

# HIV infection alters the production of both type 1 and 2 cytokines but does not induce a polarized type 1 or 2 state

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**Objective:** To test the T-helper (TH)1/TH2 cytokine paradigm in HIV infection.

**Design and methods:** Cytokine profiles in two separate studies of HIV patients and controls are presented: (i) a longitudinal study of HIV patients with CD4 counts  $> 500 \times 10^6/l$  tested at three timepoints compared with controls; (ii) a blinded cross-sectional study of controls and patients with high ( $> 500 \times 10^6/l$ ) and low ( $< 500 \times 10^6/l$ ) CD4 counts. Peripheral blood mononuclear cells (PBMC) from patients and controls were tested for the production of two type 1 [interleukin (IL)-2, interferon (IFN)- $\gamma$ ] and two type 2 (IL-4, IL-10) cytokines by enzyme-linked immunosorbent assay. Both spontaneous and mitogen-induced cytokine production was measured.

**Results:** HIV infection was noted to have the following effects on cytokine production: (i) it led to the *in vivo* activation of type 2 cytokines in a small group of individuals with high CD4 numbers characterized by the spontaneous release of IL-4 and IL-10. Longitudinal data showed high spontaneous IL-4 and IL-10 to be a consistent feature of the patient group (at each timepoint some patients were high producers) but to be variable in a given individual; (ii) HIV infection impaired the ability of PBMC to respond to stimuli (selected for their ability to optimally induce each cytokine) in terms of IL-2, IL-4 and IL-10 production in patients with both high and low CD4 cell counts; and (iii) conversely, HIV infection led to an overproduction of IFN- $\gamma$  in patients with high CD4 counts; patients with low CD4 produced normal levels of IFN- $\gamma$ .

**Conclusions:** Our observations did not suggest polarization of the type 1/type 2 cytokine profile in HIV patients. Instead, the data suggested more complex changes to type 1/type 2 cytokine patterns in HIV infection than originally proposed by the TH1/TH2 dichotomy.

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**Keywords:** T-helper 1/T-helper 2 cytokines, AIDS

## Introduction

Studies of type 1/type 2 cytokine production have been useful in establishing the nature of protective and pathogenic immune responses to pathogens [1,2]. In humans, type 1 cytokines [interferon (IFN)- $\gamma$ , inter-

leukin (IL)-2, and IL-12] induce cellular immunity [cytotoxic T lymphocytes delayed type hypersensitivity and immunoglobulin (Ig) G<sub>1</sub> antibody], whereas type 2 cytokines (IL-4, IL-6, IL-5 and IL-10) induce antibodies of the IgA, IgE and IgG<sub>4</sub> subclasses. Type 1/type 2 cytokines are reciprocally regulated, and immunity to

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many foreign antigens includes the preferential activation of one group or the other [3]. However, some pathogens have evolved mechanisms to induce a highly polarized type 1 or 2 state as a means of subverting the defence mechanisms of the host [1,2,4].

The earliest observations in HIV infection suggested that disease was associated with overproduction of type 2 cytokines and decreased production of type 1 cytokines [5,6]. However, these observations have been challenged. There is general agreement that one type 1 cytokine, IL-2, is decreased in HIV-infected individuals [7-10] and that the overall production of another type 1 cytokine, IFN- $\gamma$ , is enhanced in peripheral blood mononuclear cells (PBMC), although IFN- $\gamma$  production may be impaired at the CD4+ T-cell level [8,9,11]. There is also some agreement that the type 2 cytokine IL-10 is overproduced in some HIV-infected individuals [9,10]. However, there is controversy regarding the production of the key type 2 cytokine IL-4. Some studies have shown IL-4 to be enhanced [7,12,13], whereas others have shown this cytokine to be decreased [9,10,14]. Such diverse observations have resulted in two questions on type 1/type 2 cytokine production in HIV infection remaining unresolved. It is not clear whether (i) HIV infection results in a polarized type 1 or 2 state, and (ii) the production of type 1 or 2 cytokines governs the disease state of the individual.

In this study, we have suggested some answers to these questions. A pilot study to determine the optimal induction of key type 1/type 2 cytokines (IL-2, IFN- $\gamma$ , IL-4 and IL-10) in seronegative controls showed that PBMC of some individuals spontaneously produced IL-4 and IL-10; the optimal mitogenic stimulus (in terms of inducing the highest level of cytokine and the number of responders) for these cytokines was as follows: IL-4 and IL-10, concanavalin A (Con A); IFN- $\gamma$ , Con A or phytohaemagglutinin (PHA) plus phorbol myristate acetate (PMA); IL-2, anti-CD3 plus PMA or PHA plus PMA but not Con A. The optimal timepoint for measuring all four cytokines was 2 days (unpublished data).

