

CD28 costimulation and T lymphocyte proliferative responses in HIV-1 infection

M. CARLESIMO, O. PONTESILLI, A. R. VARANI, M. L. BERNARDI, A. M. MAZZONE, R. ROSSO, E. C. GUERRA, A. CASSONE*, R. PAGANELLI & F. AIUTI *Department of Clinical Medicine, Chair of Clinical Immunology and Allergy, University of Rome 'La Sapienza', and *Laboratory of Bacteriology and Medical Mycology, Istituto Superiore di Sanità, Rome, Italy*

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SUMMARY

To investigate whether defective costimulatory signals could be involved in the loss of T lymphocyte functions during HIV-1 infection, we tested the effect of CD28 costimulation on both T cell receptor/CD3 and HIV-1 antigen-induced proliferative responses. Although CD3-mediated responses significantly decreased with more advanced stages of HIV-1 infection, the ability of potentiating the responses through CD28 costimulation was maintained at all stages and did not differ from that of HIV-1⁻ subjects. When CD28 costimulation was studied in lymphocyte cultures stimulated with HIV-1 gp160 or p24, potentiation was seen only when a significant response was present without additional CD28 triggering, namely in subjects receiving active immunization with recombinant gp160. These results confirm the integrity of the CD28 pathway of costimulation during HIV-1 infection, and suggest that lymphocytes responding to soluble HIV-1 antigen are not deleted in HIV-1-infected patients, but do not receive significant priming during the natural course of the infection.

Keywords HIV-1 gp160 costimulation T lymphocyte proliferation

INTRODUCTION

Infection with HIV-1 results in vigorous virus-specific cytotoxic T lymphocyte (CTL) responses [1] and production of antibodies to most viral antigens [2] throughout the period of asymptomatic infection. This contrasts with relatively weak and sporadic *in vitro* lymphoproliferative responses to HIV-1 antigen at all stages of infection [3–6]. A progressive defect of T cell responses to recall antigens, allo-antigens and mitogens has been described in HIV-1 infection, independent of a significant CD4⁺ T cell loss [7,8]. In particular, *in vitro* lymphoproliferative response to CD3 MoAb decreases progressively during the infection, and is regarded as a marker of progression to AIDS independent of CD4⁺ T cell numbers [9,10].

A possible explanation for the progressive loss of accessory cell-dependent T lymphocyte function could be an altered delivery of costimulatory signals from professional antigen-presenting cells (APC), such as monocyte-derived macrophages which are selective targets of non-syncytium inducing (NSI) HIV-1 variants characteristic of the initial phases of HIV-1 infection [11]. The best known T lymphocyte costimulatory signal is derived from

the interaction between CD28, expressed on thymocytes and mature T lymphocytes, and its two ligands, CD80 and CD86 [12,13]. CD28 costimulation, in addition to ligation of T cell receptor (TCR)/CD3 complex, provides a potent signal for IL-2 production and lymphocyte proliferation [14–16]. Blocking this interaction during antigen-specific or allogeneic responses induces T cell unresponsiveness [17].

To test the hypothesis of a lack of HIV-1-specific lymphoproliferative responses as a consequence of T cell anergy we first tested the efficiency of the CD28 costimulation pathway during HIV-1 infection at various stages, and then evaluated the effect of CD28 MoAb addition to HIV-1 antigen-driven proliferative responses. The integrity of CD28-derived signals and the absence of a rescue effect of CD28 costimulation on HIV-1 antigen-specific proliferative responses, unless specific *in vivo* immunization is administered, are the major findings reported here.

PATIENTS AND METHODS

Patients

To investigate CD3 MoAb-driven lymphoproliferation we studied 36 patients at various stages of HIV-1 infection, according to 1993 CDC classification [18] (classes: A1, *n* = 10; A2, *n* = 14; B2, *n* = 1; B3, *n* = 3; C3, *n* = 8). Twenty-five HIV-1⁻ healthy subjects were tested as controls.

