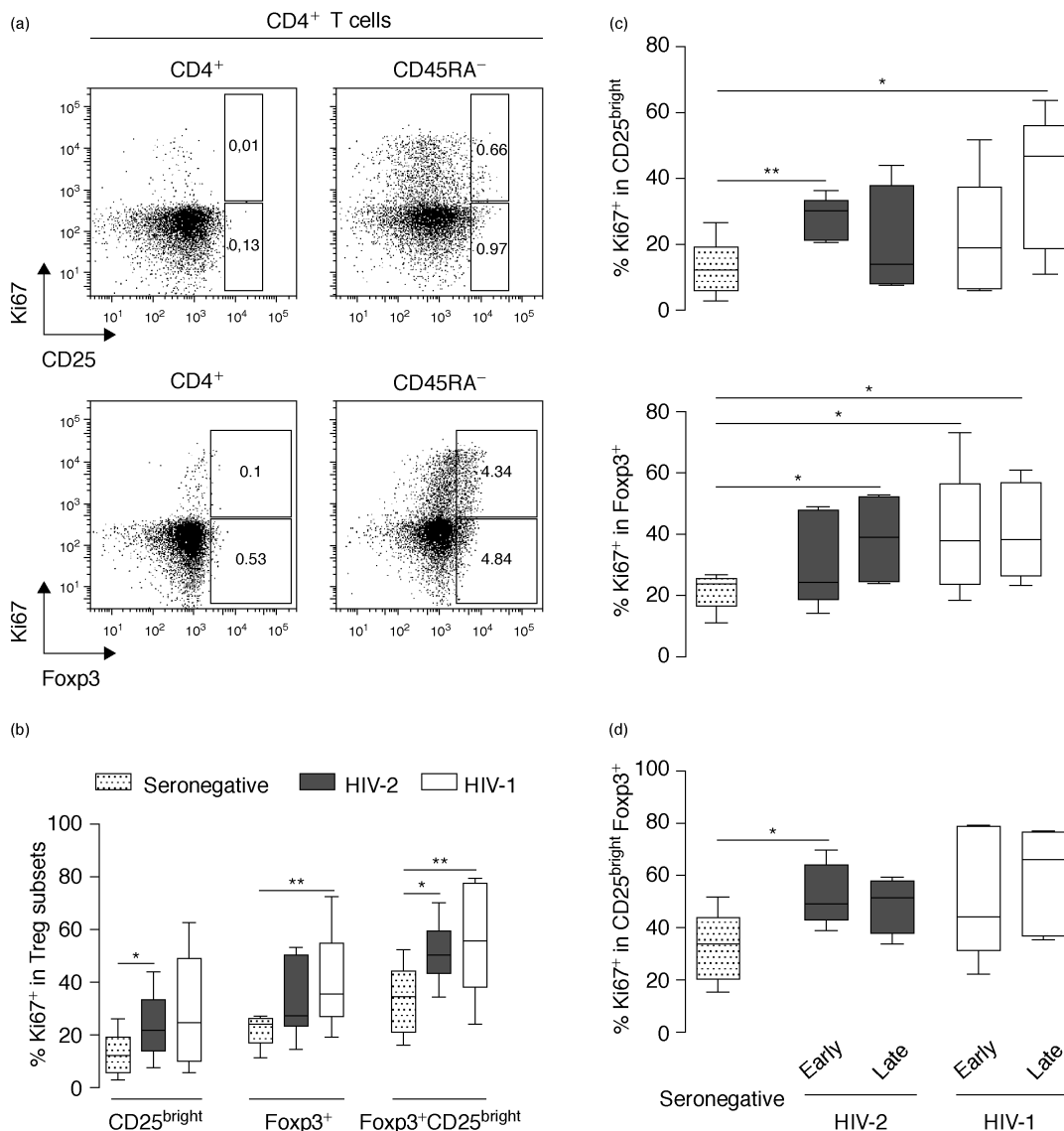


Fig. 3. Frequency of cycling regulatory T cells in untreated HIV-2 and HIV-1 cohorts. (a) Representative dot plots from an HIV-2-positive individual (a 57-year-old, therapy-naive patient with a CD4⁺ T-cell count of 480 CD4⁺ T cells/ μ l) illustrating the pattern of Ki67 staining in CD25^{bright} or Fopx3⁺ subpopulations of CD4⁺-naive (CD45RA⁺) and memory (CD45RA⁻) T cells. (b) Proportion of Ki67⁺ cells within the CD4⁺CD25^{bright}, CD4⁺Fopx3⁺ and CD4⁺CD25^{bright}Fopx3⁺ populations in seronegative controls (n=10), HIV-2 (n=9) and HIV-1 (n=10) cohorts. (c) Graphs illustrate the proportion of Ki67⁺ cells within the CD4⁺CD25^{bright} (top), CD4⁺Fopx3⁺ (middle) and CD4⁺CD25^{bright}Fopx3⁺ (bottom) populations in the HIV-2 and HIV-1 cohorts stratified according to their CD4⁺ T-cell counts into early (>350 CD4⁺ T cells/ μ l; n=5 for both the HIV-2 and HIV-1 cohorts) and late disease (<350 CD4⁺ T cells/ μ l; n=4 and 5 for the HIV-2 and HIV-1 cohorts, respectively) as compared with seronegative controls (n=10). Box and whisker plots are shown, with the upper and lower whiskers set at the 90th and 10th percentile respectively. * $P < 0.05$, and ** $P < 0.01$.

Fig. 3a, as previously reported for HIV seronegative individuals [32], and further supporting the resting nature of CD45RA⁺ Treg in the HIV-2 cohort.

