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# Decreased CD40 ligand induction in CD4 T cells and dysregulated IL-12 production during HIV infection

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**SUMMARY**

During HIV infection various cytokines are overproduced in early stages, whereas in advanced disease cytokines of the T helper 1 type (e.g. interferon-gamma (IFN-$\gamma$)) are selectively deficient. During antigenic stimulation, the production of type-1 cytokines is enhanced by IL-12, secreted by antigen-presenting cells (APC) after their interaction with activated CD4 T cells. Two factors are essential in this process: priming APC with IFN-$\gamma$ and triggering the CD40 receptor on APC by CD40 ligand (CD40L). In view of the importance of this pathway, we compared its regulation in HIV-infected and control subjects. After cross-linking of the T cell receptor (TCR)/CD3 complex, the proportional expression of CD40L was similar on $CD4^+$ T cells from controls and from patients with high circulating CD4 T counts ($>500/\mu$l), but CD40L up-regulation was significantly reduced in patients with more advanced disease. Simultaneous triggering of the costimulatory receptor CD28 on T cells through its natural ligand CD80 partly corrected the CD40L defect in patients with intermediate CD4 T counts (200–500), but not in AIDS patients. Early production of IFN-$\gamma$ was preserved in lymphocytes from $HIV^+$ patients. The expression of CD40 on peripheral monocytes from $HIV^+$ subjects was increased in a disease stage-related fashion. Stimulation of mononuclear cells through cell-bound CD40L and soluble IFN-$\gamma$ induced significantly higher IL-12 in cultures from patients with $>200$ circulating CD4 T cells, whereas IL-12 production was marginally decreased in cultures from patients with $<200$ CD4 T cells, compared with healthy control cultures. In conclusion, our data suggest that impaired CD40L induction on CD4 T cells contributes to deficient type-1 responses through decreased IL-12 production in AIDS infection, whereas enhanced CD40-mediated IL-12 production in less advanced stages might contribute to increased levels of various cytokines in early disease

**Keywords** CD40 CD40L interferon-gamma IL-12 HIV

## INTRODUCTION

IL-12 is considered as the most potent inducer of type-1 responses, characterized by dominant interferon-gamma (IFN-$\gamma$) production, together with IL-2 and tumour necrosis factor (TNF) [1]. Products of infectious agents can induce IL-12 secretion by antigen-presenting cells (APC), including monocytes and dendritic cells, either directly or indirectly through antigen-activated T cells [2–5]. A combination of two signals is essential in the latter pathway: priming of APC with IFN-$\gamma$ and subsequent triggering of CD40 on their membrane [6–8]. Sufficient levels of IFN-$\gamma$ for priming are produced early during an immune response, presumably by natural killer (NK) cells and/or T cells. Antigen-mediated cross-linking of the $\alpha\beta$ T cell receptor (TCR)–CD3 complex induces up-regulation of CD40 ligand (CD40L, CD154 or gp39) on the CD4 T cell membrane, which triggers CD40 on the APC. Co-stimulation of CD4 T cells through CD28 via the natural ligands of the B7 family (CD80 or CD86), expressed on B cells and APC, is not absolutely required but increases and prolongs the CD40L expression [9–11].

During HIV infection cytokine profiles are altered in a disease stage-related way. Early in the course of the disease, massive

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immune activation, with enhanced production of several cytokines, has been observed [12,13]. The immune deficiency of the later stages is characterized by decreased type-1 responses, including impaired IFN-$\gamma$ production [14–19]. The CD40/IL-12/IFN-$\gamma$ axis is thought to be essential in protective immunity against some intracellular 'parasites', including Leishmania, Toxoplasma and Mycobacteria, all well known opportunists in HIV-infected subjects [5,20–24].

Studying the regulation of IL-12 after stimulation with regular antigens has been limited by technical and conceptual problems. IL-12 levels induced by antigen in normal peripheral blood mononuclear cells (PBMC) are at or below the sensitivity threshold of existing ELISA. Similarly, antigen-induced CD40L up-regulation on CD4 T cells is difficult to measure, because of the low precursor frequency and the apparent recycling of this receptor. Antigen-specific responses are highly variable in healthy subjects, because they depend on the subtle differences in individual immune histories. A further level of complexity is present during HIV infection. Besides CD4 T cell depletion, HIV is associated with T cell activation by antigen-specific, cross-reactive and polyclonal stimulation, as well as with activation-induced clonal anergy and apoptosis [25,26].

