CTLA-4 upregulation during HIV infection: association with anergy and possible target for therapeutic intervention

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Objective: To study the role of cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) during HIV infection.

Methods: Intracellular CTLA-4 expression, determined by flow-cytometry, and proliferative responses to HIV antigens, were studied in peripheral blood mononuclear cells (PBMC) from 93 HIV-1-infected [HIV(+)] patients and 40 HIV-1 seronegative controls.

Results: The proportions of CTLA-4 expressing CD4+ T cells were: (1) significantly higher in HIV(+) patients, $10.95 \pm 0.66\%$, than in controls, $6 \pm 0.45\%$ ($P < 0.0001$); (2) inversely correlated to CD4+ counts ($r = -0.67$, $P < 0.005$, $n = 16$, drug-naive patients; $r = -0.57$, $P < 0.0001$, $n = 77$, HAART-treated patients); and (3) positively correlated to proportion of activated (HLA-DR+CD3+) ($r = 0.53$, $P < 0.0001$) and memory (CD45RO+CD4+) T cells ($r = 0.46$, $P < 0.001$). CD28 median fluorescence intensity in CTLA-4- cells was twice that in CTLA-4+ cells (140 ± 5.3 versus 70 ± 2.28, $P < 0.00001$), whereas cells low in CD28 and CD4, expressed more CTLA-4 ($P < 0.0001$). Higher proportion of CTLA-4+CD4+ cells expressed CCR5 and Ki-67, in comparison with CTLA-4-CD4+ cells, (65 ± 11.9 and $25 \pm 7.5\%$ versus 27 ± 8.9 and $3.7 \pm 2\%$, $P < 0.0001$ and $P < 0.01$, respectively). Among HAART-treated patients, with viral load below detectable levels, CD4+ cells increase was inversely correlated to %CTLA-4+CD4+ cells ($r = -0.5$, $P = 0.003$, $n = 39$). Proliferation of PBMC to anti-CD3, gp-120 depleted HIV-1 antigen or HIV-1 p24 stimulation was inversely correlated with CTLA-4 levels ($r = -0.68$, $P = 0.0035$; $r = -0.38$, $P = 0.04$; and $r = -0.43$, $P = 0.028$, respectively).

Conclusions: (1) CTLA-4 is upregulated during HIV infection and may therefore account for CD4 T-cell decline and anergy in HIV-1 infection. (2) Increased levels of CTLA-4 may undermine immune responses and in the HAART-treated patient-immune reconstitution. (3) Blocking of CTLA-4 may offer a novel approach for immune-based therapy in HIV infection.

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CTLA-4, AIDS, HIV-1, anergy, Ki-67, CD28, CCR5

Introduction

Progressive decline of CD4 T-helper cells, and loss of specific immune response to HIV, are two hallmarks of HIV infection. Although the mechanisms responsible for them are still not clear, we and other investigators have suggested that they are due to chronic immune activation caused by HIV infection more than due to direct cytopathic effects of the virus [1–3].

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The cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), upregulated during T-cell activation, may account for some of the main features of HIV infection, and may therefore be a central player in HIV pathogenesis. Although full activation of T-cells requires co-stimulatory signalling through the ligation of CD28 receptor with the B7-1 (CD80) or B7-2 (CD86) ligands of antigen-presenting cells, CTLA-4 engagement to B7 terminates ongoing responses and proliferation of activated helper T cells, and results in apoptosis [4]. Cross-linking of CTLA-4 reduces interleukin (IL)-2 production and arrests the cells in the G1 phase of the cell cycle [5], and downregulates T-cell responses by raising the threshold for effective T-cell activation [4–7]. Blockage of CTLA-4/B7 interactions prevents induction of peripheral T-cell tolerance upon vaccination with peptides under tolerogenic conditions [6], suggesting that CTLA-4 might be involved in the induction of anergy, present during HIV infection. CTLA-4 knockout mice have significantly higher blood levels of CD4+ T cells and CD4/CD8 ratios than normal mice, from 6 : 1 to as high as 20 : 1 [7]. Administration of monoclonal antibodies (MAbs) to CTLA-4, enhances CD4+ T-cell expansion in response to a variety of stimuli, and is a potent anti-tumour and anti-parasitic tool, in experimental animal models [8–10].

Recent studies have revealed that the majority of CTLA-4 molecules are localized in intracellular stores and CTLA-4 is only transiently expressed and rapidly endocytosed away from the cell surface following T-cell activation [11,12]. Therefore, and since the kinetics of intracellular expression of CTLA-4 after stimulation parallel those of surface expression [11,12], today CTLA-4 expression is routinely studied by using intracellular staining techniques [13,14].

In the present study, by measuring intracellular expression of CTLA-4, we show that the number of CTLA-4 molecules and the proportion of CTLA-4+CD4+ cells is significantly higher in HIV-positive [HIV(+)] individuals, and is strongly associated with HIV disease stage. The implications of these findings have prompted this report.

Materials and methods

Lymphocyte phenotype analysis

FACS analysis (FACScan®, Becton Dickinson Immunocytometry Systems, San Jose, California, USA) was performed on whole heparin-anticoagulated blood within 3 h after the blood collection. A mixture of one to three of the following MAbs conjugated with either fluorecein isothiocyanate (FITC), phycoerythrine (PE), peridin chlorophyll protein (PerCP) or cychrome directed against: CD3, CD4, CD8, HLA-DR, CD28, CD45RA, CD45RO, CD25 (Dako, Glostrup, Denmark), Ki-67 (Immunotech, Marseille, France), CCR5, CXCR4 or CTLA-4 (PharMingen, San Diego, California, USA) were used. Intracellular labelling of CTLA-4 and Ki-67 was carried out, after labelling the cells with MAb against surface markers, by fixing and permeabilizing the cells with Ortho Permeafix® (Ortho Diagnostic Systems Inc., Raritan, New Jersey, USA), according to the manufacturer's instruction (supplemented with 0.1% saponin in the washing solution in the case of CTLA-4), and incubating them with FITC-conjugated CTLA-4 MAb (PharMingen) or FITC-conjugated Ki-67 MAb (Immunotech). Mouse IgG1/IgG2a (Dako) served as isotype controls. Lymphocytes were distinguished from monocytes on the basis of their forward versus side light scatter pattern. A minimum of 10 000 cells per sample was analysed.