The proportion of cycling Treg, whether defined as CD25^{bright} and/or Foxp3⁺, was always increased relative to the proportion of cycling total CD4⁺ T cells in all three cohorts (data not shown), although both HIV cohorts featured higher levels than their seronegative counterparts (Fig. 3b). Of note, the proportions of cycling Treg did not significantly differ in HIV-2-positive and HIV-1-positive individuals (Fig. 3b). Additionally, no significant differences were observed between early and late stages of either HIV infection (Fig. 3c).

Hyperimmune activation has been shown to be an important determinant of increased cycling in CD4⁺ T cells in HIV-2 infection [23]. Of note, a direct correlation between the proportion of cycling cells within Foxp3⁺ Treg and both immune activation (as measured by the MFI of CD38 on CD8⁺ T cells; $r = 0.733$, $P = 0.0246$, $n = 9$) and the frequency of cycling cells within total CD4⁺ T cells ($r = 0.833$, $P = 0.0053$, $n = 9$) was observed in HIV-2-positive individuals, suggesting that CD4⁺Foxp3⁺ cell turnover may be directly related to hyperimmune activation in HIV-2 infection. Notably, no such correlations were observed for cycling cells within CD4⁺CD25^{bright} ($r = 0.268$, $P = 0.486$; $r = 0.109$, $P = 0.781$; for percentage Ki67⁺ CD4⁺ T cells and CD38 MFI on CD8⁺ T cells, respectively, $n = 9$) and CD4⁺CD25^{bright}Foxp3⁺ populations ($r = 0.4$, $P = 0.286$; $r = 0.217$, $P = 0.576$ for percentage Ki67⁺ CD4⁺ T cells and CD38 MFI on CD8⁺ T cells, respectively, $n = 9$). These data suggest that the turnover of Foxp3⁺, but not CD25^{bright} or CD25^{bright}Foxp3⁺, cells is more directly related to T-cell activation during HIV-2 infection.

Overall, HIV-2 disease was associated with an increase in the frequency of cycling Treg irrespective of disease stage.