In view of these limitations of antigen-specific responses, various non-physiological stimuli have been used to evaluate IL-12 and CD40L regulation in HIV-infected subjects, but with conflicting results. Stimulation with formalin-treated *Staphylococcus aureus* (SAC), with mitogens or with *Toxoplasma* induced lower levels of IL-12 in PBMC from HIV-infected subjects compared with controls [27–34]. In contrast, IL-12 levels after stimulation of whole blood with lipopolysaccharide were not different between $HIV^+$ patients and controls [35]. Brugnoni *et al*. suggested that CD4 T cells from $HIV^+$ subjects have an unimpaired ability to up-regulate CD40L after stimulation with phorbol myristate acetate (PMA) and ionomycin [36], whereas Wolthers *et al*. in a similar system showed decreased expression [37]. Chougnet *et al*. were able to correct the impaired SAC-induced IL-12 production in $HIV^+$ PBMC by addition of IFN-$\gamma$ and soluble CD40L [38].

Because of the remaining questions on CD40L and IL-12 regulation after TCR/CD3 stimulation during HIV infection, we took advantage of some models developed in our laboratories. These allow us to quantify CD40L on T cells after CD3/TCR triggering with cell-bound anti-CD3 or with superantigens, and to measure IL-12 production in PBMC after stimulation with cell-bound CD40L and IFN-$\gamma$. Our results suggest that the IL-12 production capacity of APC is rather enhanced in the early stages and not significantly decreased in AIDS patients. 'Priming' IFN-$\gamma$ production is unaltered, but CD40L up-regulation on CD4 T cells from AIDS patients is significantly decreased.

## SUBJECTS AND METHODS

*Subjects*

Thirty healthy controls were recruited at the Antwerp Blood Transfusion Centre and amongst the laboratory personnel of our Institute. Seventy HIV-infected subjects, excluding acutely ill subjects, were recruited at the out-patients clinic. All patients were suboptimally treated, according to our present standards. During the studies using OKT3 stimulation, protease inhibitors were only used in monotherapy or with one reverse transcriptase inhibitor. For the experiments on superantigen-induced CD40L, patients were investigated before starting triple therapy and thus they were protease inhibitor-naive. All patients were classified according to peripheral CD4 T counts into CDC categories: group 1, CD4 T > 500/$\mu$l; group 2, CD4 T 200–500/$\mu$l; and group 3, CD4 T < 200/$\mu$l.

*Culture conditions*

PBMC were isolated from EDTA-anti-coagulated blood, using Histopaque (Sigma, Costa Mesa, CA). The cells were resuspended in RPMI, supplemented with penicillin, streptomycin and glutamine (all from GIBCO, Paisley, UK) and with 10% bovine calf serum (Hyclone, Logan, UT).

For induction of CD40L expression on T cells, either anti-CD3 or superantigens were used to stimulate the T cells through the CD3/TCR complex. In the experiment with anti-CD3, the PBMC were first depleted of B cells and monocytes by two rounds of negative selection with Dynabeads-CD19 and -CD14 (Dynal, Oslo, Norway), according to the manufacturer's instructions. Five hundred thousand depleted PBMC were either cultured in 1 ml complete medium or with the anti-CD3 MoAb OKT3 (purified from hybridoma supernatant) at a final concentration of 1 $\mu$g/ml. To part of the cultures $10^5$ parental or CD80-expressing P815 cells were added (both kindly provided by Dr L. Lanier, DNAX, Palo Alto, CA), after pretreatment with Mitomycin C at 25 $\mu$g/ml for 30 min at 37°C. The parental P815 cells express Fc$\gamma$ receptors, which cross-link anti-CD3, bound on the T cells, thus providing a strong activation signal to the T cells. The P815, transfected with human CD80, trigger the CD28 co-receptor on the T cells and therefore provide an even stronger stimulation. After 1 day of culture at 37°C in 5% $CO_2$, cells were labelled for CD40L and supernatants stored for IFN-$\gamma$ determination.

Non-depleted PBMC ($10^6$/ml final concentration) were stimulated with the superantigen Staphylococcus enterotoxin A (SEA; Sigma) at 10 ng/ml. After 3 days of culture with or without SEA, the CD40L was measured on the CD4 T cells.

For induction of IL-12, non-depleted PBMC were cultured at a final concentration of $2 \times 10^6$/ml, in the presence of either 60 000 3T6 mouse fibroblasts, stably transfected with human CD40L (produced by one of the authors, K.T.), 100 U/ml recombinant IFN-$\gamma$ (Genzyme, Cambridge, MA) or both stimuli. After 2 days of culture, the supernatants were harvested and stored at –20°C.