M.C. and O.P. have contributed equally to this work and therefore have both to be regarded as first authors.

Correspondence: Oscar Pontesilli MD, Department of Clinical Viro-Immunology, CLB, Plesmanlaan 125, 1066 CX Amsterdam, The Netherlands.

Lymphoproliferative response to HIV-1 gp160 was studied in 44 asymptomatic HIV-1-infected patients enrolled in a double-blind placebo controlled trial of post-exposure immunization with recombinant gp160 with aluminium hydroxide (VaxSyn: Protein Sciences Corp., formerly MicroGeneSys Inc., Meriden, CT). CD4⁺ T cell numbers ranged from 261 to 862 cells/ μ l (median 439 cells/ μ l). After completion of the trial, treatment codes were disclosed and 27 of them were shown to have received VaxSyn. The remaining 17 patients had received aluminium hydroxide placebo injections and oral zidovudine (250 mg b.d.). Details and results of the immunization trial will be published elsewhere ([19], [19a]). Twenty-four patients in this group were studied also for lymphoproliferative responses to HIV-1 p24. Fourteen HIV-1⁻ healthy subjects were included as controls for lymphoproliferative responses to HIV-1 antigen.

T cell phenotyping

CD3⁺ and CD4⁺ T lymphocytes were quantified in EDTA anticoagulated whole blood samples by direct immunofluorescence and flow cytometry according to current standard protocols.

Lymphoproliferation assays

Whole blood cultures were set up to test lymphocyte reactivity to TCR/CD3 ligation essentially as described elsewhere [9,10]. In brief, 150 μ l of heparinized whole blood diluted 1:10 with RPMI 1640 were seeded in each U-bottomed microtitre well (Falcon, Lincoln Park, NY); 15 μ l of CD3 MoAb (CLB/T3-4E; CLB, Amsterdam, The Netherlands; 1:10 000 dilution of ascitic fluid) or phytohaemagglutinin (PHA; Murex Diagnostics, Dartford, UK) at a final concentration of 5 μ g/ml were added. CD28 MoAb (CLB-CD28/1; CLB; 10 μ l/well of a 1:5000 dilution of ascitic fluid) was also added to CD3 MoAb-stimulated cultures where appropriate. Lymphoproliferative responses to CD3 and CD28 MoAbs were studied in the frame of the European Community Concerted Action on Progression Markers in HIV Infection, co-ordinated by Dr F. Miedema (Department of Clinical Viro-Immunology, CLB, Amsterdam, The Netherlands).

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood by density gradient centrifugation on Lymphoprep (Nycomed AS, Oslo, Norway) at 800 *g* for 30 min. Separated PBMC (1×10^6 cells/ml) were cultured in 200 μ l of RPMI 1640 with 10% AB human serum tested negative for anti-HIV-1 antibodies (HS) (N.A.B.I., Miami, FL) in 96-well flat-bottomed cell culture plates (Falcon) in the presence of baculovirus-expressed recombinant gp160 of HIV-1_{IIIB} (Protein Sciences Corp., formerly MicroGeneSys Inc.) or *Escherichia coli*-derived recombinant p24 of HIV-1_{IIIB} (Intracel Corp., Cambridge, MA) at a concentration of 2 μ g/ml. CD28 MoAb was added to appropriate wells 24 h later.

All cultures were set up in triplicate. After 6 days of culture at 37°C in humidified 5% CO₂ atmosphere (4 days for cultures with CD3 MoAb or PHA), 0.5 μ Ci of tritiated thymidine (³H-TdR; specific activity 25 Ci/mmol; Amersham, Aylesbury, UK) was added to each well and after a further 24 h cells were harvested onto glassfibre filters by a cell harvester. Incorporated radioactivity was measured in a scintillation counter and expressed as ct/min.