Characterization of regulatory T cells in antiretroviral therapy-discordant HIV-1-positive individuals and comparison with untreated HIV-2-positive individuals in late-stage disease

More than 10% of ART-treated HIV-1-positive individuals exhibit low CD4 cell counts (immunological failure) despite a maintained reduction in viremia to undetectable levels (virologic response) [25,41,42]. These individuals (ART-discordant individuals) are comparable to HIV-2-positive individuals with marked CD4⁺ T-cell depletion in the absence of viremia. We assessed the proportion of CD25^{bright} cells within the CD4⁺ T-cell subset in our previously described ART-discordant and ART-responsive HIV-1-positive cohorts [28] and found a significant increase in the former as compared with the latter (3.5 ± 0.47 vs. $2 \pm 0.25\%$, $P = 0.0256$). When the relationship between CD4⁺ T-cell levels and the proportion of CD25^{bright} within the CD4⁺ T-cell subset

in this treated HIV-1-positive cohort as a whole was assessed, we observed negative correlations with both the number ($r = -0.4671$, $P = 0.0284$) and percentage ($r = -0.6217$, $P = 0.002$) of CD4⁺ T cells, further supporting an association between relative Treg expansion and CD4 lymphopenia rather than viral load.

The cross-sectional nature of this study precluded the definition of the causal relationship between immunological failure and increased frequency of CD25^{bright}, as it was not possible to determine whether this population existed prior to, or emerged after, starting ART. However, comparison with a second time-point in five of the ART-discordant individuals, with an intersampling mean of 42.6 ± 1.8 months, revealed a stable proportion of CD25^{bright} within total, naive and memory CD4⁺ T cells despite the considerable time between sampling and the tendency for CD4⁺ T cells to increase (Fig. 4a). However, the naive/memory imbalances persisted in both total CD4⁺ and Treg subsets (Fig. 4a), which was in marked contrast to data from a subgroup of ART-responder individuals, also evaluated at the same time, that exhibited a clear increase in the proportion of CD45RA⁺ within CD25^{bright} Treg (8.38 ± 2.41 vs. 21.19 ± 5.56 , $P = 0.0625$, mean intersampling period 42 ± 1.5 months, $n = 5$).

Of note, despite the increased proportion of CD25^{bright} within CD4⁺ T cells in ART-discordant individuals, the expression of Ki67 in this subset, as in bulk CD4⁺ T cells, was significantly lower in HIV-1-positive ART-discordant individuals as compared with seronegative and untreated late-stage HIV-1-positive or HIV-2-positive individuals (Fig. 4b).

Thus, in contrast to untreated HIV-2 and HIV-1 infections with a similar degree of CD4 depletion, a reduced frequency of cycling Treg was found in ART-discordant individuals (Fig. 4b), suggesting a possible role of increased survival rather than proliferation in maintaining CD25^{bright} Treg in these individuals.

Discussion

This study, the first to assess Treg in HIV-2-positive individuals, revealed a progressive increase in Treg frequency, in parallel with CD4 decline, which reached significantly elevated levels in advanced disease, similar to those found in HIV-1 individuals, despite the distinct viremia and prognosis of these two infections. Of note, both HIV infections were associated with significantly increased proportions of cycling Treg as compared with seronegative controls, suggesting a contribution of increased cell turn over to the maintenance of the Treg pool.

ART-treated HIV-1-positive individuals that suppress viremia, but fail to increase/restore their CD4⁺ T-cell