Cell preparations and cultures

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood with Histopaque (Sigma, Rehovet, Israel), washed and resuspended in RPMI 1640 medium with 10% human AB serum (Sigma) and antibiotics and cultured at 37°C under 5% CO_2. The PBMC (500×10^6 cells/l), cultured in 96-well plates (Corning, New York, USA) for 3 days with 0.5 μg/ml anti-CD3 MAb (UCHT1, R&D Systems, Minneapolis, Minnesota, USA) or for 6 days in the presence of 10 μg/ml HIV-1 p24 or gp120-depleted HIV-1 (Remune: a generous gift of Dr. Ronald Moss, The Immune Response Corporation, California, USA), were pulsed at the last day of culture with 0.5 μCi [^{3}H]thymidine (Amersham Pharmacia Biotech, Buckinghamshire, UK). Sixteen hours later the cells were harvested on Whatman 934-AH glass micro-fibre filters (Whatman, Maidstone, UK) and the radioactivity was measured by a β-counter. All experiments were carried out in triplicate.

Determination of viral load

The HIV plasma viral load was determined by automated Amplicor® polymerase chain reaction and Amplilink Software (Cobas Amplicor, Roche Diagnostics, Branchberg, New Jersey, USA).

Statistical analysis

Student's t-tests, correlations and regression analysis were performed by using SigmaPlot software (SPSS®, Chicago, Illinois, USA).

Results

Characterization of the studied HIV-1 infected individuals

Ninety-three HIV(+) individuals (37 ± 10 years old) and 40 HIV-1 seronegative [HIV(−)] individuals (30 ± 4.2 years old) were studied. Seventy-seven HIV(+) individuals were treated by highly active antiretroviral treatment (HAART), with mean blood CD4 levels of $258 \pm 167 \times 10^6$ cells/l. These patients were either asymptomatic ($n = 42$; $300 \pm 165 \times 10^6$ CD4+ cells/l), or had advanced HIV clinical symptoms or AIDS ($n = 35$; $202 \pm 141 \times 10^6$ CD4+ cells/l). Sixteen patients were drug-naive and asymptomatic, with mean blood CD4 levels of $436 \pm 236 \times 10^6$ cells/l. The patients were also stratified according to their CD4+ levels into three groups: less than 200×10^6 (HIV < 200), 200 to 400×10^6 (HIV 200–400) or above 400×10^6 CD4+ cells/l (HIV > 400). The immune profile was determined in all participants of the study. The %HLA-DR+CD3+ (activated T cells) cells were significantly higher ($P < 0.0001$) and the %CD28+CD8+ cells were significantly lower ($P < 0.0001$) in all HIV(+) groups than in the HIV(−) group. In the HIV < 200 group, the proportions of CD28+CD4+ cells and CD45RA+CD4+ (naive T cells) cells were also significantly lower ($P < 0.01$), whereas the proportions of CD45RO+CD4+ (memory T cells) cells were significantly higher ($P < 0.05$), than in the HIV(−) group.

Increased CTLA-4 expression in T cells from HIV-1-infected individuals: correlation with CD4+ T-cell decline and disease stage

As depicted in Fig. 1a the proportion of CTLA-4+CD4+ T cells was significantly higher in HIV(+) individuals, $10.95 \pm 6.5\%$, in both drug-naive and HAART-treated HIV(+) patients, in comparison to HIV(−) healthy controls, $6 \pm 2.9\%$ ($P < 0.0001$). In contrast to the significantly lower immune activation