*Staining procedures*

The expression levels of CD40 on MO were evaluated using a standard whole blood procedure. EDTA–blood (50 $\mu$l) was incubated with a combination of 5 $\mu$l anti-CD40–FITC or IgG1–FITC control MoAb (Pharmingen, Erembodegem, Belgium), 5 $\mu$l anti-CD4–PE and 5 $\mu$l anti-CD3–peridinin chlorophyll protein (PerCP) (all from Becton Dickinson, Erembodegem, Belgium) for 30 min at 4°C. Afterwards, the erythrocytes were lysed (Becton Dickinson's lysing solution), and the leucocytes washed with PBS–bovine serum albumin (BSA) and fixed with 1% paraformaldehyde. Ten thousand events were acquired in a FACScan and the results were analysed with the LYSYS I software. Monocytes were gated based on a combination of physical characteristics and their expression of CD4, but not CD3. The mean fluorescence intensity (MFI) of the anti-CD40–FITC binding on the monocytes, expressed in relative fluorescence units (RFU), is used as a parameter of CD40 density, in comparison with the RFU of the control IgG–FITC.

The OKT3- and/or P815-stimulated cells were stained after overnight culture with 5 $\mu$l anti-CD40L–PE, 5 $\mu$l anti-CD4–FITC and 5 $\mu$l anti-CD8–PerCP for 30 min at 4°C. The percentage of

$CD40L^+$ cells was measured in the bright $CD4^+$ and bright $CD8^+$ lymphocytes. The threshold for positivity, based on isotypic controls, was 40 RFU.

SEA-stimulated PBMC were incubated with 5 $\mu$l anti-CD40L–PE, anti-CD25–PE or IgG1–PE control MoAb, together with 5 $\mu$l anti-CD4–FITC and 5 $\mu$l anti-CD3–PerCP (all from Becton Dickinson) at 37°C in complete culture medium for various amounts of time. After a wash with PBS–BSA, the cells were fixed and acquired in the FACScan. Results are expressed as the percentage of $CD40L^+$ cells within the $CD4^+CD3^+$ lymphocytes. The positivity threshold, based on the binding of IgG1–PE, was 70 RFU.

*Cytokine determinations*

The concentration of IFN-$\gamma$ was determined, using an ELISA kit from Eurogenetics (Tessenderlo, Belgium) with a sensitivity of 0·1 U/ml. For IL-12 p70 determination, antibody pairs and the standard from R&D Systems (Immunosource, Zoersel, Belgium) were used (lower limit 10–20 pg/ml).

*Statistical analysis*

The various data were processed using SPSS software. Non-parametric analyses were used throughout. Results are reported as the median and the confidence intervals; differences between groups were calculated with the Mann–Whitney *U*-test and correlations between variables with the Spearman's rank test.

## RESULTS

*Strategies to measure CD40L up-regulation on T cells*

PBMC were stimulated with OKT3 or SEA for 1–3 days and membrane expression of CD40L was evaluated by immunofluorescence after staining with anti-CD40L for 30 min at 4°C. Under these standard conditions, only low levels (< 5%) of CD40L expressing CD4 T cells were observed (data not shown), which might be explained by release of CD40L into the supernatant and/or by a continuous recycling of CD40L between the cell membrane and an intracellular compartment, induced by CD40 on monocytes and B cells. To circumvent the latter phenomenon, we developed two strategies: (i) prevention of CD40L down-regulation by a preceding depletion of $CD40^+$ cells (the B cells and monocytes); (ii) 'catching' the cycling receptor by prolonged incubation of stimulated PBMC with anti-CD40L at 37°C.

The first approach is illustrated in Fig. 1. Stimulation of entire PBMC with soluble anti-CD3 (OKT3) induced limited CD40L expression (2·8%) that was only slightly enhanced by cross-linking of the T cell-bound OKT3 through Fc$\gamma$R-expressing parental P815 (7·7%) and further increased by costimulation of CD28 on T cells with CD80-transfected P815 (11·7%). After preliminary depletion of B cells and monocytes, the CD4 T cells became unresponsive to soluble OKT3 (only 0·3% CD40L), since this pretreatment removes the main accessory cells. Addition of Fc$\gamma$R-expressing P815 induced a much higher CD40L expression (48·9%), which was further enhanced when using the CD80 transfectants (60·6%).