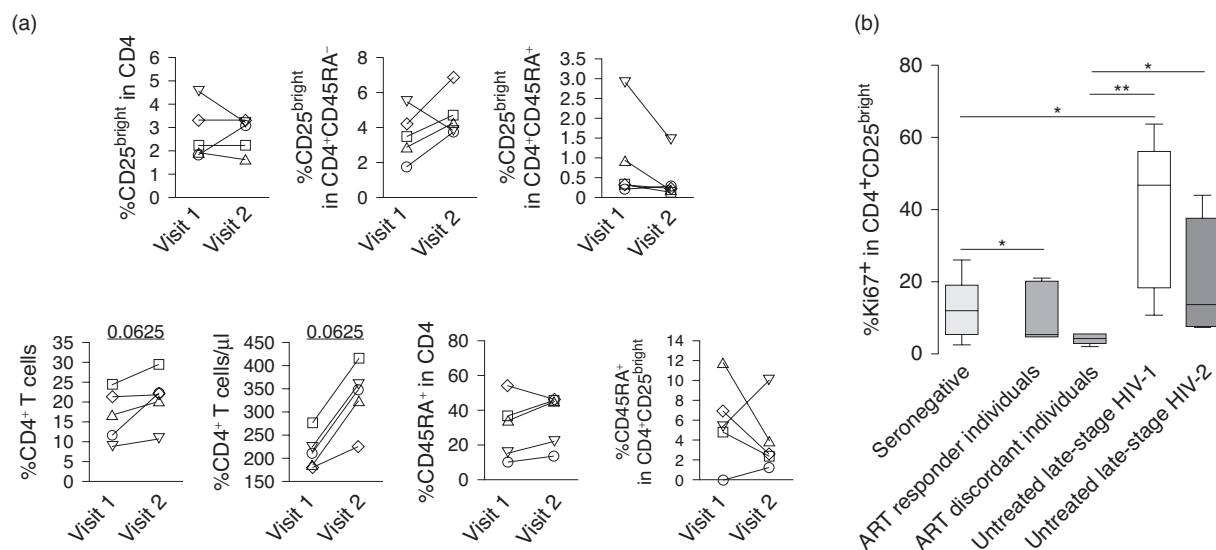


Fig. 4. Increased frequency of regulatory T cells in HIV-1-positive antiretroviral therapy-discordant infected patients as compared with untreated HIV-2-positive individuals. (a) ART-discordant individuals were assessed at two time-points with a mean interval of 42.6 ± 1.8 months. Graphs illustrate data collected at the two time-points in terms of: proportion of CD25^{bright} within CD4⁺ T cells, proportion of CD25^{bright} cells in memory (CD45RA⁻) and naive (CD45RA⁺) CD4⁺ T cells, frequency and absolute numbers of circulating CD4⁺ T cells and proportions of CD45RA⁺ cells within total CD4⁺ and CD4⁺CD25^{bright} T cells as indicated in the different graphs. Each dot represents one individual. (b) Frequency of cycling (Ki67⁺) cells in ART-discordant individuals ($n = 5$) within CD25^{bright}CD4⁺ T-cell subset in comparison with cohorts of ART-responder individuals ($n = 5$), as well as seronegative controls ($n = 10$), and untreated HIV-1-positive ($n = 5$) and HIV-2-positive ($n = 4$) individuals with less than 350 CD4⁺ T cells/ μ l. Box and whisker plots are shown, with the upper and lower whiskers set at the 90th and 10th percentile, respectively. ART, antiretroviral therapy. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

population (ART-discordant individuals), provide a comparable clinical model to ART-naïve HIV-2-positive individuals, as both feature marked CD4 depletion in the absence of detectable viremia [28]. We noted a significant increase in Treg frequency in ART-discordant individuals, suggesting that immunological failure under ART is associated with an expansion of this population. Longitudinal assessment of a subset of these individuals revealed that Treg were maintained at high levels over time, and that this was unlikely to be due to increased cell cycling given the low levels of Ki67 expression.

Overall, we showed that Treg frequency was increased in all lymphopenic HIV-1-positive and HIV-2-positive individuals irrespective of the presence or absence of viremia or ART suggesting that the relative expansion of Treg we observed was more directly related to the degree of CD4 depletion than to viremia.

Several mechanisms likely to contribute to the relative expansion of the Treg pool during HIV-2 disease progression.

Regarding early HIV-2 infection, it is possible that the significant reduction in the proportion of circulating Treg we observed as compared with seronegative controls reflects increased tissue homing [13,14]. Thus, an important complementary study would be the evaluation

of Treg frequency in lymphoid tissues from untreated HIV-2-positive individuals at an early stage of disease.

On the other hand, our analysis of Treg within the CD45RA⁺ and CD45RA⁻ subsets of CD4⁺ T cells suggested that the observed progressive increase in Treg frequency within the CD4⁺ T-cell subset in HIV-2 infection cannot be solely attributed to the relative expansion of CD4⁺ memory T cells associated with disease progression [43].

We observed a relative expansion of Treg within the naive CD4⁺ T-cell pool that increased with CD4⁺ T-cell decline both in HIV-2 and HIV-1 infections. This is of particular interest given that the proportion of naive CD4⁺ T cells decreased in parallel with CD4⁺ T-cell loss. Moreover, as the proportion of CD45RA⁺ within Treg was similar in HIV cohorts as compared with seronegative individuals and remained stable as CD4 frequency declined in both HIV-1-positive and HIV-2-positive individuals, it would appear that this population, unlike other naive CD4⁺ T-cell subsets, remains relatively unaffected in both HIV-1 and HIV-2 infection.