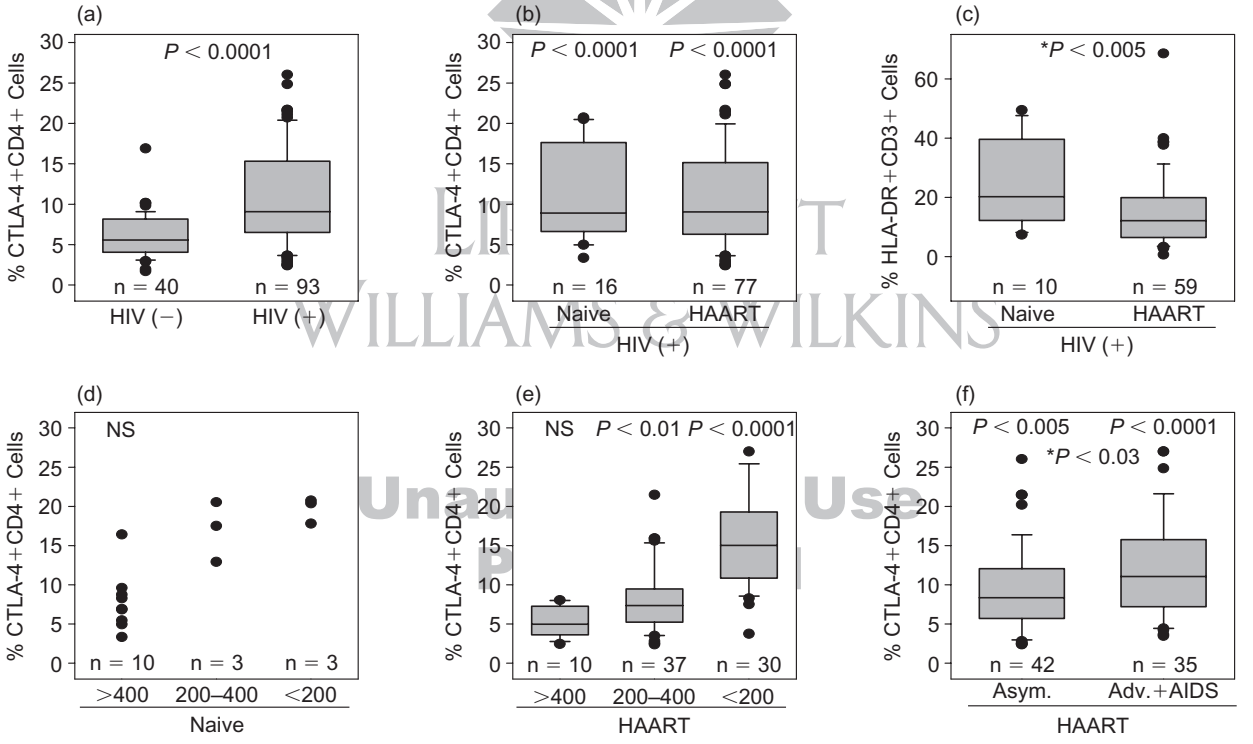

Fig. 1. Increased CTLA-4 expression in HIV-positive [HIV(+)] individuals is associated with disease stage. The proportion of CTLA-4+CD4+ cells in HIV-negative [HIV(−)] and HIV(+) individuals was determined by surface staining of CD4 and intracellular staining of CTLA-4, followed by FACS analysis. (a) Comparison between HIV(−) and HIV(+) groups. (b) Comparison between drug-naive and highly active antiretroviral therapy (HAART)-treated HIV(+) patients. (c) Comparison between the proportions of HLA-DR+CD3+ cells in drug-naive or HAART-treated HIV(+) patients. (d) CTLA-4 expression in HIV(+) drug-naive patients according to their CD4 counts. (e) CTLA-4 expression in HIV(+) HAART-treated patients according to their CD4 counts. (f) CTLA-4 expression in asymptomatic HAART-treated patients and HAART-treated patients with advanced clinical symptoms or AIDS. The number of individuals (n) tested in each group and the statistical difference (*t*-test) between each HIV(+) group and the HIV(−) group (*P*), or between the two groups appearing in the panel (* *P*), are shown. The boxes represent the middle 50% of the data values. The horizontal line across the box marks the median value. The error bars show the 10th and 90th percentiles of the population. Individual data-points falling beyond these boundaries are shown as dots. NS, not significant.

found in the HAART-treated patients than in the drug-naive patients (Fig. 1c, $P < 0.05$), the %CTLA-4+CD4+ cells was similar in both group of patients (Fig. 1b). The proportion of CTLA-4+CD4+ cells in the HIV > 400 group, in both drug-naive and HAART-treated patients, was not significantly different from that found in the HIV(−) group (Fig. 1d,e). However, in both drug-naive and HAART-treated patients, in the HIV 200–400 and HIV < 200 groups, the proportions of CTLA-4+CD4+ cells were significantly higher than in the HIV(−) group (Fig 1d,e). In HAART-treated patients, CTLA-4 expression was significantly higher in both asymptomatic and advanced disease or AIDS patients, than in the HIV(−) controls (Fig. 1f, $P < 0.005$). However, CTLA-4 expression in HAART-treated patients with advanced disease or AIDS were significantly higher than in asymptomatic HAART-treated patients (Fig. 1f, $P = 0.03$). A highly significant inverse correlation was found between proportions of CTLA-4+CD4+ cells and percentage (data not shown), number of CD4+ cells (Fig. 2) or CD4/CD8 ratio (Fig. 2), in both drug-naive and HAART-treated patients. The proportion of CTLA-4+CD4+ cells was weakly correlated to HIV plasma viral load ($r = 0.3$, $P < 0.01$), but strongly correlated to the proportions of either HLA-DR+CD3+ cells ($r = 0.53$, $P < 0.0001$) or CD45RO+CD4+ cells ($r = 0.46$, $P < 0.001$), and inversely correlated to the proportions of CD45RA+CD4+ cells ($r = -0.35$, $P < 0.01$) and to CD28+CD8+ cells ($r = -0.44$, $P < 0.01$). There was no correlation between the age of the patients and CTLA-4 expression.

Importantly, the increase of CD4+ cells levels during 6 months of follow-up, among 39 HAART-treated patients with undetectable levels of plasma viral RNA, was inversely correlated to the proportions of CTLA-4+CD4+ (Fig. 3, $r = -0.5$, $P = 0.003$), but not to the proportions of HLA-DR+CD3+ cells ($r = -0.28$, $P = 0.12$). Accordingly, the increase in CD4+ cells during this time period was significantly higher in those patients with less than 9% CTLA-4+CD4+ cells than in those patients with more than 9% CTLA-4+CD4+ cells (Fig. 3 inset, $P < 0.001$). The value of 9% was chosen as a cutoff, since this value is one SD and 50% above the mean of CTLA-4+CD4+ levels found in HIV(−) individuals.

The proportion of CTLA-4+CD8+ cells was also higher in the HIV(+) group than in the HIV(−) group (1.5 ± 0.19 versus $0.96 \pm 0.21\%$, $P = 0.07$), and was correlated to the proportion'of CTLA-4+CD4+ cells ($r = 0.49$, $P < 0.05$). In both HIV(+) and HIV(−) groups, however, the proportion of CTLA+CD8+ cells was significantly lower than the proportion of CTLA-4+CD4+ cells (1.5 ± 0.2 versus $10.95 \pm 0.66\%$, $P < 0.0001$ and 0.96 ± 0.2 versus $6 \pm 0.45\%$, $P < 0.0001$, respectively).

Interaction between CTLA-4, CD28 and CD4

Since the overall regulation of T-cell levels and response may represent the balance between positive signals through CD28 and negative signals through CTLA-4 [4], the ratio between CTLA-4 and CD28 expression may be more representative of the effects HIV has on the immune system. As depicted in Fig. 4a, the ratio between the percentage of CTLA-4+CD4+ cells and of CD28+CD4+ cells, increases with HIV-1 disease progression, being significantly higher in the HIV < 200 group than in HIV(−) individuals. Furthermore, this ratio was inversely correlated to the percentage of CD4 cells ($r = -0.57$, $P < 0.0001$), CD4/CD8 ratio ($r = -0.49$, $P = 0.0001$), %CD28+CD8+ cells ($r = -0.47$, $P = 0.003$) and %CD45RA+CD4+ cells ($r = -0.32$, $P < 0.02$), and positively correlated to %HLA-DR+CD3+ cells ($r = 0.59$, $P < 0.0001$) and %CD 45RO+CD4+ cells ($r = 0.44$, $P < 0.001$). These correlations were similar to those found with CTLA-4 expression itself (shown above). We then compared the relative amount of CTLA-4 and CD28 expression on CD4+ cells by triple staining (CD4, CD28 and CTLA-4) of PBMC obtained from 36 HIV(+) patients and also measured the median fluorescence intensity (MFI) of intracellular CTLA-4 and membrane CD28. As depicted in Fig. 4b, the MFI of CD28 in the CTLA-4- cells was twice as high as the MFI of CD28 on CTLA-4+ cells (140 ± 5.3 versus 70 ± 2.28, $P < 0.00001$). Interestingly, a very significant correlation was found between the intensity of CD28, CD4, and CTLA-4 staining for a given cell (Fig. 4c,d). Gating cells with low CD28 and CD4 revealed that the proportion of CTLA-4+ cells among this population is significantly higher than its proportion among cells expressing high CD28 and CD4 molecules (21.7 ± 8.7 versus 8.32 ± 4.9, $P < 0.0001$; B versus A). A very clear and strong positive correlation is seen in CD28+CD4+ cells, between CD28 and CD4 expression (Fig. 4c). The more CD28 molecules there are in a given CD4 cell, the more CD4 molecules are expressed in this cell. Notoriously, the percentage of CTLA-4+ cells is significantly higher in those cells expressing low CD4 and low CD28, than in those cells expressing high CD4 and CD28 molecules, in both HIV(+) and HIV(−) individuals.