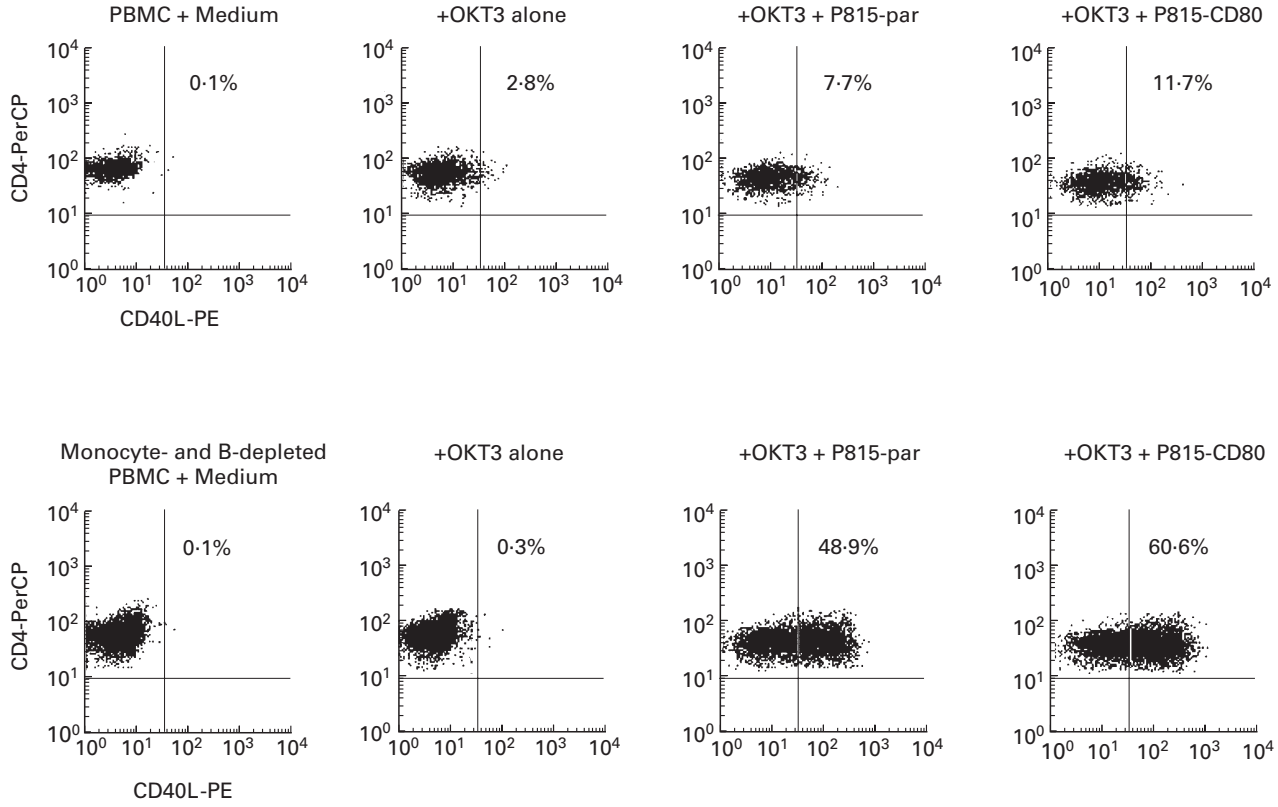



**Fig. 1.** OKT3-induced CD40L expression on normal CD4 T cells: influence of preliminary B cells and monocyte depletion. Peripheral blood mononuclear cells (PBMC) from one control subject were either sham-treated or depleted of B cells and monocytes. Afterwards they were stimulated overnight with either medium, OKT3 alone or OKT3 in combination with parental or CD80-transfected P815 accessory cells. The T cells were stained at 4°C with anti-CD4, anti-CD3 and anti-CD40L and analysed by FACS. Dot plots of gated CD4 T cells show the expression of CD40L in the various conditions.

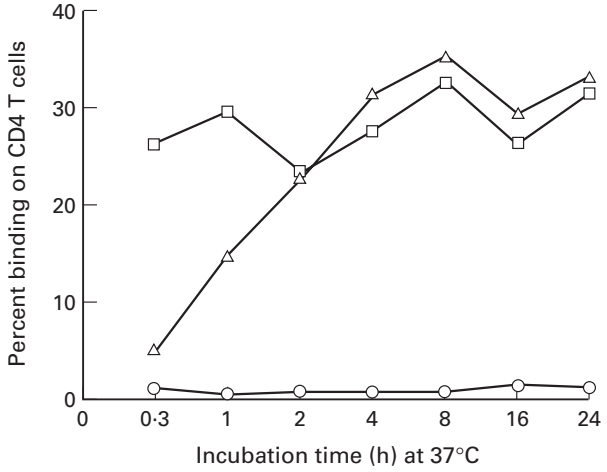



**Fig. 2.** Influence of incubation time on the measurement of Staphylococcus enterotoxin A (SEA)-induced CD40L on CD4 T cells. Peripheral blood mononuclear cells (PBMC) from one control subject were cultured with 10 ng/ml SEA for 3 days. The cells were stained thereafter with anti-CD4–FITC and one of the following PE-labelled MoAbs: isotypic control (○), anti-CD25 (□), or anti-CD40L (Δ). The labelling was performed in complete medium at 37°C. The binding kinetics of the PE-labelled MoAbs on gated CD4 T cells is shown.