On the basis of this study, we compared the levels of IL-2, IL-4, IL-10 and IFN- $\gamma$  in PBMC cultures of HIV-infected patients and seronegative controls. Two groups of patients were studied: those with high ( $> 500 \times 10^6/l$ ) and those with low ( $< 500 \times 10^6/l$ ) CD4 counts. Cytokine levels in some of the patients with high CD4 were monitored over three timepoints. We asked the following questions. (i) Do patients and controls differ in cytokine production, and is the difference linked to CD4 number? (ii) Is there a difference in spontaneous and stimulated cytokine production between patients and controls? (iii) What is the variation over time in cytokine production in a given patient? Our data suggested that unlike other infectious

diseases, HIV infection, despite causing the altered production of both type 1 and type 2 cytokines, did not lead to a polarized type 1 or 2 state that could be correlated with disease.

## Materials and methods

### Patients and controls

Two groups of HIV-seropositive patients were studied. Twenty health patients [Centers for Disease Control and Prevention (CDC) 1987 criteria stage II/III] with CD4 counts greater than  $500 \times 10^6/l$ . These patients were HIV p24 antigen-negative and had received no prior antiviral chemotherapy. Longitudinal studies were performed on PBMC from patients in this group because 16 patients were recruited as part of a trial of *Mycobacterium vaccae* immunotherapy. Heparinized blood samples were taken at three timepoints (0, 14 and 28 days) prior to immunotherapy. Seven patients with CD4 counts below  $500 \times 10^6/l$  were also studied. The control group consisted of 33 HIV-seronegative individuals. Eleven healthy HIV-seronegative individuals were recruited from an outpatient clinic, and 22 were individual blood donors. Blood from these individuals was collected in citrate phosphate dextrose buffer (blood packs from St George's Medical School, Tooting, London, UK) or collected in heparinized tubes.

### Cell cultures

Heparinized blood (50 ml) diluted 1 : 1 with Hank's balanced salt solution (HBSS; Gibco, Paisley, Scotland, UK) was layered on 50 ml Ficoll-Paque (Pharmacia Biotech, St Albans, Hertfordshire, UK) and centrifuged at 500 g, for 30 min at room temperature. The interface containing mononuclear cells was harvested and washed three times in RPMI-1640 medium (Gibco), plus 2% human serum (First Link UK, Brierly Hill, West Midlands, UK). PBMC ( $1 \times 10^6$ ) were cultured at 37°C in a final volume of 500  $\mu$ l in RPMI plus 10% human serum medium in 24-well tissue culture plates in the presence and absence of 20  $\mu$ g/ml concanavalin A (Con A; Sigma, Poole, Dorset, UK) or anti-CD3 antibody (final concentration 1 : 100 tissue culture supernatant; kind gift from Prof. P.C.L. Beverley, UCL Medical School, London, UK) plus 1 ng/ml PMA (Sigma) or 1  $\mu$ g/ml PHA (Sigma) plus 1 ng/ml PMA. Cell-free supernatants were harvested at 2 days post-stimulation and stored at  $-80^\circ\text{C}$  until analysed.

### Cytokine assays

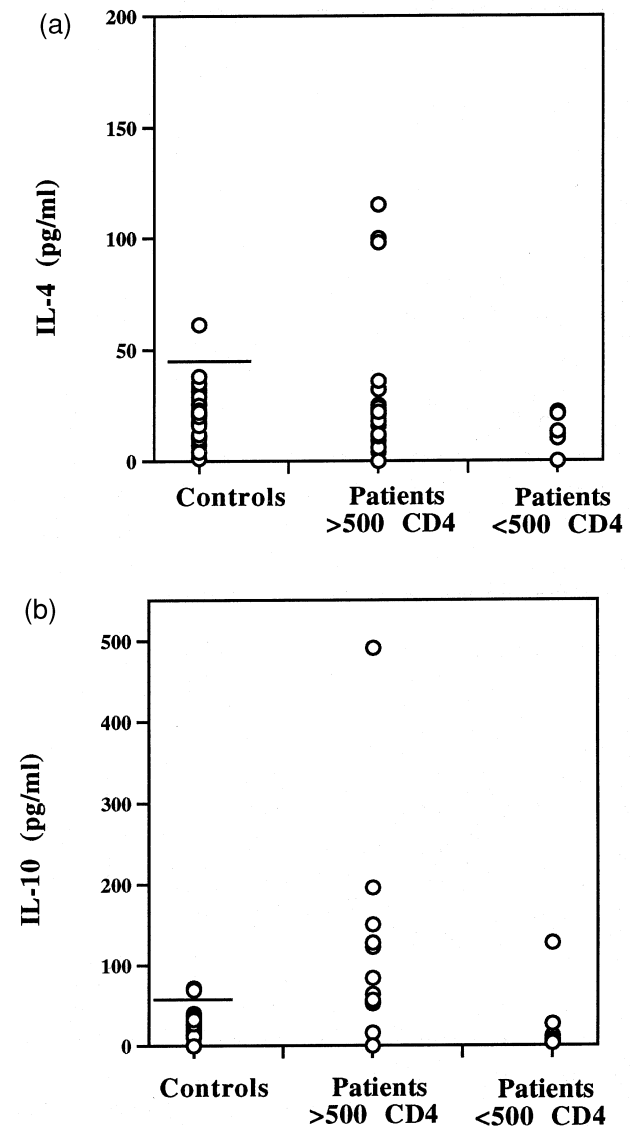
Four cytokines were measured using commercially available paired antibodies: IL-4 and IFN- $\gamma$  (AMS Biotechnology, Whitney, Oxfordshire, UK), IL-10 (Pharmingen, Cambridge Bioscience, Cambridge, UK),