Evaluation of lymphoproliferative responses

³H-TdR uptake in whole blood cultures was corrected for the number of CD3⁺ lymphocytes contained in the culture and expressed as ct/min per 10³ T cells. Antigen-driven responses of separated PBMC were expressed as stimulation index (SI), calcu-

lated dividing the mean of ct/min of stimulated cultures by that of unstimulated ones. Lymphoproliferative responses to HIV-1 were considered positive for SI > 2. This cutoff value was chosen by considering that the mean + 2 s.d. of SI obtained in cultures of PBMC from HIV-1⁻ controls was < 2. The same cutoff was employed by others in similar assays with HIV-1 antigen [20].

Statistical analysis

Data from different groups of subjects were compared by Mann-Whitney *U*-test. Paired data were compared by Wilcoxon signed-rank test.

RESULTS

Effect of CD28 costimulation on CD3 MoAb-induced proliferative responses

To assess the function of the CD28 costimulation pathway in all T cells of HIV-1-infected subjects at various stages of disease, the addition of CD28 MoAb to CD3 MoAb-stimulated whole blood cultures was studied. Average ³H-TdR incorporation in whole blood cultures is shown in Fig. 1a, which depicts the increase of proliferative responses to CD3 MoAb when CD28 costimulation is provided. Given the variable number of lymphocytes in whole blood cultures, ct/min were corrected for the number of T cells contained in each culture well, to allow comparison of responses among groups of subjects. CD3 MoAb-induced proliferation in samples from HIV-1-infected patients was lower than that found in HIV-1⁻ healthy controls. The difference was not statistically significant in those patients with CD4⁺ T cell numbers > 500 cells/ μ l (CDC group 1) (median (range) = 412 (44–2762) ct/min per 10³ T cells versus 1588 (105–4652) ct/min per 10³ T cells, median (range) of controls), but the result was significant in the group of patients with CD4⁺ T cell counts between 200 and 500 cells/ μ l (CDC group 2) (650 (14–6961) ct/min per 10³ T cells; *P* < 0.01 versus controls). The lowest responses to CD3 MoAb were found in the group of patients with CD4⁺ T cell counts < 200 cells/ μ l (CDC group 3) (50 (12–351) ct/min per 10³ T cells; *P* < 0.001 versus controls) (Fig. 1b). When PHA was used, only the proliferative responses in samples from patients in group 3 were significantly lower than those of controls (*P* < 0.05) (Fig. 1b).

The effect of CD28 MoAb addition to CD3 MoAb-containing cultures ranged from a median 2.5-fold increase of the proliferation corrected for the number of T cells in CDC group 3 to a median 4.1-fold increase in CDC group 2, resulting in significantly higher proliferative responses than those with CD3 MoAb alone in all groups of subjects (Fig. 1b). The differences in increase of proliferation in the four groups were not statistically significant, and as a consequence, responses to the combination of CD3 and CD28 MoAbs in HIV-1-infected patients differed from those obtained in control samples to the same extent as the responses to CD3 MoAb alone. Compared with responses of HIV-1⁻ controls (median (range) 5583 (1059–10 792) ct/min per 10³ T cells), patients in CDC group 3 showed the lowest response to the combination of CD3 and CD28 MoAbs (median (range) = 168 (18–3520) ct/min per 10³ T cells; *P* < 0.001 versus controls), followed by CDC group 1 (2391 (738–12 159) ct/min per 10³ T cells; *P* < 0.05 versus controls), and CDC group 2 (2438 (199–22 102) ct/min per 10³ T cells; *P* < 0.01 versus controls).