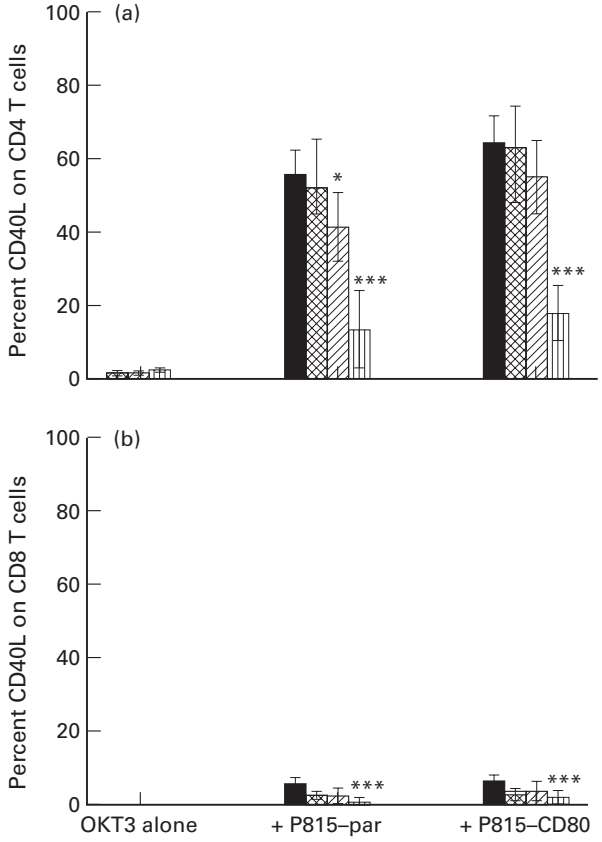



**Fig. 3.** OKT3-induced expression of CD40L on CD4 and CD8 T cells from patients and controls. Peripheral blood mononuclear cells (PBMC), depleted of B cells and monocytes, were incubated with either OKT3 alone or in combination with parental or CD80-transfected P815. The cells was harvested after an overnight culture and stained with anti-CD4–FITC, anti-CD40L–PE and anti-CD8–PerCP for 30 min at 4°C. The proportional expression levels of CD40L on CD4 T cells (a) and CD8 T cells (b) after various stimulations are represented as medians ± confidence intervals. The subject groups are as follows: 10 healthy controls (■), seven HIV-infected subjects with CD4 T counts > 500/$\mu$l (□), 10 patients with CD4 T counts 200–500/$\mu$l (oblique hatched), and seven patients with CD4 T counts < 200/$\mu$l (vertical hatched). Levels of significance of differences between patient groups and controls: *$P < 0{\cdot}05$; ***$P < 0{\cdot}001$.

Apparently, the depletion of $CD40^+$ B cells and monocytes prevents down-regulation of CD40L on optimally stimulated T cells.

The second procedure was applied in the model of PBMC stimulation with SEA. A kinetic study indicated that a 3-day stimulation period was best suited to induce both CD25 (IL-2R$\alpha$) and CD40L on CD4 T cells, but the requirements to measure these receptors optimally were quite different. Incubation of the stimulated cells with anti-CD25–PE, specific for the IL-2 receptor $\alpha$-chain, for 20 min either at 4°C (not shown) or 37°C (Fig. 2) was sufficient to quantify accurately CD25 on gated CD4 T cells and prolonged incubation at 37°C failed to reveal additional IL-2R$\alpha$. In contrast, maximal binding of anti-CD40L only occurred after 4 h of incubation at 37°C (Fig. 2). The latter finding can tentatively be explained by a progressive labelling of the cycling CD40L. The observed CD40L staining was specific, since it could be blocked by unlabelled anti-CD40L, but not by isotypic control MoAb (data not shown).

*Anti-CD3 ± CD80 stimulation of T cells from patients with advanced HIV infection results in lowered early CD40L but preserved early IFN-γ*

After overnight stimulation with OKT3 + parental P815 cells, CD40L could be induced in about 50% of control CD4 T cells. The extent of CD40L up-regulation was similar in $HIV^+$ subjects with high circulating CD4 T counts (group 1: CD4 T > 500/$\mu$l); it was significantly reduced in patients with intermediate levels of CD4 T cells (group 2: CD4 T 200–500/$\mu$l), and it was dramatically impaired in AIDS patients. Costimulation through CD28, using CD80-expressing P815, increased the expression of CD40L on CD4 T cells from controls and group 1 patients by 9% and even by 13% in group 2 patients ($P < 0{\cdot}05$). In contrast, the response of CD4 T cells from AIDS patients was not improved by CD80 costimulation (Fig. 3a).