and IL-2 (Medginex, Lifescreen Ltd, Watford, Hertfordshire, UK). A detailed description of the enzyme-linked immunoabsorbent assay (ELISA) conditions is described. Maxisorp plates (96-well; Nunc, Gibco, Paisley, Scotland) were coated at 4°C overnight with the following antibody concentrations: IL-2, 2.5 µg/ml in phosphate-buffered saline (PBS); IL-10, 2 µg/ml in 0.1 M NaHCO<sub>3</sub> (pH 8.2); IL-4, 1 mg/ml PBS; IFN-γ 0.5 mg/ml PBS. The plates were washed twice in PBS plus 0.05% Tween-20 (Sigma) and blocked for 2 h at room temperature with PBS plus 2% bovine serum albumin (BSA) with the exception of IL-10, which was blocked with PBS plus 10% fetal calf serum (FCS). Standards consisted of twofold serial dilutions (in PBS plus 2% BSA) of recombinant cytokines from the following sources: IL-4 (R&D Systems, Abingdon, Oxfordshire, UK), IL-10 (AMS Biotechnology), IFN-γ and IL-2 (UK AIDS Repository, National Institute for Biological Standards and Control, Potters Bar, Hertfordshire, UK; reference number ADP902 and ADP901, respectively). Plates were incubated with 100 µl test sample and standards at 4°C overnight, with the exception of IL-2, which was incubated for 2 h at room temperature on a plate shaker at 700 rpm. For the IL-2 assay, the detection antibody was added at the same time as the standard and test samples at a final concentration of 1 µg/ml in 2% PBS-BSA. For all other assays, plates were washed four times after the overnight incubation with samples/standards and 100 µl of detection antibody added as follows: IL-10, 1 µg/ml in PBS plus 10% FCS, IL-4, 1 µg/ml in 2% BSA-PBS, IFN-γ 0.5 µg/ml in 2% BSA-PBS. Plates were incubated for 1 h at room temperature, washed four times, and 100 µl of either streptavidin-alkaline phosphatase (AMS Biotechnology; used at 1 : 1000 in PBS plus 2% BSA) for IL-4 and IFN-γ or avidin-peroxidase (1 : 400, 1 µg/ml; Sigma) for IL-2 and IL-10. Plates were incubated at room temperature for between 30 min and 1 h followed by a further four washes and 100 µl substrate solution (Sigma substrate tablet sets) was added. The substrates used and the timings at which the plates were read on an UV<sub>max</sub> ELISA reader (Molecular Devices, Coulter Electronics, Luton, Bedfordshire, UK) are as follows: IL-2, tetramethylbenzidine dihydrochloride stopped at 30 min and read at 450 nm (reference wavelength, 650 nm); IL-10, o-phenylenediamine dihydrochloride stopped at 30 min and read at 490 nm; IL-4 and IFN-γ, p-nitrophenyl phosphate read at 405 nm after 1 h for IL-4 and 30 min for IFN-γ. The IL-2 and IL-10 assays were stopped by the addition of 50 µl 2M H<sub>2</sub>SO<sub>4</sub>.

