

# Demonstration of the Th1 to Th2 cytokine shift during the course of HIV-1 infection using cytoplasmic cytokine detection on single cell level by flow cytometry

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**Objective:** To characterize changes of Th1/Th2 cytokine production by peripheral blood mononuclear cells (PBMC) that occur during the course of HIV infection by cytoplasmic cytokine staining on single cell level.

**Design and methods:** Mitogen-stimulated PBMC from 16 healthy donors, 18 HIV-1-infected individuals without AIDS and 14 patients with AIDS were stained intracellularly with fluorescein-labelled MAb against interleukin (IL)-2, IL-4, IL-10 and interferon (IFN)- $\gamma$ . Additionally, co-staining of CD4+ T-cell, CD8+ T-cell, natural killer (NK) cell, B-cell and monocytic markers was performed. Fluorescence staining was analysed by three-colour flow-cytometry.

**Results:** A reduced percentage of IL-2 and IFN- $\gamma$  (Th1 type)-producing cells among CD4+ T cells from HIV-1-infected individuals could be demonstrated. There was a continuous decrease of IFN- $\gamma$ -producing CD4+ T cells in the course of HIV infection and a dramatic reduction of IL-2-expressing cells among CD4+ T cells in patients with AIDS. In contrast to Th1 cytokines, the frequency of Th2 cytokine expressing cells among CD4+ T cells increased in HIV-infected individuals. The maximum frequency of IL-4-expressing cells among CD4+ T cells was seen in HIV-infected individuals without AIDS, whereas the rate of IL-10-producing cells was highest in patients with AIDS. In HIV-infected individuals no significant proportion of Th0 cells expressing both Th1 and Th2 cytokines was detectable. In CD8+ T cells the percentage of IL-2 expressing cells decreased continuously accompanied by a strong increase of the frequency of IFN- $\gamma$ -producing cells.

**Conclusion:** The decreased percentage of cells expressing IL-2 and IFN- $\gamma$  in conjunction with an increased proportion of IL-4- and IL-10-producing cells among the CD4+ T cells in HIV-1-infected individuals demonstrate a Th1 to Th2 cytokine shift in the course of HIV infection on a single cell level. There was no evidence of a Th1 to Th0 cytokine shift. In addition to the loss of CD4+ T cells in HIV infection, the qualitative changes of Th1/Th2 cytokine expression may serve as a marker for progressive failure of cell-mediated immunity.

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## Introduction

Apart from the loss of CD4+ T cells, defects in T-cell immune function such as a reduced expression of Th1 cytokines can be detected in HIV-infected individuals [1–5]. Moreover, the regular occurrence of a polyclonal B-cell activation and hypergammaglobulinaemia suggests that the Th2-type cytokine expression may be increased. However, changes in the pattern of Th1- and Th2-type cytokine expression in the course of HIV infection are discussed controversially. Whereas Clerici and others [6–12] observed a Th1 to Th2 cytokine shift, several authors have proposed either a Th1 to Th0 shift [13–17] or contradict a change of Th1/Th2 cytokine pattern [18].

A dichotomy between Th1 and Th2 has been identified in murine CD4+ T cells [19]. Analysis of T-cell clones in humans have shown an analogous, although not identical, cytokine synthesis heterogeneity [20–23]. Th1 and Th2 CD4+ T cells are characterized exclusively by differences in cytokine expression: Th1 cells produce interleukin (IL)-2, IL-12 and interferon (IFN)- $\gamma$ , whereas Th2 cells express IL-4, IL-5, IL-6, IL-10 and IL-13 [20]. Moreover, it has been shown that the Th1/Th2 cytokine heterogeneity is not only restricted to CD4+ T cells [20]. Cell types such as CD8+ T cells or natural killer (NK) cells contribute to the Th1/Th2 cytokine heterogeneity as well. Thus, the terms Th1-type or Th2-type cytokine or cell are used to characterize the cytokine profile of the different cell types.

Due to the different methods used for assessing cytokine expression [e.g., enzyme-linked immunosorbent assay (ELISA), reverse transcriptase (RT)-polymerase chain reaction (PCR) or T-cell-cloning and differences between the examined T-cell populations obtained from peripheral blood or lymph nodes, the results of Th1/Th2-cytokine expression in HIV-infected individuals are contradictory. None of the conventional methods for measuring cytokine expression used in earlier reports facilitates characterization of cytokine production of T cells directly. To circumvent this problem, we employed the method of cytoplasmic cytokine staining to determine whether the suggested Th1 to Th2 cytokine-shift indeed occurs in course of HIV infection. Using this method we were able to characterize and distinguish cells which are solely defined by their cytokine production such as Th1- and Th2-type T cells. In addition, it is possible to detect cytokine production of cells on single cell level without previous cell sorting.

## Material and methods

### Study population

Peripheral blood was obtained with informed consent

from 16 healthy adult donors, from 18 HIV-1-infected donors classified Centers for Disease Control and Prevention (CDC) 1993 criteria stage A1, A2, B1, and B2 and from 14 HIV-1-infected individuals in CDC stage C3. Patients suffering from malignancies (except Kaposi's sarcoma) and individuals receiving chemotherapy were excluded.

### Cell preparation and culture

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Histopaque (Sigma, Deisenhofen, Germany) density gradient centrifugation. Cells were adjusted to a concentration of  $1 \times 10^6$  cells/ml in RPMI 1640 medium (Gibco, Karlsruhe, Germany) containing heat inactivated fetal calf serum (Gibco), 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin and 1 mM glutamine (Biochrom, Berlin, Germany).