Effect of CD28 costimulation on HIV-1-specific proliferative responses

Lymphoproliferative responses to gp160 were measured in 34

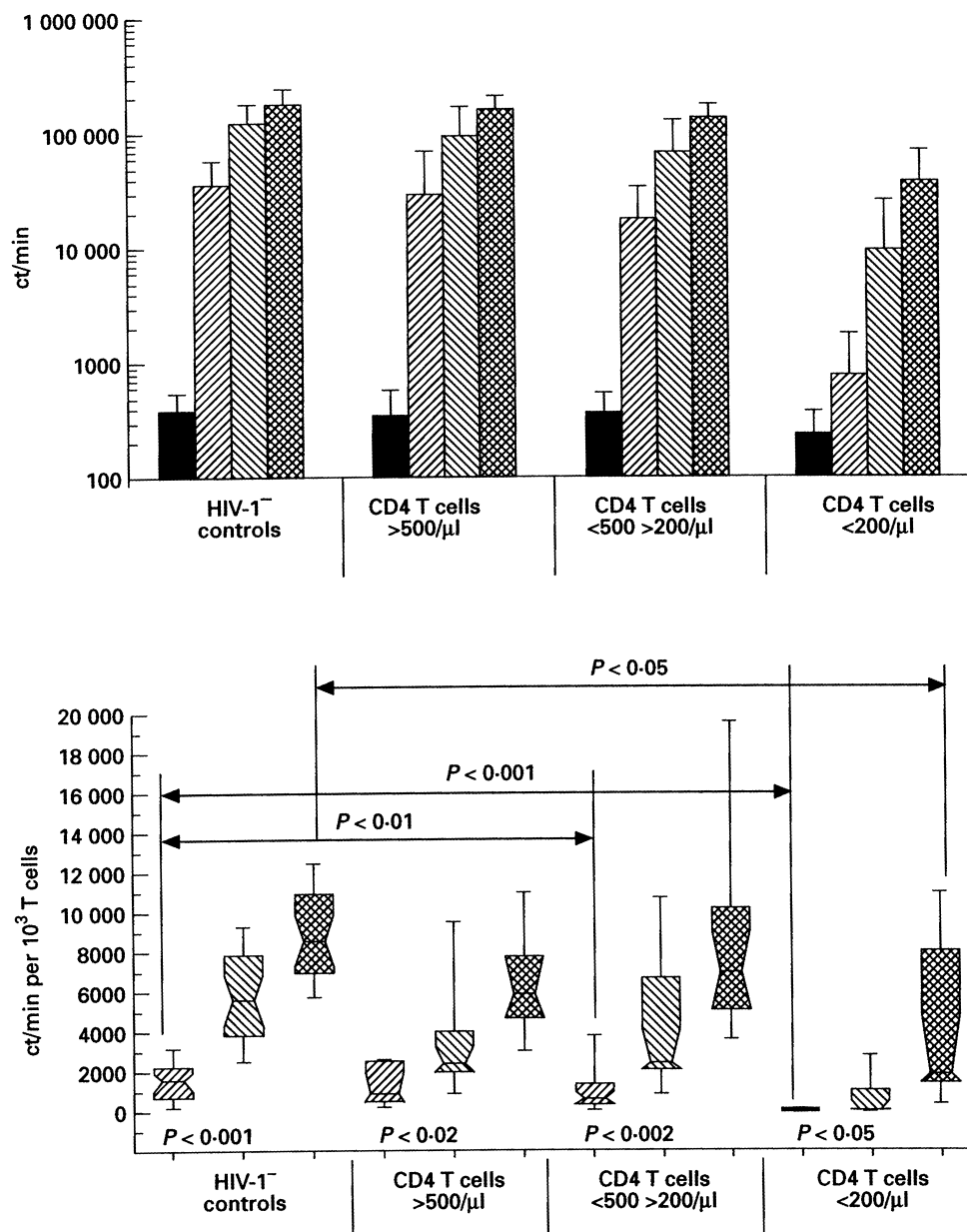


Fig. 1. (a) Lymphocyte thymidine (³H-TdR) incorporation in response to CD3 MoAb alone, CD3 and CD28 MoAbs, and phytohaemagglutinin (PHA) in the following groups of subjects: HIV-1⁻ healthy controls ($n = 25$), median CD4⁺ T cell count = 799 cells/ μ l, range 465–1347; CDC A1 HIV-1-infected patients (CD4⁺ T cell count >500 cells/ μ l; $n = 10$), median CD4⁺ T cell count = 602 cells/ μ l, range 532–1737; CDC A, B2 HIV-1-infected patients (200 < CD4⁺ T cell count <500 cells/ μ l; $n = 15$), median CD4⁺ T cell count = 349 cells/ μ l, range 225–493; CDC B, C3 HIV-1-infected patients (CD4⁺ T cell count <200 cells/ μ l; $n = 11$), median CD4⁺ T cell count = 15 cells/ μ l, range 4–181. Results are expressed as mean ct/min; error bars represent s.d. ■, Unstimulated; ▨, CD3; ▩, CD3 + CD28; ▤, PHA. (b) Lymphocyte proliferative responses in the four groups of subjects expressed as ct/min per 10³ T cells and depicted as box plots representing the 10th, 25th, 40th, 50th (median), 60th, 75th and 90th percentiles.