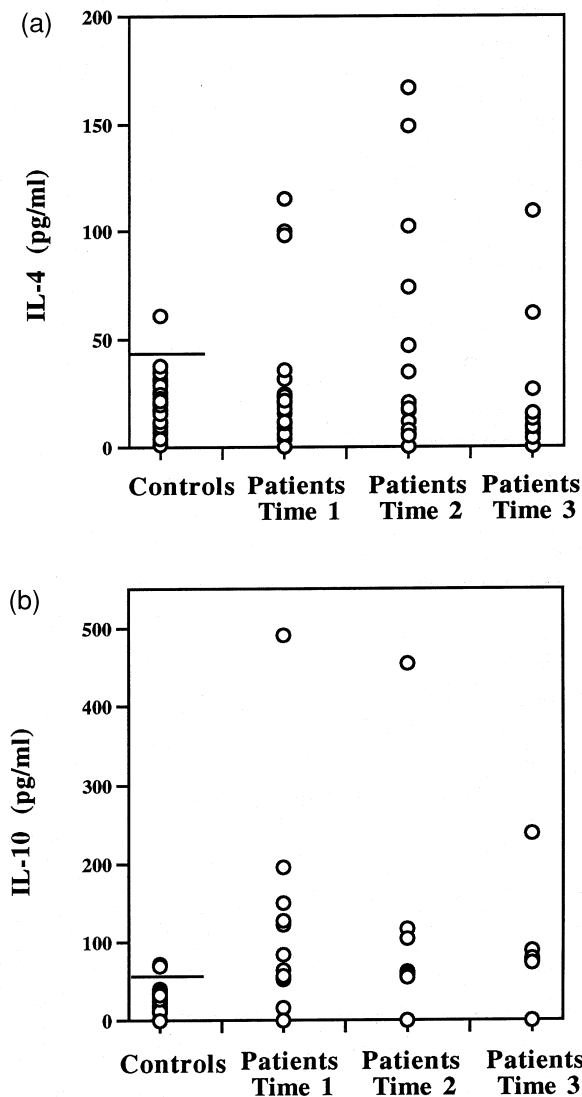
### Statistical analysis

A non-parametric (Mann-Whitney U test) was used to analyse differences between cytokine levels in the HIV-infected groups and seronegative controls. Fisher's exact test was used to analyse differences in the number

of high cytokine producers (calculated as those who produced greater than the mean + 2 SD of the control) in the patient versus control groups as well as determining the number of responders and non-responders to each mitogen. Responders were defined as those individuals whose PBMC produced more than twice the (same individuals) spontaneous cytokine level.



**Fig. 1.** Spontaneous interleukin (IL)-4 and IL-10 production by patients and controls. (a) IL-4 levels in culture supernatants harvested at 2 days from peripheral blood mononuclear cells (PBMC) of controls ( $n = 33$ ) and patients with CD4 counts greater ( $n = 20$ ) or less than ( $n = 7$ )  $500 \times 10^6/l$ . (b) IL-10 levels in culture supernatants harvested at 2 days from PBMC of controls ( $n = 32$ ) and patients with CD4 greater ( $n = 16$ ) or less than ( $n = 6$ )  $500 \times 10^6/l$ . Cells were cultured without stimulation.



**Fig. 2.** Spontaneous interleukin (IL)-4 and IL-10 production over time by patients with high CD4 count. (a) Spontaneous production of IL-4 by patients with CD4  $>500 \times 10^6/l$  tested on three separate occasions (time 1, day 0; time 2, day 14; time 3, day 28) compared with controls. Spontaneous production of IL-10 by patients with CD4  $>500 \times 10^6/l$  tested on three separate occasions (time 1, day 0; time 2, day 14; time 3, day 28) compared with controls. The line across the control group in each case represents the mean + 2 SD of IL-4 or IL-10 produced by the control group. Cytokine production over this level indicates high producers.

## Results

### Type 2 cytokines: spontaneous IL-4 and IL-10 is enhanced in a subgroup of HIV patients

One indication of HIV infection directly inducing cytokine synthesis is the spontaneous secretion of cytokines (in the absence of any *in vitro* activation signals) from patients compared with controls. Figure 1 indicates the spontaneous levels of IL-4 and IL-10 in

cultures of controls and patients with high ( $> 500 \times 10^6/l$ ) and low ( $< 500 \times 10^6/l$ ) CD4 counts. High producers were identified as those who produced more than the mean + 2 SD of the control group as a whole. For IL-4, the number of high producers ( $> 44$  pg/ml) were one out of 33 controls, three out of 20 patients with CD4 counts  $> 500 \times 10^6/l$ , and none out of seven patients with CD4  $< 500 \times 10^6/l$ . For IL-10, the number of high producers ( $> 61$  pg/ml) were three out of 32 controls, seven out of 16 patients with CD4 count  $> 500 \times 10^6/l$ , and one out of six patients with CD4 counts  $< 500 \times 10^6/l$ .