Treg ontogeny remains the subject of intense debate [32,44]. If these cells have a thymic origin, as has been suggested [32,45], our data raise the possibility of preferential and/or maintained production of the Treg

lineage in thymus of HIV-positive individuals, an idea supported by recently published data [46].

It has been suggested that CD45RA⁺ Treg represent a long-lived 'naive resting' subpopulation with the ability to replenish a cycling pool of short-lived CD45RA⁻ 'activated' Treg [32], and thus may play a critical role in Treg homeostasis. Given the relatively normal frequencies of naive Treg we observed in our HIV cohorts, it may be that, despite increased levels of Treg cycling in both these infections, the overall Treg homeostasis remains largely unperturbed. Of note, no significant differences in the proportion of cycling Treg, as assessed by Ki67, were observed in early-stage and late-stage HIV infection, indicating that factors other than cell cycling may play a role in Treg maintenance in late-stage HIV-2 and HIV-1 disease.

In this respect, the marked increase in Treg within CD4⁺ T cells we observed in ART-discordant individuals is of interest, particularly as it appears to be preserved over time. A previous cross-sectional study [47] reported similarly increased frequencies of CD4⁺CD25^{bright} lymphocytes to those observed in our ART-discordant group. The persistence of naive/memory imbalances in CD4⁺ T cells is in agreement with the reduced hematopoietic regenerative capacity, both at the level of the bone marrow [48] and thymus [25,26] documented in ART-discordant individuals. Despite a reduced level of turn over, Treg frequencies remained at a similarly increased level over time in these individuals, suggesting that these cells are maintained by improved survival rather than active expansion, which, in turn, may reflect an alternative mechanism to preserve Treg in the context of impaired thymic function.

Overall, our data provide evidence that CD4 depletion is associated with a relative expansion of Treg irrespective of the presence or absence of circulating virus, supporting an apparently better preservation of circulating naive and memory Treg as compared with other CD4⁺ T-cell subsets in HIV/AIDS.

Acknowledgements

We gratefully acknowledge Andreia Fonseca for the multivariate analysis and the clinical collaboration of the following colleagues: A. Ribeiro, E. Valadas, F. Antunes, M. Doroana, M. Lucas, R. Dutchman, and R. Marçal. This work was supported by grants from 'Fundação para a Ciência e a Tecnologia' (FCT) and by 'Programa Operacional Ciência e Inovação 2010' (POCI2010), as well as from Fundação Calouste Gulbenkian to A.E.S. R.B.F., R.S.S., A.S.A., A.P.B., R.C. and R.T. received scholarships from FCT.

Author contributions: R.B.F. designed and performed experiments, analyzed data and wrote the paper;

A.S.A. performed experiments and analyzed data regarding the discordant cohort; R.S.S., A.P.B., R.C., R.T. and P.G. performed experiments; R.M.M.V. contributed to the design of the study and data interpretation; A.E.S. designed the research and wrote the paper.

Conflicts of interest

The authors have no conflicting financial interests.