HIV-1-infected asymptomatic patients with CD4⁺ T cell numbers between 261 and 862 cells/ μ l (median 439 cells/ μ l) and in 14 HIV-1⁻ healthy controls. In the group of HIV-1-infected patients, 27 were undergoing post-exposure immunization with gp160. Average ³H-TdR incorporation in PBMC cultures stimulated with HIV-1 recombinant antigens, in the presence or absence of CD28 MoAb, is shown in Fig. 2. No increase of ³H-TdR incorporation with respect to unstimulated cultures was seen with the addition of CD28 MoAb and/or HIV-1 antigen, unless

patients were receiving immunization with rgp160 and gp160 was used as stimulus. All but three patients in the group receiving post-exposure immunization had significant lymphoproliferative response to gp160 *in vitro*, as assessed by SI > 2 (median SI = 8.8, range 0.6–145.9). No significant responses to gp160 were found in the untreated group of HIV-1-infected patients (median SI = 0.9, range 0.2–1.9) and in HIV-1⁻ controls (median SI = 1.1, range 0.8–2.0) (Fig. 3). The addition of CD28 MoAb augmented significantly the *in vitro* proliferative response to gp160 (median

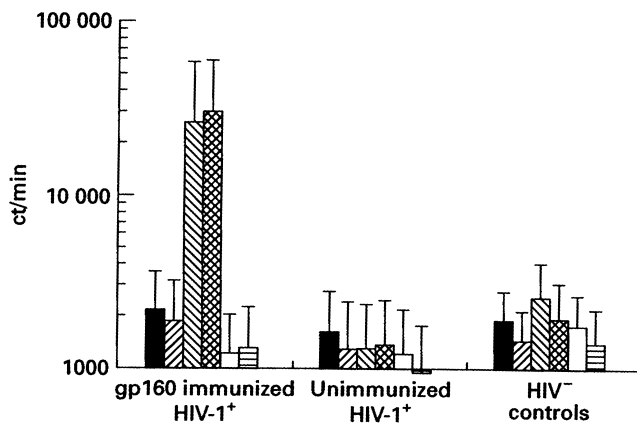


Fig. 2. Lymphocyte thymidine (³H-TdR) incorporation in response to HIV-1 antigen with or without addition of CD28 MoAb in HIV-1-infected patients receiving or not active immunization with VaxSyn and in HIV-1⁻ healthy controls. Results are expressed as mean ct/min; error bars represent s.d. ■, unstimulated; medium only (bkg); ▨, bkg + CD28; ▩, gp160; □, gp160 + CD28.