To establish whether high constitutive IL-4 and IL-10 synthesis was a feature of patients as opposed to controls, a group of patients with high CD4 counts were followed for IL-4 and IL-10 production on a further two occasions (Fig. 2). Although the same control individual was not studied on three separate occasions, the levels represent data from cultures set up at four separate timepoints consisting of cells from a group of six, eight, nine and 11 healthy individuals. Analysis of the control data showed no significant difference in any of the cytokines between the timepoints (data not shown), and therefore the data from the four timepoints was combined. Fig. 2 and Table 1 show high spontaneous IL-4 and IL-10 production to be a consistent feature of the patient group; at each timepoint some HIV-infected patients were identified as high producers. Statistical analysis based on the number of high producers confirmed differences between patients and controls to be significant ( $P < 0.05$ ) on one of the three timepoints for IL-4, whereas differences in IL-10 were significant at all three timepoints (Table 1). It was noted, however, that cytokine production in a given individual varied over time. Thus, of the six high IL-4 producers, four produced high levels on two of three timepoints, while two individuals produced high IL-4 only on one of the three timepoints. None of the six patients produced high IL-4 on all three timepoints. Of the 10 high IL-10 producers, seven did so on two of the three timepoints, whereas the rest produced high levels of IL-10 on only one of the three timepoints.

Having identified a small number of individuals who overproduced IL-4 and IL-10 spontaneously, it was important to establish whether these individuals were predisposed to a faster rate of CD4 decline. Serial CD4 counts performed over 18 months showed no apparent differences in rates of decline in patients with high compared with those with low spontaneous IL-4 production. The mean slopes in the two groups were  $-0.12$  and  $0.03$ , respectively (data not shown).

### Type 2 cytokines: mitogen-induced IL-4 and IL-10 production is impaired in HIV patients

Cytokine levels in Con A-stimulated cultures were measured to establish whether the enhanced sponta-

**Table 1.** Cytokine overproduction by HIV-infected patients.

Group	Cytokine levels [median (range)]	<i>P</i> (Mann–Whitney)	High producers (n/total)	<i>P</i> (Fisher's exact test)
Spontaneous IL-4 (pg/ml)				
Control	18.00 (1–61.00)		1/33	
Patients > 500 × 10 <sup>6</sup> /l CD4				
Time 1	19.5 (0–115.0)	0.6011	3/20	0.1
Time 2	17.5 (0–167.0)	0.8902	5/16	0.01
Time 3	8.5 (0–109.0)	0.0297	2/16	0.2
Patients < 500 × 10 <sup>6</sup> /l CD4	12.0 (0–22.0)	0.2127	0/7	1.0
Spontaneous IL-10 (pg/ml)				
Control	16.0 (0–72.0)		3/32	
Patients > 500 × 10 <sup>6</sup> /l CD4				
Time 1	57.0 (0–491.0)	0.0223	7/16	0.01
Time 2	57.0 (0–455.0)	0.2969	5/12	0.02
Time 3	36.5 (0–239.0)	0.5636	5/11	0.02
Patients < 500 × 10 <sup>6</sup> /l CD4	12.0 (3–127.0)	0.8072	1/6	0.5
Con A-induced IFN-γ (IU/ml)				
Control	98.0 (0–646.0)		1/34	
Patients > 500 × 10 <sup>6</sup> /l CD4				
Time 1	644.0 (2–2926.0)	0.0019	10/19	0.0004
Time 2	656.5 (1–2894.0)	0.0210	10/16	0.00007
Time 3	95.0 (4–2558.0)	0.4860	7/16	0.0007
Patients < 500 × 10 <sup>6</sup> /l CD4	161.0 (0–267.0)	0.1363	0/7	1.0

Data expressed as the median (range) levels of spontaneous interleukin (IL)-4, spontaneous IL-10 and concanavalin A (Con A)-induced interferon (IFN)-γ in the control and patient groups. Patients with high CD4 count were studied at three timepoints. In addition, the number of individuals in the control and patient groups who produced high levels of IL-4 (> 44 pg/ml), IL-10 (> 61 pg/ml) and IFN-γ (>367 IU/ml) is shown. High producers were identified as those who produced greater than the mean + 2 SD of the control level of a given cytokine. Tests for significance between the patient and control groups were calculated using Mann–Whitney U test or by Fisher's exact test.

neous production of IL-4 and IL-10 by HIV-infected patients was associated with cytokine overproduction in response to stimuli. In contrast to spontaneous cytokine production, Fig. 3 and Table 2 show that stimulation of patient cells (irrespective of CD4 number) led to the reduced production of IL-4 (Fig. 3a) and IL-10 (Fig. 3b). Not only was the overall level of both cytokines lower in cultures of patient compared with control cells, but fewer patients responded to Con A (response was measured as twofold increase over the same individuals spontaneous production). In addition, the reduced IL-4 and IL-10 response was consistent over time (Table 2).