Increased CCR5 and Ki-67 expression in CTLA-4+CD4+ cells

The two main co-receptors for HIV-1 entry into CD4+ cells are the β-chemokines receptors CCR5 and CXCR4. The proportion of cells expressing CCR5 was higher in CTLA-4+CD4+ cells than in CTLA-4-CD4+ cells, (65 ± 11.9 versus $27 \pm 8.9\%$, $P < 0.0001$). Similarly, the number of CCR5 molecules/cell, as determined by the MFI of CCR5, was four-fold higher in CTLA-4+ cells, compared with CTLA-4- cells (26.1 ± 8.6 versus 6.5 ± 2.3, $P < 0.0001$). The percentage of CTLA-4+CD4+ and

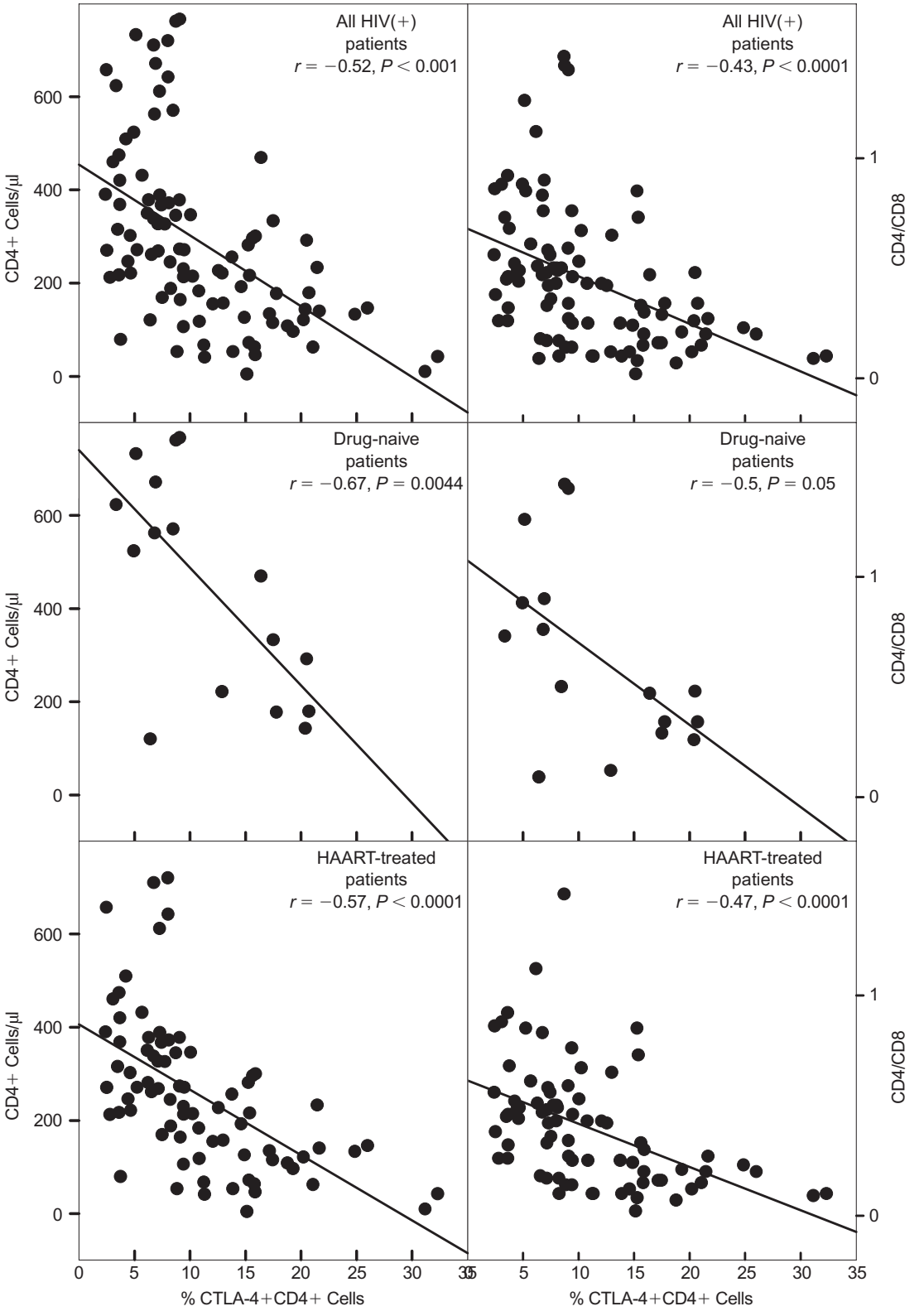

Fig. 2. Inverse correlation between CTLA-4 expression and CD4 counts and CD4/CD8 ratio, in both naive and highly active antiretroviral therapy (HAART)-treated HIV-1-infected patients. The correlations between the proportions of CTLA-4+CD4+ cells and CD4 levels or CD4/CD8 ratio were determined by using SigmaPlot software (SPSS®). The correlations (*r*) and their statistical significance (*P*) obtained by using the Pearson Product Moment Test are shown.