The proportional expression of CD40L on optimally stimulated CD8 T cells was limited to 6–7% in controls and it was significantly lower in AIDS patients (< 3%). There was no enhancement by CD80 (Fig. 3b).

IFN-γ was measured after overnight stimulation of monocytes/B-depleted PBMC with OKT3 ± P815, as a parameter of the early availability of the 'IL-12 priming signal'. Soluble OKT3 or P815 alone were unable to induce IFN-γ, whereas combined OKT3 + parental P815 induced around 15 U/ml IFN-γ in cultures from both control and patient cells. If CD80-expressing P815 + OKT3 was used, the IFN-γ level was almost tripled in control supernatants ($P < 0{\cdot}05$); it was even slightly (but NS) higher in supernatants from group 1 patients, but not enhanced in those from group 2/3 patients (Fig. 4).

Taken together, the most significant finding seems to be that the early CD40L expression on CD4 T cells from subjects with advanced HIV infection is impaired after stimulation with anti-CD3, even if optimal costimulation through CD80 is provided.

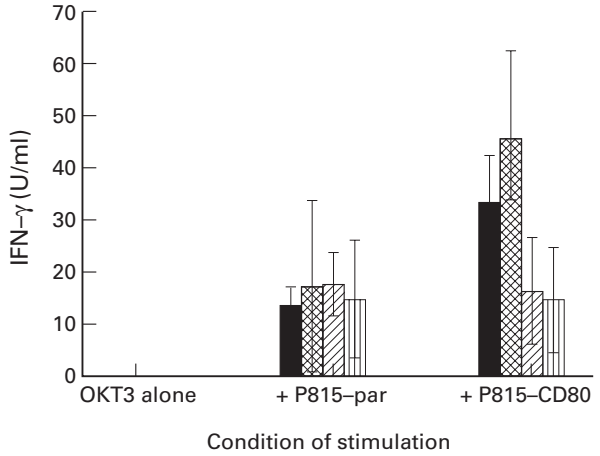



**Fig. 4.** Early OKT3-induced IFN-γ production by lymphocytes from HIV$^-$ and HIV$^+$ subjects. In the experiment described in Fig. 3, the supernatants were harvested after overnight stimulation and IFN-γ was determined. No significant differences were noted between the subject groups.

*Impaired SEA-induced CD40L on CD4 T cells from AIDS patients*

In a second series of experiments, the effect of HIV infection on CD40L induced by SEA stimulation of complete PBMC was examined. Superantigens, including SEA, are known to involve similar molecular interactions to regular antigens in T cell activation: they bind to HLA class II on APC and to the TCR [39–41]. In addition, they are dependent on costimulation through B7–CD28 interaction [42]. The advantage of superantigens over recall antigen is that the former bind to a specific set of V$\beta$ chains and therefore directly activate a substantial and rather similar proportion of T cells in different individuals [39].

In order to exclude the possible influence of intensive antiretroviral therapy, we selected a limited group of protease inhibitor-naive subjects. Using the prolonged staining protocol, it was found that the proportional CD40L expression on CD4 T cells was decreased in the three AIDS patients and in one patient of group 2, who had only 213 CD4 T cells/$\mu$l blood. CD40L expression on CD4 T cells from the other non-AIDS patients (all with CD4 T counts >250) was indistinguishable from control subjects (Fig. 5). Thus, a model antigenic stimulus, which is dependent on TCR and B7–CD28 interactions, results in impaired CD40L up-regulation selectively in patients with severe immunodeficiency.

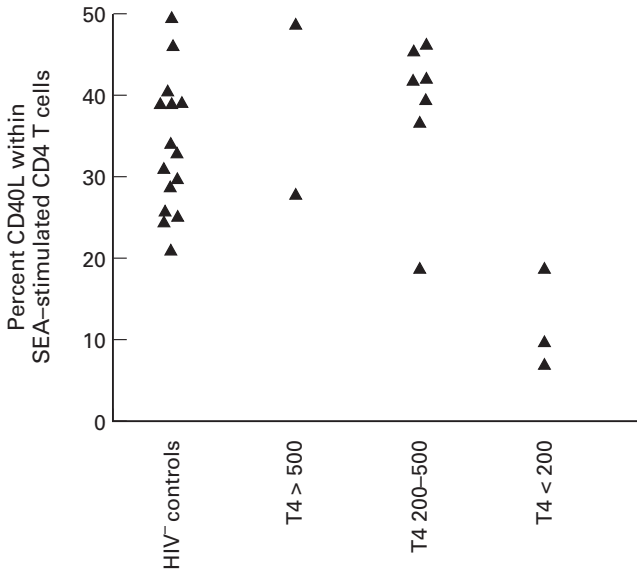
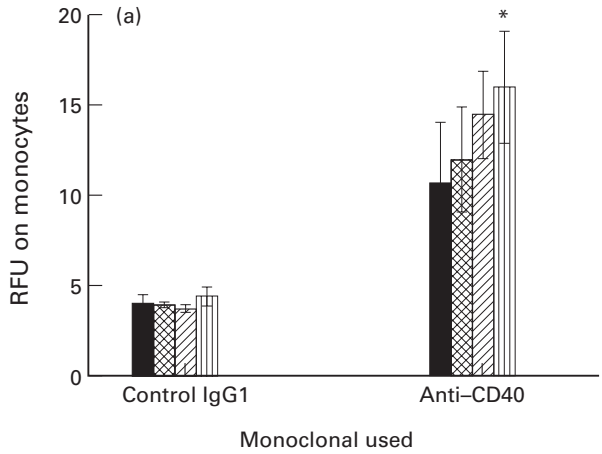