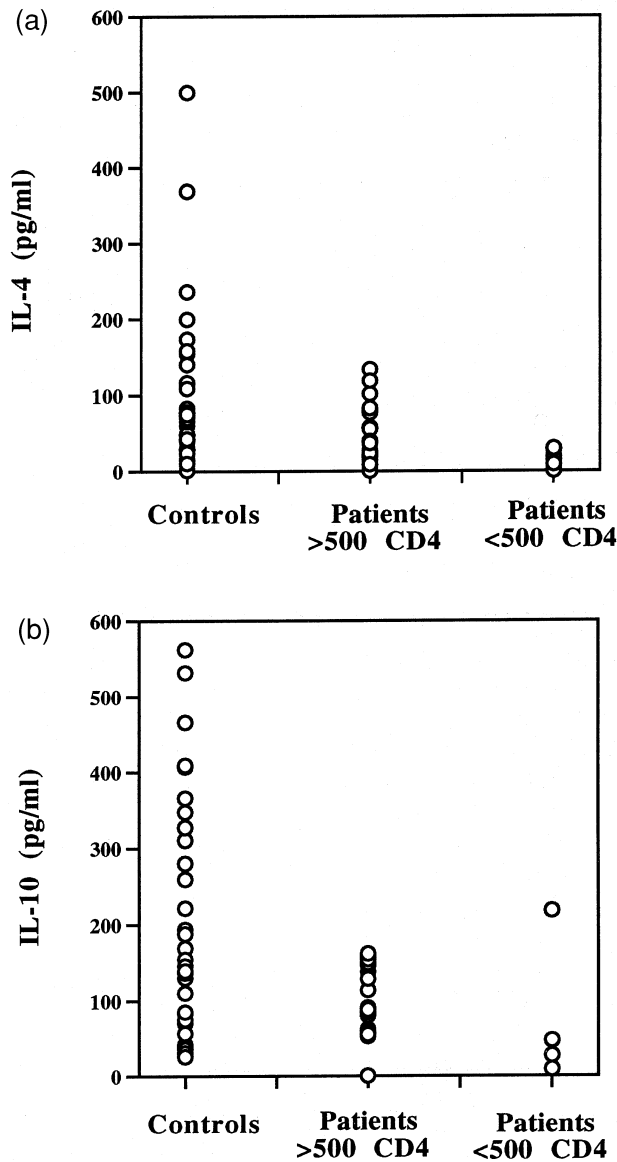
### Type 1 cytokines: IL-2 production is impaired but IFN-γ is over-produced in patients with normal CD4 counts

The production of IL-2 and IFN-γ (type 1 cytokines), was measured in parallel to the two type 2 cytokines. Unlike type 2 cytokines, HIV-infected patients did not spontaneously produce IL-2 or IFN-γ (data not shown). The ability to produce these cytokines upon activation was studied by measuring IFN-γ production in Con A-stimulated cultures and IL-2 in anti-CD3 plus PMA-stimulated cultures.

IFN-γ production was associated with patients' CD4 cell counts. Patients with high CD4 counts produced six- to eightfold higher levels of IFN-γ than controls.

By comparison, patients with low levels of CD4 cells produced similar levels to controls (Table 1). Thus, patients with high CD4 cell counts showed signs of immune activation by spontaneously secreting high levels of IL-4 and IL-10 and overproducing IFN-γ in response to stimulation (Table 1). The increased production of IFN-γ by patients was consistent over time. A group of patients with high CD4 counts were followed for IFN-γ over three timepoints. Differences between the control and patient groups calculated on the basis of the frequency of high producers (identified as those who produced greater than the mean + 2 SD of the control, >347 IU/ml) was significant ( $P < 0.05$ ) at all three timepoints (Table 1) despite differences in the level of overall IFN-γ being significant on only two of the three timepoints (Table 1).

Several studies [7–10] have recorded reduced IL-2 production in patients with low CD4 counts, so we therefore focused on IL-2 production by patients with high CD4 counts. IL-2 production was significantly lower in patients compared to controls (Table 2). The mean overall level secreted by patients' cells was 2.5- to fourfold lower than control cells. Longitudinal data of IL-2 production over three timepoints showed these observations to be consistent over time. Not only was the overall level of IL-2 lower in patients than controls, but fewer patients responded to anti-CD3 plus PMA stimulation compared with the control group (Table 2).