References

- Shen S, Ding Y, Tadokoro CE, Olivares-Villagomez D, Camps-Ramirez M, Curotto de Lafaille MA, Lafaille JJ. **Control of homeostatic proliferation by regulatory T cells.** *J Clin Invest* 2005; **115**:3517–3526.
- Belkaid Y. **Regulatory T cells and infection: a dangerous necessity.** *Nat Rev Immunol* 2007; **7**:875–888.
- Boettler T, Spangenberg HC, Neumann-Haefelin C, Panther E, Urbani S, Ferrari C, *et al.* **T cells with a CD4+CD25+ regulatory phenotype suppress in vitro proliferation of virus-specific CD8+ T cells during chronic hepatitis C virus infection.** *J Virol* 2005; **79**:7860–7867.
- Bolacchi F, Sinistro A, Ciaprin C, Demin F, Capozzi M, Carducci FC, *et al.* **Increased hepatitis C virus (HCV)-specific CD4+CD25+ regulatory T lymphocytes and reduced HCV-specific CD4+ T cell response in HCV-infected patients with normal versus abnormal alanine aminotransferase levels.** *Clin Exp Immunol* 2006; **144**:188–196.
- Deeks SG, Kitchen CM, Liu L, Guo H, Gascon R, Narvaez AB, *et al.* **Immune activation set point during early HIV infection predicts subsequent CD4+ T-cell changes independent of viral load.** *Blood* 2004; **104**:942–947.
- Hazenberg MD, Otto SA, van Benthem BH, Roos MT, Coutinho RA, Lange JM, *et al.* **Persistent immune activation in HIV-1 infection is associated with progression to AIDS.** *AIDS* 2003; **17**:1881–1888.
- Rodriguez B, Sethi AK, Cheruvu VK, Mackay W, Bosch RJ, Kitahata M, *et al.* **Predictive value of plasma HIV RNA level on rate of CD4 T-cell decline in untreated HIV infection.** *JAMA* 2006; **296**:1498–1506.
- Aandahl EM, Michaelsson J, Moretto WJ, Hecht FM, Nixon DF. **Human CD4+ CD25+ regulatory T cells control T-cell responses to human immunodeficiency virus and cytomegalovirus antigens.** *J Virol* 2004; **78**:2454–2459.
- Kinter AL, Hennessey M, Bell A, Kern S, Lin Y, Daucher M, *et al.* **CD25(+)CD4(+) regulatory T cells from the peripheral blood of asymptomatic HIV-infected individuals regulate CD4(+) and CD8(+) HIV-specific T cell immune responses in vitro and are associated with favorable clinical markers of disease status.** *J Exp Med* 2004; **200**:331–343.
- Weiss L, Donkova-Petrini V, Caccavelli L, Balbo M, Carbonneil C, Levy Y. **Human immunodeficiency virus-driven expansion of CD4+CD25+ regulatory T cells, which suppress HIV-specific CD4 T-cell responses in HIV-infected patients.** *Blood* 2004; **104**:3249–3256.
- Kinter AL, Horak R, Sion M, Riggan L, McNally J, Lin Y, *et al.* **CD25+ regulatory T cells isolated from HIV-infected individuals suppress the cytolytic and nonlytic antiviral activity of HIV-specific CD8+ T cells in vitro.** *AIDS Res Hum Retroviruses* 2007; **23**:438–450.
- Kinter A, McNally J, Riggan L, Jackson R, Roby G, Fauci AS. **Suppression of HIV-specific T cell activity by lymph node CD25+ regulatory T cells from HIV-infected individuals.** *Proc Natl Acad Sci U S A* 2007; **104**:3390–3395.
- Eppe HJ, Loddenkemper C, Kunkel D, Troger H, Maul J, Moos V, *et al.* **Mucosal but not peripheral FOXP3+ regulatory T cells are highly increased in untreated HIV infection and normalize after suppressive HAART.** *Blood* 2006; **108**:3072–3078.
- Nilsson J, Boasso A, Velilla PA, Zhang R, Vaccari M, Franchini G, *et al.* **HIV-1-driven regulatory T-cell accumulation in lymphoid tissues is associated with disease progression in HIV/AIDS.** *Blood* 2006; **108**:3808–3817.