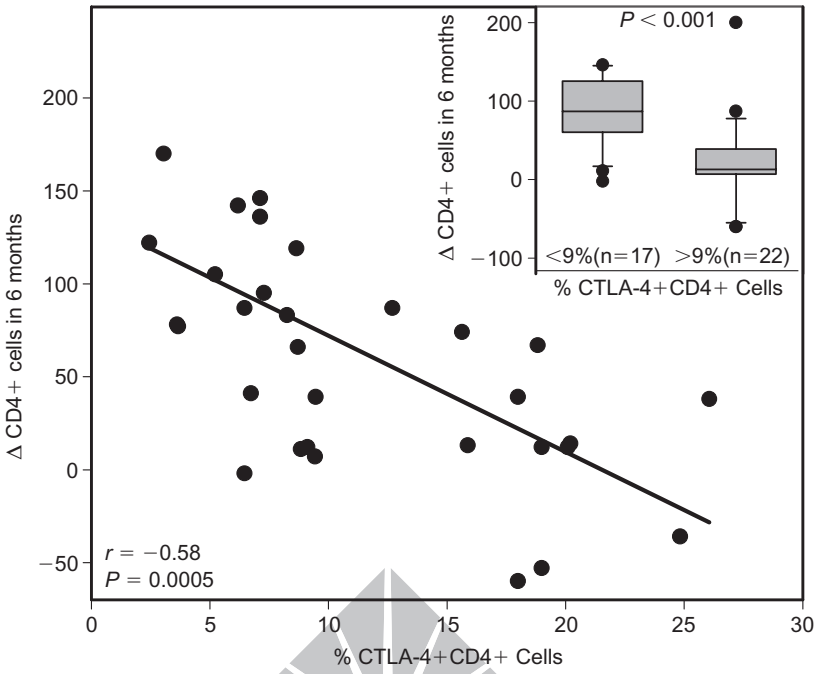

Fig 3. Increase in CD4 levels in highly active antiretroviral therapy (HAART)-treated individuals with viral load below detectable levels, is inversely correlated to proportion of CTLA-4+CD4+ cells. The number of CD4+ cells $\times 10^6$/l of HAART-treated patients with viral load below detectable levels, was followed-up for 6 months. There is an inverse correlation between the increase in CD4 counts and the proportion of CTLA-4+CD4+ cells ($r = -0.5$, $P = 0.003$). The CD4 levels increased significantly more in those patients with less than 9% of CTLA-4+CD4+ cells (inset).

CTLA-4-CD4+ cells expressing CXCR4 was similar (~70%), although the number of CXCR4 molecules/cell was significantly higher in the CTLA-4-CD4+ compartment (MFI of 18.8 ± 4.7 versus 33.4 ± 4.6, $P < 0.0001$).

Ki-67, a marker for dividing cells, is expressed in cells during the late G1, M and S phases of the cell cycle [15]. Activated cells remaining in G1 phase and not dividing may still bear this marker [15]. This was clearly shown in a study of chronically HIV-1 infected patients, in whom $92 \pm 5\%$ of the CD4+CD45 RO+Ki67+ cells were in the G1 phase of the cell cycle [16]. Since CTLA-4 is present in cells that are activated but arrested at the G1 stage of proliferation [17], the use of both markers could help distinguish between truly dividing cells and cells activated but frozen in the G1 phase. It was found that $25 \pm 7.5\%$ of the CTLA-4+CD4+ cells expressed Ki-67, whereas only $3.7 \pm 2\%$ of the CTLA-4-CD4+ cells expressed Ki-67 ($P < 0.01$). Similarly, the MFI of Ki-67 in CTLA-4+CD4+ cells was significantly higher than the MFI of Ki-67 in CTLA-4-CD4+ cells (23 ± 5.1 versus 8.85 ± 1.7, $P < 0.0001$).

Inverse correlation between proliferation of PBMC of HIV(+) individuals to anti-CD3 or HIV antigens and levels of CTLA-4+CD4+ cells

One of the main characteristics of HIV infection is anergy and lack of specific proliferative responses. Since CTLA-4 could mediate such anergy, we examined the correlation between the capacity of PBMC of HIV-1 infected patients to respond to non-specific or HIV-1 specific stimuli and the intracellular CTLA-4 levels in CD4+ cells. There was a strong inverse correlation between the %CTLA-4+CD4+ cells and the capacity of PBMC to proliferate following stimulation with anti-CD3 antibodies ($r = -0.68$, $P = 0.0035$, Fig. 5a). Similar results were obtained with PBMC taken from HIV(−) individuals (data not shown). Proliferation of the PBMC following stimulation with gp120-depleted HIV-1 antigen (Remune) or p24 (Fig. 5b,c) was also inversely correlated to CTLA-4 levels in CD4+ cells ($r = -0.38$, $P = 0.04$ and $r = -0.43$, $P = 0.028$, respectively).

Correlation between CTLA-4 and CD25 expression

As shown in Figures 6a and b, there is a clear correlation between CTLA-4 and CD25 expression for a given cell in both HIV(+) and HIV(−) individuals. Although only 4–6% of CD4+CTLA-4- cells were CD25+, 30–40% of CD4+CTLA-4+ cells were also CD25+ cells, in both HIV(+) and HIV(−) individuals (Fig. 6c,d).