**Fig. 3.** Concanavalin A (Con A)-induced interleukin (IL)-4 and IL-10 in patients and controls. (a) IL-4 levels in culture supernatants harvested at 2 days from peripheral blood mononuclear cells (PBMC) of controls ( $n = 33$ ) and patients with CD4 greater ( $n = 20$ ) or less than ( $n = 7$ )  $500 \times 10^6/l$ . (b) IL-10 levels in culture supernatants harvested at 2 days from PBMC of controls ( $n = 32$ ) and patients with CD4 greater ( $n = 16$ ) or less than ( $n = 6$ )  $500 \times 10^6/l$ . Cells were cultured with 20  $\mu g/ml$  Con A.

## Discussion

A polarized type 1/type 2 cytokine state is characterized by two features: First, by the overproduction of type 1 or 2 cytokines (usually more than one of each category), which can be measured by an increase in the expression of cytokine-specific mRNA or protein, or

both, before and after *in vitro* stimulation of cell mixtures (e.g., PBMC). Second, as the classification of the type 1/type 2 cytokine system was originally based on CD4+ T cells, polarization would lead to a shift from a predominantly large number of type 0 CD4+ T-cell clones to an increase in the number of either type 1 or 2 CD4+ T cells in the infected individual, and to an associated increase in the production of those cytokines by freshly isolated CD4+ T cells. Three studies have shown that HIV infection induces a shift to a type 2 or an activated type 0 state at the CD4+ T-cell level and to the overproduction of the type 2 cytokine IL-4 [7,12,13], whereas four studies have suggested reduced production of IL-4 [9,10,14,15]. Only one study has found no change in IL-4 production in HIV [16]. Our data paradoxically supported both opposing views. On the one hand, the spontaneous increase in IL-4 and IL-10 production supported increased type 2 cytokine production in HIV, and on the other, the decreased production of both these cytokines in response to stimulation supported the opposite view. Part of the answer to this paradox lies in how cells are cultured. We provide two novel observations regarding the production of type 2 cytokines. First, these cytokines can be spontaneously secreted (in controls and patients), with the frequency of high producers being higher in the patient group. Second, we have noted that both these cytokines are optimally induced by Con A as opposed to anti-CD3 plus PMA or PHA plus PMA (unpublished observation). Optimal stimulation of cells would be critical in comparing cytokine production between patients and controls. It is therefore possible that the failure to do so might have contributed to some of the contradictory observations in the literature.

The paradox of why cytokines activated *in vivo* (e.g., IL-4 and IL-10) are not overproduced when the cells are stimulated might be explained by the high levels of activation-induced cell death (AICD) observed in HIV-infected cultures [17]. HIV proteins such as Tat and gp120 enhance AICD of CD4+ T cells through the CD95 pathway [18]; this would explain why stimulation of cells from HIV-infected patients leads to the reduced production of type 1 (IL-2) and type 2 cytokines (IL-4, IL-10). The cytokines that are not dramatically lost in HIV infection, such as IFN- $\gamma$ , are most likely to be produced by cells other than CD4 [e.g., CD8+ T cells or natural killer (NK) cells]; indeed, CD8+ T cells expressing high levels of IFN- $\gamma$  mRNA have been found in the lymph nodes of HIV-infected individuals [19].

On the basis of the arguments presented above, we concluded that the cytokine profile of HIV-infected individuals does not fit a hypothesis based on a straight T-helper (TH)1 1/TH2 dichotomy, and that HIV infection is unlikely to lead to a clear polarization of the type 1/type 2 cytokine response. There are several