15. Andersson J, Boasso A, Nilsson J, Zhang R, Shire NJ, Lindback S, *et al.* **The prevalence of regulatory T cells in lymphoid tissue is correlated with viral load in HIV-infected patients.** *J Immunol* 2005; **174**:3143–3147.
16. Baker CA, Clark R, Ventura F, Jones NG, Guzman D, Bangsberg DR, Cao H. **Peripheral CD4 loss of regulatory T cells is associated with persistent viraemia in chronic HIV infection.** *Clin Exp Immunol* 2007; **147**:533–539.
17. Eggena MP, Barugahare B, Jones N, Okello M, Mutalya S, Kityo C, *et al.* **Depletion of regulatory T cells in HIV infection is associated with immune activation.** *J Immunol* 2005; **174**:4407–4414.
18. Schulze Zur Wiesch J, Thomssen A, Hartjen P, Toth I, Lehmann C, Meyer-Olson D, *et al.* **Comprehensive analysis of frequency and phenotype of T regulatory cells in HIV infection: CD39 expression of FoxP3+ T regulatory cells correlates with progressive disease.** *J Virol* 2011; **85**:1287–1297.
19. Popper SJ, Sarr AD, Gueye-Ndiaye A, Mboup S, Essex ME, Kanki PJ. **Low plasma human immunodeficiency virus type 2 viral load is independent of proviral load: low virus production in vivo.** *J Virol* 2000; **74**:1554–1557.
20. Drylewicz J, Matheron S, Lazaro E, Damond F, Bonnet F, Simon F, *et al.* **Comparison of viro-immunological marker changes between HIV-1 and HIV-2-infected patients in France.** *AIDS* 2008; **22**:457–468.
21. Marlink R, Kanki P, Thior I, Travers K, Eisen G, Siby T, *et al.* **Reduced rate of disease development after HIV-2 infection as compared to HIV-1.** *Science* 1994; **265**:1587–1590.
22. Poulsen AG, Aaby P, Larsen O, Jensen H, Nauclder A, Lisse IM, *et al.* **9-year HIV-2-associated mortality in an urban community in Bissau, west Africa.** *Lancet* 1997; **349**:911–914.
23. Sousa AE, Carneiro J, Meier-Schellersheim M, Grossman Z, Victorino RM. **CD4 T cell depletion is linked directly to immune activation in the pathogenesis of HIV-1 and HIV-2 but only indirectly to the viral load.** *J Immunol* 2002; **169**:3400–3406.
24. Grossman Z, Meier-Schellersheim M, Sousa AE, Victorino RM, Paul WE. **CD4+ T-cell depletion in HIV infection: are we closer to understanding the cause?** *Nat Med* 2002; **8**:319–323.
25. Aiuti F, Mezzaroma I. **Failure to reconstitute CD4+ T-cells despite suppression of HIV replication under HAART.** *AIDS Rev* 2006; **8**:88–97.
26. Marchetti G, Gori A, Casabianca A, Magnani M, Franzetti F, Clerici M, *et al.* **Comparative analysis of T-cell turnover and homeostatic parameters in HIV-infected patients with discordant immune-virological responses to HAART.** *AIDS* 2006; **20**:1727–1736.
27. Soares R, Foxall R, Albuquerque AS, Cortesao C, Garcia M, Victorino RM, Sousa AE. **Increased frequency of circulating CCR5+ CD4+ T cells in human immunodeficiency virus type 2 infection.** *J Virol* 2006; **80**:12425–12429.
28. Albuquerque AS, Foxall RB, Cortesao CS, Soares RS, Doroana M, Ribeiro A, *et al.* **Low CD4 T-cell counts despite low levels of circulating HIV: insights from the comparison of HIV-1 infected patients with a discordant response to antiretroviral therapy to patients with untreated advanced HIV-2 disease.** *Clin Immunol* 2007; **125**:67–75.
29. Foxall RB, Soares RS, Albuquerque AS, Cortesao CS, Victorino RM, Sousa AE. **Increased frequency of CD25dimCD4+ T-cells in HIV-2 infection, a naturally occurring attenuated form of HIV-1.** *Clin Immunol* 2008; **127**:158–167.
30. Soriano V, Gomes P, Heneine W, Holguin A, Doruana M, Antunes R, *et al.* **Human immunodeficiency virus type 2 (HIV-2) in Portugal: clinical spectrum, circulating subtypes, virus isolation, and plasma viral load.** *J Med Virol* 2000; **61**:111–116.
31. Baecher-Allan C, Brown JA, Freeman GJ, Hafler DA. **CD4+CD25high regulatory cells in human peripheral blood.** *J Immunol* 2001; **167**:1245–1253.