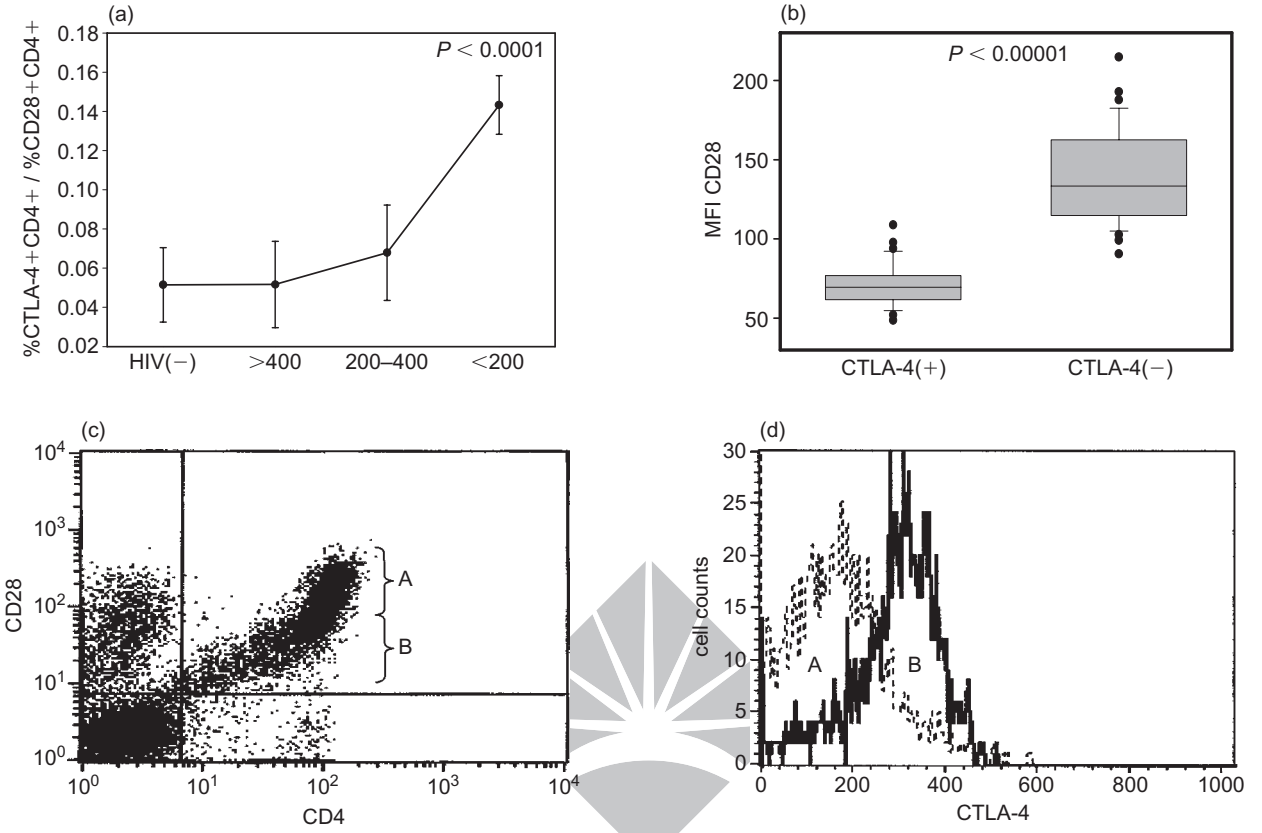

Fig 4. Relationship between CTLA-4, CD28, CD4 and HIV disease stage. (a) The proportion of CTLA-4+ and CD28+ cells in the CD4 T-cell compartment in 65 HIV-positive [HIV(+)] and 24 HIV-negative [HIV(–)] individuals was determined by FACS analysis. The ratio between the percentage of CTLA-4+CD4+ cells/percentage of CD28+CD4+ cells, was significantly higher in the HIV(+) individuals with less than 200 $\times$ 10^6 CD4+ cells/l than the HIV(–) control. (b) Peripheral blood mononuclear cells of 36 HIV(+) patients were stained with fluorecein isothiocyanate (FITC)-labelled anti-CD4 and phycoerythrine (PE)-labelled anti-CD28 Abs, fixed, permeabilized and stained with cychrome-labelled anti-CTLA-4 Ab. CD4+ cells were gated to those expressing CTLA-4 and to those that did not, and the median fluorescence intensity (MFI) of surface CD28 was measured. (c) A representative example of triple staining with anti CD4, CD28 and CTLA-4 Abs and FACS analysis. The lower is the CD28 expression, the lower is the CD4 expression. (d) Histogram of CTLA-4 expression in gated CD4+ with low or high CD28 expression from (c).

Discussion

The results of this study clearly indicate that CTLA-4 plays an important role during HIV-1 infection, through the following main observations: (1) the proportion of CTLA-4+CD4+ cells is significantly higher in HIV(+) individuals in comparison to HIV(-) controls; (2) intracellular CTLA-4 levels are inversely correlated to CD4+ levels and to CD4/CD8 ratio; (3) CTLA-4 levels are higher in HIV(+) patients with advanced clinical symptoms or AIDS than in asymptomatic patients; (4) CD4 cell counts increase in HAART-treated patients with undetectable viral load, and is inversely correlated to the proportions of CTLA-4+CD4+ cells; (5) CTLA-4 expression and the ratio between the proportion of CTLA-4+CD4+ cells and that of CD28+CD4+ cells, is correlated with disease stage and with immune activation; (6) CTLA-4+ cells have very low expression of the co-stimulatory molecule CD28; (7) CCR5 and Ki-67 are expressed significantly more in CTLA-4+ cells; and (8) the capacity of PBMC of HIV-1 infected patients to respond to non-specific or HIV-1-specific stimuli was inversely correlated to the levels of CTLA-4+CD4+ cells.

The upregulation and increased expression of CTLA-4 that we found in HIV(+) individuals may account for some of the major disturbances that characterize HIV infection. CTLA-4 expression plays a key role in maintenance of peripheral CD4+ and CD8+ homeostasis [7], CTLA-4 knockout mice have greatly increased levels of CD4 cells [7], and CTLA-4 mediates antigen-specific human T-cell apoptosis [18]. CTLA-4 is important in induction of T-cell anergy [4–6], and apoptosis and anergy are highly important characteristics of advanced HIV infection and chronic immune activation [19,20]. In addition, blockage of CTLA-4