**Fig. 5.** Staphylococcus enterotoxin A (SEA)-induced CD40L on CD4 T cells from patients and controls. Peripheral blood mononuclear cells (PBMC) from 15 HIV$^-$ controls and 12 protease inhibitor-naive patients were incubated for 3 days with SEA and stained with anti-CD4–FITC and anti-CD40L–PE for 4 h at 37°C. Individual values of CD40L are represented as closed triangles.



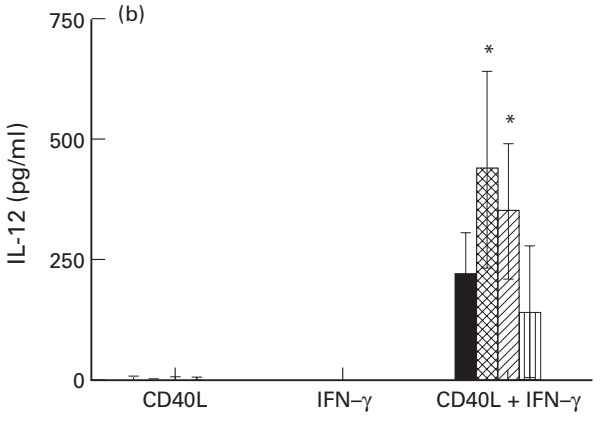




**Fig. 6.** Expression and function of CD40 on peripheral monocytes from HIV-infected subjects and controls. (a) Whole blood was stained with anti-CD4–PE, anti-CD3–PerCP and either an FITC-labelled isotypic control or anti-CD40–FITC. Monocytes were gated as CD4$^+$CD3$^-$ cells and the binding of the FITC MoAb was expressed in relative fluorescence units (RFU). Ten controls and 27 patients were studied: nine belonged to group 1, eight to group 2 and 10 to group 4. Symbols are as in Figs 3 and 4. (b) Two million PBMC from 20 controls and from 34 HIV$^+$ subjects (10 in group 1, 13 in group 2 and 11 in group 3) were incubated with CD40L-expressing 3T6 fibroblasts, IFN-γ or a combination of both stimuli for 2 days. Bioactive (p70) IL-12 was measured in the supernatant. The representation and the symbols are as in Fig. 3.

*Increased CD40 expression and altered CD40 function in monocytes from HIV-infected subjects*

Median CD40 expression was measured on monocytes in whole blood from control and $HIV^+$ subjects. CD40 expression was significantly higher in the HIV-infected subjects and increased according to disease stage (Fig. 6a).

The PBMC from these individuals were incubated with either IFN-$\gamma$, CD40L-expressing mouse fibroblasts (3T6) or the combination of both stimuli, and IL-12 p70 was assayed in day 2 supernatants. As shown in Fig. 6b, each stimulus alone was unable to induce IL-12, but both stimuli combined induced a median of 220 pg/ml IL-12 in the control cultures. Median IL-12 production in supernatants from patients with > 500 CD4 T cells/$\mu$l amounted to twice the control levels, and in supernatants from group 2 patients IL-12 was 50% higher than the control value ($P < 0{\cdot}05$). In AIDS patients, however, IL-12 production was marginally (but NS) decreased.

Although normal B cells express even higher levels of CD40 than monocytes, the former fail to secrete IL-12 [43]. CD40 stimulation of PBMC thus primarily evaluates the IL-12 production capacity of the monocytes. However, the percentage of monocytes within the PBMC could not explain the differences in cytokine output between patients and controls. In the patients, no correlation was found between the production of IL-12 and the percentage of monocytes or the level of monocytic CD40 expression. A possible influence of IL-12 production by dendritic cells was not excluded.