**Table 2.** Reduced cytokine production by HIV-infected patients.

Group	Cytokine levels [median (range)]	<i>P</i> (Mann–Whitney)	Responders (n/total)	<i>P</i> (Fisher’s exact test)
Con A-induced IL-4 (pg/ml)				
Control	70.0 (1–500.0)		29/33	
Patients > 500 × 10 <sup>6</sup> /l CD4				
Time 1	30.25 (0–134.0)	0.03	5/20	0.00001
Time 2	34.5 (0–223.0)	0.046	5/16	0.0001
Time 3	13.5 (2–73.0)	0.0001	6/16	0.005
Patients < 500 × 10 <sup>6</sup> /l CD4	19.0 (0–30.0)	0.0017	2/7	0.003
Con A-induced IL-10 (pg/ml)				
Control	155.0 (25–562.0)		29/32	
Patients > 500 × 10 <sup>6</sup> /l CD4				
Time 1	89.5 (0–162.0)	0.0258	4/16	0.00001
Time 2	63.5 (0–210.0)	0.0018	3/12	0.00005
Time 3	66.5 (0–239.0)	0.0114	2/11	0.0001
Patients < 500 × 10 <sup>6</sup> /l CD4	36.5 (9–218.0)	0.0113	3/6	0.04
Anti-CD3+PMA-induced IL-2 (IU/ml)				
Control	50.05 (20.2–86.6)		10/10	
Patients > 500 × 10 <sup>6</sup> /l CD4				
Time 1	1.05 (0–99.9)	0.002	8/16	0.01
Time 2	3.65 (0.3–102.8)	0.0038	10/16	0.05
Time 3	0.85 (0–40.1)	0.0002	5/13	0.02

Data expressed as the median (range) levels of concanavalin A (Con A)-induced interleukin (IL)-4, Con A-induced IL-10 and anti-CD3 plus phorbolmyristate acetate IL-2 in the control and patient groups. Some patients with high CD4 counts were studied at three timepoints. In addition, the number of individuals in the control and patient groups who responded to stimulation by producing a minimum of twofold increase over spontaneous cytokine release is shown. Tests for significance between the patient and control groups were calculated using Mann–Whitney U test or by Fisher’s exact test.

reasons for this. In addition to increased AICD (see above), recent data enumerating cytokine production at the single cell level by flow cytometry suggests that unlike the mouse, human memory cells can have complex cytokine patterns [20] and include cells that present a broad spectrum of cytokines as opposed to cells that fall into two clear subsets [20,21]. In particular, human TH1/TH2 cells cannot be classified on the basis of IL-10 production because they both can produce this cytokine [22]. Finally, cytokine profiles are likely to be governed by the differential replication of HIV in CD4+ T-cell subsets [13,23]. We have shown that CD4+ T-cell clones that fall in the type 1 category, unlike TH2 clones, fail to support HIV replication efficiently [23], are intrinsically more prone to AICD [24] (consistent with murine observations) [25], and are more prone to apoptosis when exposed to HIV [24]. Type 1 CD4+ T cells would therefore be most susceptible to apoptosis in HIV-infected individuals and a loss of this population would be consistent with a fall in IL-2 production. A fall in type 1 CD4+ T cells would also be consistent with an increase in type 2 cytokines [7,13]. However, we suggest that a loss of type 1 CD4+ T cells in HIV infection is unlikely to lead to the overproduction of type 2 cytokines. This could be due partly to the increased production of IFN- $\gamma$ , which can downregulate IL-4 and IL-10, and partly to the reduced production of type 2 cytokines in response to stimulation.

A recent study by Meyaard *et al.* [26] lends support to the above discussion. Consistent with our findings, Meyaard *et al.* [26] noted that IL-2, IL-4 and IL-10 production (in response to stimulation) was impaired by PBMC of HIV-infected patients. By single cell analysis after intracytoplasmic cytokine staining. They found no differences between patients and controls in the number of T cells that produced IL-4. However, unlike our data, they observed reduced IFN- $\gamma$  in patient cultures and fewer CD4 cells that produced this cytokine. Two possibilities might account for the disparity between our IFN- $\gamma$  data and those of Meyaard *et al.* [26]. First, the stimulus used in the two studies was different. Secondly, they did not measure the total number of IFN- $\gamma$ -producing cells. It is possible that although the frequency of IFN- $\gamma$  producing CD4 cells was decreased, the same might not have occurred in other IFN- $\gamma$  producing cell types (e.g., CD8+ T cells and NK cells). Taken together, their data indicated that reduced cytokine production in HIV is not necessarily due to a reduced frequency of CD4+ T cells that produce those cytokines (e.g., IL-4 production). In turn, this supports our suggestion that a polarization of the type 1/type 2 cytokine response does not occur in HIV infection.

Finally, in view of the accepted importance of type 1/type 2 cytokines in inducing different arms of the immune response, it is worth considering whether this factor should be taken into account in developing

strategies for prophylactic immunization against HIV [27]. Our data and those reviewed above [28,29] indicate that further studies are needed to answer this question. In particular, further data on the frequency and phenotype of cells that produce key type 1 and 2 cytokines in HIV infection is needed. Correlating, at the single cell level, cytokine production [21,26] with virus production (p24 staining) and apoptosis is likely to resolve some of the paradoxes in cytokine production in HIV infection. Such a study might also provide important information on those cytokines that are associated with disease and those associated with a disease-free state.

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