32. Miyara M, Yoshioka Y, Kitoh A, Shima T, Wing K, Niwa A, *et al.* **Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor.** *Immunity* 2009; **30**:899–911.
33. Hori S, Nomura T, Sakaguchi S. **Control of regulatory T cell development by the transcription factor Foxp3.** *Science* 2003; **299**:1057–1061.
34. Tran DQ, Ramsey H, Shevach EM. **Induction of FOXP3 expression in naive human CD4+FOXP3 T cells by T-cell receptor stimulation is transforming growth factor-beta dependent but does not confer a regulatory phenotype.** *Blood* 2007; **110**:2983–2990.
35. Wang J, Ioan-Facsinay A, van der Voort EI, Huizinga TW, Toes RE. **Transient expression of FOXP3 in human activated non-regulatory CD4+ T cells.** *Eur J Immunol* 2007; **37**:129–138.
36. Del Pozo-Balado Mdel M, Leal M, Mendez-Lagares G, Pacheco YM. **CD4(+)CD25(+/hi)CD127(lo) phenotype does not accurately identify regulatory T cells in all populations of HIV-infected persons.** *J Infect Dis* 2010; **201**:331–335.
37. Albuquerque AS, Cortesao CS, Foxall RB, Soares RS, Victorino RM, Sousa AE. **Rate of increase in circulating IL-7 and loss of IL-7Ralpha expression differ in HIV-1 and HIV-2 infections: two lymphopenic diseases with similar hyperimmune activation but distinct outcomes.** *J Immunol* 2007; **178**:3252–3259.
38. Grabar S, Selinger-Leneman H, Abgrall S, Pialoux G, Weiss L, Costagliola D. **Prevalence and comparative characteristics of long-term nonprogressors and HIV controller patients in the French Hospital Database on HIV.** *AIDS* 2009; **23**:1163–1169.
39. Giorgi JV, Hultin LE, McKeating JA, Johnson TD, Owens B, Jacobson LP, *et al.* **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* 1999; **179**:859–870.
40. Booth NJ, McQuaid AJ, Sobande T, Kissane S, Agius E, Jackson SE, *et al.* **Different proliferative potential and migratory characteristics of human CD4+ regulatory T cells that express either CD45RA or CD45RO.** *J Immunol* 2010; **184**:4317–4326.
41. Benveniste O, Flahault A, Rollot F, Elbim C, Estaquier J, Pedron B, *et al.* **Mechanisms involved in the low-level regeneration of CD4+ cells in HIV-1-infected patients receiving highly active antiretroviral therapy who have prolonged undetectable plasma viral loads.** *J Infect Dis* 2005; **191**:1670–1679.
42. Teixeira L, Valdez H, McCune JM, Koup RA, Badley AD, Hellerstein MK, *et al.* **Poor CD4 T cell restoration after suppression of HIV-1 replication may reflect lower thymic function.** *AIDS* 2001; **15**:1749–1756.
43. Roederer M, Dubs JG, Anderson MT, Raju PA, Herzenberg LA. **CD8 naive T cell counts decrease progressively in HIV-infected adults.** *J Clin Invest* 1995; **95**:2061–2066.
44. Vukmanovic-Stejić M, Zhang Y, Cook JE, Fletcher JM, McQuaid A, Masters JE, *et al.* **Human CD4+ CD25hi Foxp3+ regulatory T cells are derived by rapid turnover of memory populations in vivo.** *J Clin Invest* 2006; **116**:2423–2433.
45. Mabarrack NH, Turner NL, Mayrhofer G. **Recent thymic origin, differentiation, and turnover of regulatory T cells.** *J Leukoc Biol* 2008; **84**:1287–1297.
46. Bandera A, Ferrario G, Saresella M, Marventano I, Soria A, Zanini F, *et al.* **CD4+ T cell depletion, immune activation and increased production of regulatory T cells in the thymus of HIV-infected individuals.** *PLoS One* 2010; **5**:e10788.
47. Marziali M, De Santis W, Carello R, Leti W, Esposito A, Isgro A, *et al.* **T-cell homeostasis alteration in HIV-1 infected subjects with low CD4 T-cell count despite undetectable virus load during HAART.** *AIDS* 2006; **20**:2033–2041.
48. Isgro A, Leti W, De Santis W, Marziali M, Esposito A, Fimiani C, *et al.* **Altered clonogenic capability and stromal cell function characterize bone marrow of HIV-infected subjects with low CD4+ T cell counts despite viral suppression during HAART.** *Clin Infect Dis* 2008; **46**:1902–1910.