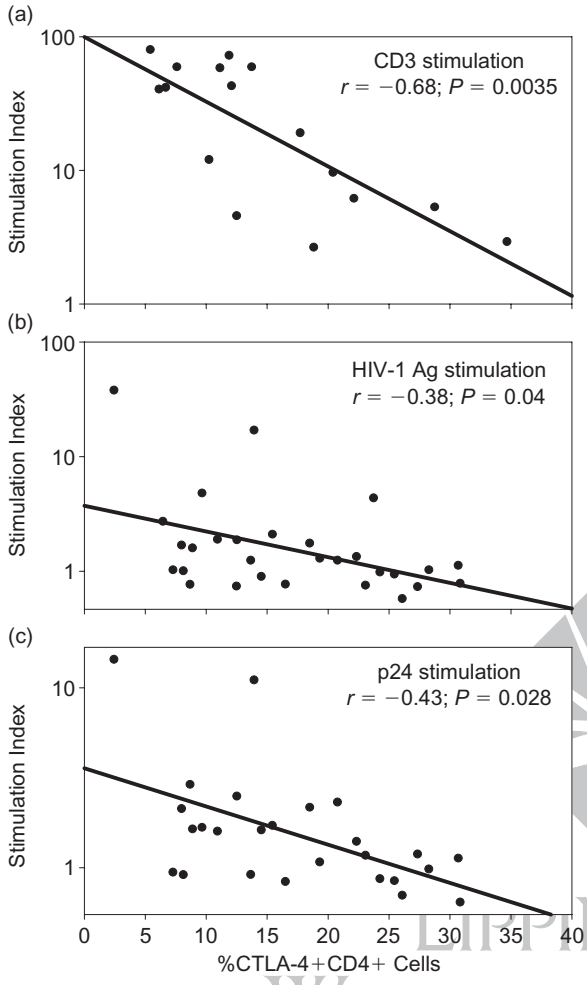

Fig 5. Inverse correlation between CTLA-4 expression and peripheral blood mononuclear cells (PBMC) proliferative response to (a) anti CD3 antibodies, (b) gp120-depleted HIV-1 (Remune) antigen or (c) p24 HIV-1 antigen, in HIV-1-infected patients. PBMC of HIV(+) individuals were cultured at 37°C under 5% CO_2 for 2 days with 0.5 mg/ml anti-CD3 MAb or 5 days with 10 mg/ml gp120-depleted HIV-1 or HIV-1 p24 antigen, before being pulsed overnight with 0.5 mCi [^{3}H]thymidine. As control cells were incubated with medium only.

binding to its ligand B7 increases antigen-specific immune responses [8,10]. Our results, although they do not directly establish CTLA-4 expression as a mechanism of anergy, show an inverse correlation between CTLA-4 levels and proliferative responses of PBMC of HIV(+) patients stimulated with anti-CD3 Ab or HIV-1 antigens, supporting the central role of CTLA-4 in inducing such anergy in these patients. Furthermore, since 30–40% of CTLA-4+CD4+ cells are also CD25+ cells, recently shown to be suppressor/regulators cells (for example [21] and [22]), an increase in CTLA-4+CD4+ cells would mean an increase also in CD4+CD25+ suppressor/regulator cells. Thus, a small increase in the proportion of CTLA-4+CD4+, which include suppressor cells, causes anergy or suppresses immune responses in CTLA-4 negative cells as well. We are currently further investigating the role of CD25+CTLA-4+CD4+ cells in HIV-1 disease.

Cell surface CTLA-4 expression is very low, transient and rapidly cleared by endocytosis [11,12]. Surface CTLA-4 expression is therefore almost undetected by regular flow cytometry staining, and in our hands less than 1% of CD4+ cells were positive for CTLA-4 surface expression in both HIV(+) patients and HIV(−) controls (not shown). Thus, although there is an increase in the transient levels of CTLA-4 surface expression in cells with high intracellular pools of CTLA-4, this is often missed and the differences between the surface levels in cells with intermediate or high CTLA-4 levels are not easily detectable. Steiner and colleagues [23], by amplifying the staining signal of antibodies bound to surface CTLA-4, also found increased expression of CTLA-4 on CD4+ T-cells obtained from 27 HIV(+) patients. However, they did not find any association between CTLA-4 expression and disease stage, as we describe. The measurement by us of intracellular pools of CTLA-4, and not of the transient surface expression of CTLA-4, most probably accounts for this discrepancy.

The most likely reason for CTLA-4 upregulation in HIV infection is the immune activation caused by HIV antigens. This is supported by: (1) the proportion of CTLA-4+ cells in HIV infection is strongly correlated with other immune activation markers such as HLA-DR+CD3+ cell levels; (2) in early HIV infection, when the immune activation is low, CTLA-4 expression is low; and (3) in another chronic immune-activation state, caused by helminthic infections, we found similar increase in CTLA-4 expression together with CD4 diminution [24]. However, in HAART-treated people, although we found significant decrease in immune activation, there was no such similar noticeable decrease in CTLA-4 expression. Furthermore, in HAART-treated individuals with no plasma viraemia, the changes of CD4+ levels were inversely correlated with CTLA-4+CD4+ levels, but not with immune activation, as determined by the levels of HLA-DR+CD3+ cells.

Increased levels of CTLA-4 result in down-regulation of ongoing T-cell responses, and in higher threshold for effective T-cell activation [4], both of which may contribute to the impaired ability of the host to contain the infection. Since CTLA-4 bind to B7-1 or B7-2 with 20- to 100-fold higher affinity than CD28 [25], and by doing so it down-regulates the immune response, the CD28/CTLA-4 ratio, may be a relevant parameter for assessment of the immune response. The importance of this ratio in making cells more susceptible to HIV infection [26] is supported by our findings of increased CCR5 expression in CTLA-4+ cells. The