SI = 15.2, range 0.44–315.8; $P < 0.05$, when compared with paired SI obtained with gp160 alone) in patients receiving immunotherapy with gp160. In this group, the gp160 SI increased from 0.7 to 5.8 times when CD28 MoAb was added (median = 1.5-fold increase). Only five patients in this group, including those with no detectable response to gp160 alone, failed to show any response

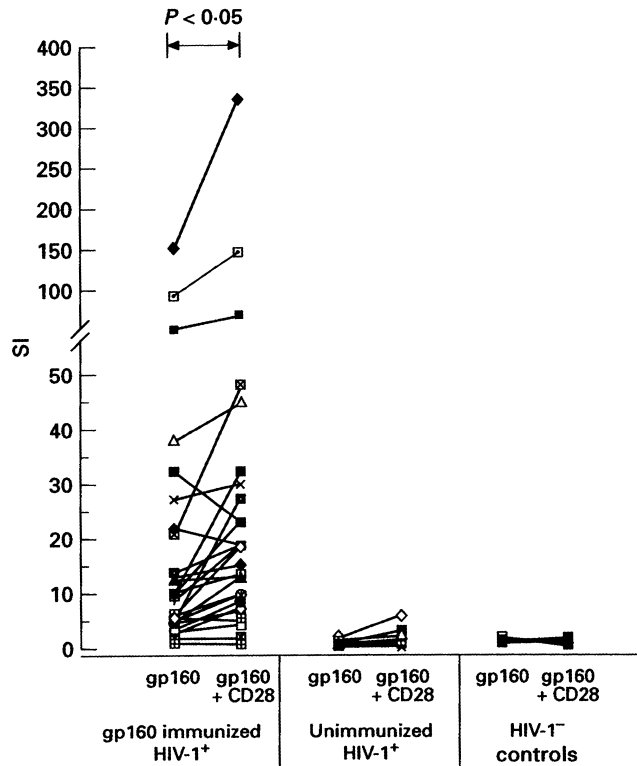


Fig. 3. Effect of CD28 costimulation on HIV-1 gp160-specific proliferative responses, expressed as stimulation index (SI), in HIV-1-infected patients receiving or not active immunization with VaxSyn and in HIV-1⁻ healthy controls.

increase when CD28 MoAb was added. No significant lymphocyte proliferation was induced by the addition of CD28 MoAb to the cultures from the remaining two groups of subjects, regardless of their HIV-1 infection status.

To evaluate the effect of CD28 costimulation on a second HIV-1 antigen, unrelated to the immunization regimen, responses to p24 were studied in the same fashion (Fig. 2). Addition of CD28 MoAb did not induce significant responses in PBMC cultures stimulated with HIV-1 p24 both from 24 HIV-1-infected patients, of whom only one showed a moderate response to p24 alone (SI = 2.3), and 14 HIV-1⁻ healthy controls.

DISCUSSION

T lymphocyte defects in the course of HIV-1 infection independent of CD4⁺ T cell loss have been previously described [7,21]. The possibility of circumventing these defects with appropriate costimulatory signals or cytokines could suggest immunotherapeutic approaches aimed at correcting immune disturbances early in HIV-1 infection. Abnormalities in CD28 costimulation, due to either defective interaction with its ligands CD80 and CD86 on APC or intrinsic defects of the lymphocyte CD28 signalling pathway, may be involved in defective T cell responses during HIV-1 infection. Triggering of CD28 can rescue T cells from anergy induced by the delivery of a single activation signal [17], and it has been recently shown to partially reverse the *in vitro* inhibitory effect of HIV-1 gp120 on T cell responses [22]. To rule out defects of CD28-associated signalling in T lymphocytes during HIV-1 infection, we tested the effect of CD28 MoAb addition to CD3 MoAb-stimulated lymphocytes. Augmentation of CD3 MoAb-induced responses was observed in all categories of HIV-1-infected patients, including those with markedly depressed basal responses. This is in agreement with the finding of intact CD28 and CD27 costimulation pathways in HIV-1 infection, reported by Meyaard *et al.* [23], and confirms the data of increased CD3 MoAb-induced proliferation in PBMC from HIV-1-infected subjects reported by Roos *et al.* [24].