## DISCUSSION

Earlier studies by other authors already indicated that normal CD3/TCR-induced IL-12 production is regulated by CD40L–CD40 interactions [9–11], and that IL-12 and type-1 responses are decreased in PBMC from $HIV^+$ subjects with advanced disease [23,24,27–32]. The physiological regulation of IL-12 production during HIV infection was, however, not investigated, and that was the aim of the present study. The most significant finding was a decreased induction of CD40L on CD4 T cells from patients with advanced disease, which was evident both in cultures containing endogenous APC (SEA stimulation) and in cultures of APC-depleted lymphocytes (OKT3 experiments). Moreover, the AIDS-related CD40L deficiency could not be corrected with optimal costimulation through CD80. Other determinants of IL-12 production were apparently not deficient in PBMC from AIDS patients. The early (priming) IFN-$\gamma$ secretion was relatively unaltered and the expression of CD40 on monocytes was even increased. Moreover, after stimulation with fixed concentrations of IFN-$\gamma$ and cell-bound CD40L, PBMC from AIDS patients produced only slightly (non-significantly) lower amounts of bioactive (p70) IL-12 compared with controls.

Taken together, our present data suggest two main reasons for the deficiency in CD40L-dependent IL-12 and type-1 responses in advanced HIV infection: the reduced CD4 T count *per se* (with consequent lowered precursor frequencies of antigen-reactive T cells), and the intrinsic defect in CD40L up-regulation in the remaining T cells. Obviously, it is likely that HIV-related structural and functional damage to lymphoid organs (e.g. thymus and lymph nodes) as well as dysfunction of other molecular interactions (e.g. the response to IL-12 or to IFN-$\gamma$) also contribute to reduced cellular immunity. According to our own previous data, however, the CD28 costimulatory pathway is not involved in intrinsic CD4 T dysfunction, since CD28 expression was only marginally lowered on CD4 T cells from HIV-infected subjects, and triggering through either anti-CD28, CD80 or CD86 resulted in normal responses [44–46].

In less advanced stages, and especially in subjects with > 500 circulating CD4 T cells, the up-regulation of CD40L was preserved and the output of bioactive IL-12 after CD40-mediated triggering was even significantly increased. This hyper-responsiveness might well be related to the relatively preserved cell-mediated immunity and even increased cytokine responses, including IFN-$\gamma$, present in early disease [12,13,15,18].

The present study confirms that CD40L up-regulation is largely restricted to CD4 T cells. The 10 times lower CD40L levels on CD8 T cells probably do not significantly contribute to CD40-triggered IL-12 production. Nevertheless, CD8 T cells are sensitive to IL-12, and they significantly contribute to IFN-$\gamma$ production and more specifically to the increased IFN-$\gamma$ levels in the early stages of HIV infection [18,19].

Our data do not confirm the observations of Brugnoni *et al*. [36], suggesting, but not formally proving, preserved CD40L induction on CD4 T cells from HIV-infected subjects, after stimulation with PMA and ionomycin. Wolthers *et al*., however, using the same stimulus, clearly showed decreased CD40L induction in CD4 T cells and provided experimental evidence that lowered CD40L could impair T-dependent B cell activation [37], which is another important function of CD40L indeed, but which involves IL-4 rather than IFN-$\gamma$ [11]. Chougnet *et al*. demonstrated that IL-12 production, triggered by fixed *Staphylococcus* SAC, was reduced in PBMC from $HIV^+$ subjects, but this defect could largely be corrected with soluble CD40L and IFN-$\gamma$ [38]. Their data thus already suggested that the responsiveness to combined CD40L and IFN-$\gamma$ may be preserved in HIV-infected patients, which is more directly confirmed in our study. In all three studies more artificial stimuli were used, while our culture conditions were closer to antigenic stimulation. Another weakness in the former papers is that the patients were not systematically classified for disease stage, while we clearly showed that different types of dysfunction are present in various stages of the disease.

In conclusion, the present data suggest that enhanced cytokine production during the early stage and the AIDS-related decreased type-1 responses can partly be explained by a dysfunction of the CD40L–CD40 interaction. In the asymptomatic stage, the capacity of the CD4 T cells to up-regulate CD40L is preserved and IL-12 production by APC upon CD40 triggering is clearly increased. In AIDS patients, in contrast, both the low number of (antigen-specific) CD4 T cells and their dramatically impaired CD40L expression preclude efficient IL-12 induction. Therapeutic interventions therefore should be tailored according to the stage of the disease. It could be beneficial to increase antigen-induced IL-12 in AIDS patients in order to enhance type-1 responses, but it might also be desirable to reduce over-production of various cytokines, including IL-12, in the early stage, in order to counteract excessive immune activation, which is known to increase HIV replication [47].

## ACKNOWLEDGMENTS

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