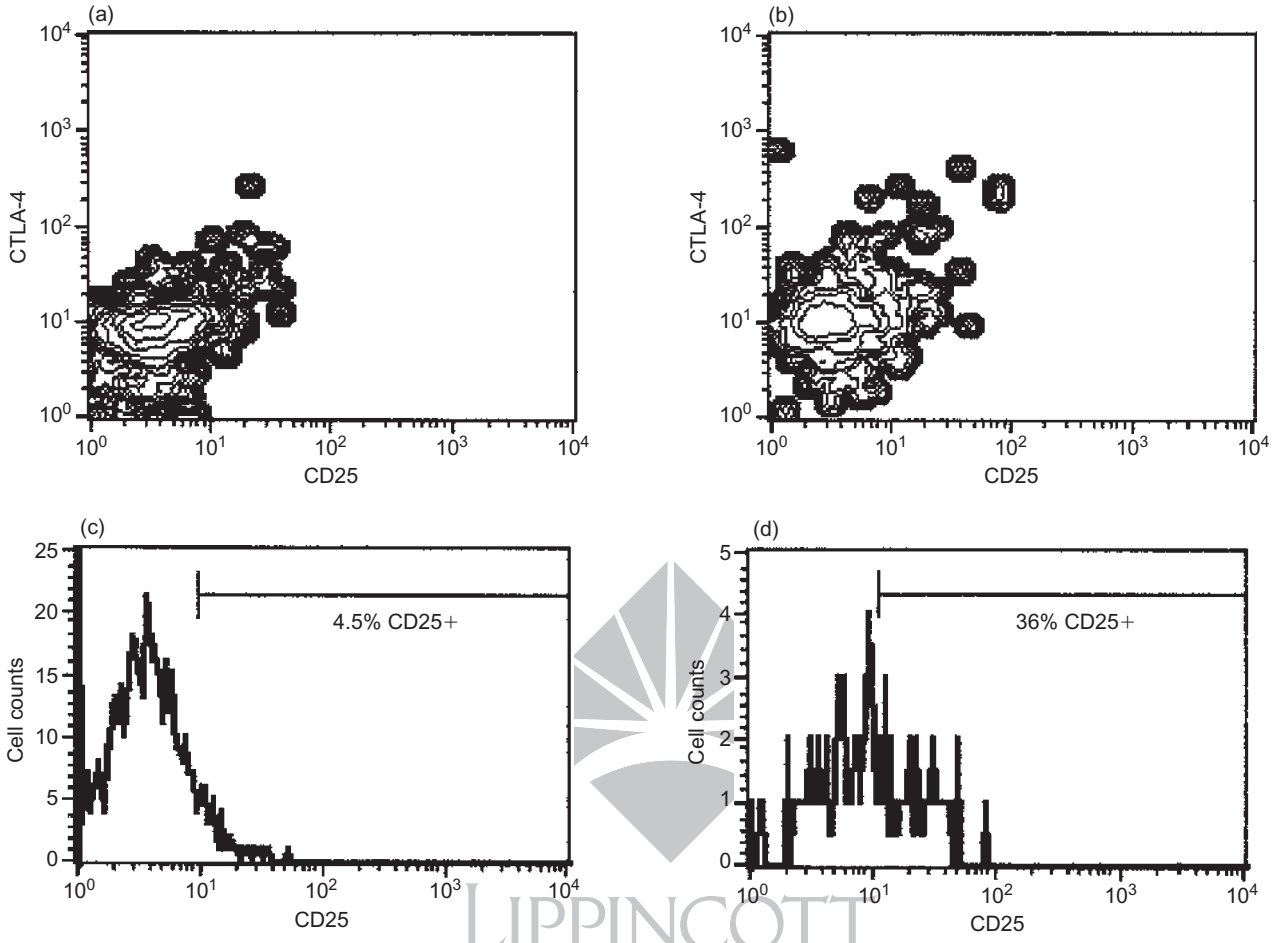

Fig 6. Correlation between CTLA-4+ and CD25+ expression on CD4+ cells. Whole blood of 20 HIV-positive [HIV(+)] patients and 15 HIV-negative [HIV(–)] individuals were stained with peridin chlorophyll protein (PerCP)-labelled anti-CD4 and fluorecein isothiocyanate (FITC)-labelled anti-CD25 Abs, fixed, permeabilized and stained with phycoerythrine (PE)-labelled anti-CTLA-4 Ab. A representative contour plot of CTLA-4 and CD25 expression of gated CD4+ cells from an (a) HIV(–) individual and (b) HIV(+) patient. A representative histogram of CD25 expression of (c) gated CD4+CTLA-4- cells or (d) gated CD4+CTLA-4+ of an HIV(+) patient.

increased ratio of CTLA-4/CD28 that we have found in HIV(+) individuals is mainly due to the increased expression of CTLA-4 in CD4+ cells, and not due to the reduction of CD28 expression on CD4+ cells (data not shown).

Is this increased expression of CTLA-4 also the cause of CD4 decrease? CTLA-4 has been shown to play an important role controlling the production of CD4+ cells, as demonstrated in CTLA-4 knockout mice [7]. Here we show interesting mutual associative relationships between CTLA-4, CD4 and CD28. The diminished expression of CD28 on CTLA-4+ cells and the clear association of CD28 with CD4 expression raises the possibility that CTLA-4 indirectly downregulates CD4 expression through downregulating CD28 expression and maybe CD4 production as well.

The observation, that CD4 counts increase significantly more among HAART-treated individuals with undetectable viral load, who have low proportions of CTLA-4+CD4+ cells, indicate that elevated CTLA-4 expression may be an important factor that impedes immune reconstitution in many HAART-treated patients. Further studies of intracellular CTLA-4 expression in HIV(+) patients, before and after HAART treatment, are required to help clarify the dissociation found in some patients on HAART, between virus suppression, and recovery or rise of CD4+ cells. Such studies may clarify the role of CTLA-4 in immune restoration following HAART, and indicate the clinical value of CTLA-4 monitoring and treatment in HIV infection.

The effect of HAART on the immune system and particularly on the rise of CD4+ T-cells has been ascribed to increased proliferation of CD4+ T-cells, as determined by increased proportion of Ki-67+CD4+

cells following HAART [27,28]. Recent studies have suggested that the rise of CD4+ T-cells following HAART is mainly due to redistribution of cells, rather than to massive proliferation of CD4+ T-cells [29,30]. Our findings that approximately 50% of the Ki-67+CD4+ cells in HIV(+) individuals are CTLA-4+ cells [3], and that approximately 25% of the CTLA-4+CD4+ cells are Ki-67+, and those reported by Autran and colleagues [16], showing that almost all CD4+CD45RO+Ki67+ cells are in the G1 phase of the cell cycle, clearly support the redistribution notion. This indicates that Ki-67+ cells are not necessarily proliferating cells [15–17], and suggests that significant proportions of activated cells during HIV infection are anergic and non-proliferating cells.

Previous studies with antibodies to CTLA-4 in other systems have shown the potential importance and implications of this approach, including the clinical use of such antibodies. In particular, the enhancement of CD4+ T-cell expansion in response to peptide antigens, superantigens and parasites [14], and their use in transplantation [31]. Although the benefits of CTLA-4 blockage in HIV-1 infection and immune reconstitution in HIV(+) patients has to be studied, the clear correlation between HIV-1 disease progression and CTLA-4 expression, raise the possibility of using anti-CTLA-4 antibodies for immunotherapy during HIV infection, probably in combination with antiretroviral therapy. There are several supportive arguments for this approach. The most important one, is the potential role of anergy and CD4 cell anergy in particular, in the diminished immune response of the HIV(+) individuals. Such anergy may be responsible for the diminished specific cytotoxic function of CD8 cells during HIV infection that is dependent on CD4 help which is essential in the ability of the host to contain HIV infection. Even in successfully HAART-treated patients there seems to be insufficient immune reconstitution and the response to HIV antigens is usually missing. CD8 T-cytotoxicity against tumour cells in mice can be enhanced by blockade of CTLA-4 only in the presence of CD4 T-cells, whereas CTL activity is lost in the absence of CD4 T-cells [8], supporting the idea that functional CD4 T-cells are essential for CD8 CTL activity. Thus, even a small increase in dysfunctional CD4 cells, namely an increase in the proportion of CTLA-4+CD4+ cells, may have dramatic effects on other compartments of the immune system, including on the capacity of CD8 cells to specifically target HIV-infected cells.

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