CD28 costimulation has been reported to increase dramatically the *in vitro* proliferative response to a recall antigen, namely tetanus toxoid, of PBMC from HIV-1-infected subjects in the same fashion as the response to pokeweed mitogen [25]. Lymphoproliferative responses to HIV-1 antigen are characteristically weak and uncommon [6] in HIV-1-infected subjects, possibly as a reflection of incomplete activation signals delivered from APC. To test this hypothesis, we extended the study of CD28 costimulation to the lymphoproliferative responses to HIV-1 antigen, namely gp160 and p24 in patients with asymptomatic HIV-1 infection. In patients exhibiting significant responses to gp160 as a result of active *in vivo* immunization with the recombinant protein, *in vitro* CD28 costimulation resulted in an increase in lymphoproliferation, as observed for CD3 MoAb-induced responses. When no significant response was observed to either gp160 or p24 alone, addition of CD28 MoAb did not exert any effect, suggesting that the frequency of circulating cells able to proliferate in response to HIV-1 antigens in HIV-1-infected patients is comparable to that of HIV-1⁻ controls. Analogous experiments have been performed by others adding IL-12 to PBMC cultures stimulated with HIV-1 Env peptides. Restoration of responses to Env peptides after the *in vitro* addition of IL-12 was described [26], but an extension of the analysis to a group of HIV-1-infected patients showed it in only a minority of patients, the HIV-1-specific lymphoproliferative

responses remaining undetectable in more than 70% of patients studied [27].

Assuming that CD4⁺ T cells, likely to be the ones reacting to processed soluble antigen, are primed to respond to HIV-1 antigen during the course of infection, several mechanisms can be hypothesized to explain their apparent disappearance from the circulation. Virus-specific T cells could be segregated in tissues where viral replication takes place, becoming preferential targets of infection and being eliminated. Preferential contact with infected APC may induce anergy [28], which is apparently not overcome by CD28 triggering. Engagement of costimulation pathways other than CD28 might be necessary to rescue HIV-1-specific T cells or, alternatively, CD28 is not available on this particular cell population. Decrease of the proportion of CD28-expressing T cells has been described. It is most pronounced in the CD8⁺ population, but also the proportion of CD28⁻ CD4⁺ T cells increases with disease progression [24,29]. Even though the decrease of CD28⁺ T cells does not significantly affect the reactivity to CD28 MoAb when all T cells are activated via CD3, it could be possible that a preferential loss of CD28 in HIV-1 antigen-specific T cells could render them refractory to this type of costimulation. In support of the hypothesis of a deletion or anergy of primed virus-specific CD4⁺ T cells, virus-specific lymphoproliferative responses were detected in macaques infected with the non-pathogenic HIV-2 but not when infected with pathogenic simian immunodeficiency virus (SIV). Moreover, SIV-specific responses induced by immunization with non-infectious antigen were lost after challenge with infectious SIV [30].

Alternatively, the possibility that HIV-1-specific CD4⁺ T cells undergo limited or no priming during the natural infection has to be considered. The susceptibility to infection of professional APC could result in a prevalent presentation of endogenously synthesized antigen in the context of MHC class I, leading to activation of CD8⁺ T lymphocytes. In this view, the vigorous lymphoproliferative responses to soluble HIV-1 antigen seen during post-exposure immunization with HIV-1 subunits, as performed in some of the patients included in the present study, have to be regarded as primary responses, as are those obtained in HIV-1 naive subjects [31].

In conclusion, the present data demonstrate that lymphocytes responding to soluble HIV-1 antigen are not deleted in HIV-1-infected patients, being detectable after specific *in vivo* stimulation, and are able to respond to costimulatory signals: the relevance of these responses to the containment of HIV-1 replication in view of therapeutic approaches has to be established.

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