PBMC were cultured using a modification of the conditions reported by Jung *et al.* [24]: cells were stimulated with 5 ng/ml PMA (phorbol 12-myristate 13-acetate) in combination with 0.75  $\mu$ g/ml ionomycin (Sigma) in round bottom tissue culture tubes (Greiner, Nürtingen, Germany). Monensine (Sigma) was added in a concentration of 3  $\mu$ M to prevent cytokine release. Cultures without the addition of monensine and without exogenous stimuli were included as controls. The optimal duration of incubation for induction of each cytokine had been determined previously in time kinetic experiments. The detection of IFN- $\gamma$ , IL-2 and IL-4 was optimal after a culture time of 5 h in contrast to 10 h for IL-10.

### Fixation, permeabilization and cytokine-staining

After incubation the cells were stained according to methods described elsewhere, with slight modifications [24–26]. The cells were washed twice in Hank's balanced salt solution (HBSS) and fixed with 4% paraformaldehyde in HBSS at 4°C for 10 minutes. After two further washing steps in HBSS, the cells were resuspended at a concentration of  $2 \times 10^5$ /tube in permeabilization buffer (HBSS containing 0.1% saponin, 0.01 M HEPES buffer and 5% type AB serum) and incubated for 5 min. The cells were then pelleted and resuspended in the remaining buffer after decantation. Anti-cytokine-specific monoclonal antibodies (MAb) were added in concentrations ranging from 0.1 to 1  $\mu$ g/ml for 20 min at 20°C followed by two further washing steps in permeabilization buffer.

The following cytokine-specific MAb were used: unlabelled anti-IFN- $\gamma$  mouse IgG2a (Genzyme, Boston, Massachusetts, USA), anti-IFN- $\gamma$ -fluorescein isothiocyanate (FITC) (Hölzel Diagnostika, Cologne, Germany), anti IL-2-biotin mouse IgG2a, clone N7.48A (Hölzel), anti IL-4-biotin mouse IgG1, clone 8F12 (Hölzel) and anti-IL10-phycoerythrin (PE) rat IgG1 clone JES3-9D7 (PharMingen, San Diego, California,

USA). Isotype controls were unlabelled or labelled with FITC, PE, or biotin (Dianova, Hamburg, Germany).

To ensure specificity of MAb staining, the binding of anti-cytokine-specific antibodies was blocked with a molar excess of the corresponding recombinant cytokine.

Cells stained with unlabelled or biotinylated MAb were pelleted again and incubated with a second FITC or PE-labelled antibody (Dianova) or a streptavidin-PE-conjugate (Serotec, Wiesbaden, Germany) for 20 min followed by two further washing steps in permeabilization buffer. After indirect antibody-staining the cells were washed twice with permeabilization buffer.

As a last step, surface phenotyping was performed with FITC, PE or peridone chlorophyll a protein (PerCP)-labelled MAb against CD3, CD4, CD8, CD56, CD20 and CD14 (Becton Dickinson, Heidelberg, Germany).

### Flow cytometry

Fluorescence staining was analysed by three-colour flow cytometry on a FACScan flow cytometer using Lysys II software (Becton Dickinson). Routinely,  $1 \times 10^4$  PBMC were analysed. For evaluation of cytokine expression in less frequent cell types such as CD4+ T cells in HIV-infected individuals, at least 2000 cells were counted. Cell populations < 1% (< 20 events) were not regarded as significant. For analysis of double cytokine staining the acquisition of CD4+ or CD8+ T cells was performed using a life gate. The cut-off point, at which a cytokine-specific signal was considered to be positive was determined using unstimulated cells and cells stained with isotype-specific antibodies. The average amount of cytokine production per cell was evaluated by measuring the cytokine-specific intensity of fluorescence. Due to the high day-to-day and even inter-experimental variability of fluorescence intensity the absolute values of fluorescence intensity cannot be used for quantification of cytokine production. To facilitate a comparison of the cytokine expression in various cell types from different donors, a ratio of the mean intensity of fluorescence staining per cell expressing a certain cytokine and of the fluorescence intensity at the cut-off point determined for

this cytokine was calculated. Statistical analysis was performed using the Wilcoxon-Mann-Whitney test.

## Results

PBMC from 16 healthy donors, 18 HIV-1-infected individuals without AIDS and 14 patients with AIDS were stimulated *in vitro* for cytokine production. After a 5 h or 10 h incubation permeabilized cells were stained intracellularly with cytokine-specific MAb against IL-2, IL-4, IL-10 and IFN- $\gamma$  in combination with MAb-staining of surface markers for CD4+ T cells, CD8+ T cells, NK cells, B cells and monocytes in order to determine the frequency of Th1- or Th2-type cytokine expression in these cell types.

### Th1-type cytokines

Analysis of the Th1-type cytokines IL-2 and IFN- $\gamma$  in CD4+ T cells revealed that the percentage of cells expressing these cytokines decreased with disease progression. The percentage of CD4+ T cells producing IFN- $\gamma$  decreased continuously from  $19.6 \pm 3.0$  in healthy donors to  $14.0 \pm 1.8$  in HIV-1-infected individuals classified CDC stages A1, B1, A2 and B2 and to  $10.6 \pm 1.9$  in patients with AIDS (CDC C3). A dramatic reduction in the proportion of IL-2-expressing cells starting from  $35.2 \pm 5.5\%$  among CD4+ T cells from HIV-1-infected individuals classified CDC A1, B1, A2 and B2 to  $9.6 \pm 1.5\%$  in patients with AIDS was detectable (Table 1, Fig. 1). In contrast to the CD4+ T cells, the percentage of IFN- $\gamma$ -expressing cells was higher among CD8+ T cells obtained from patients classified CDC C3 ( $51.4 \pm 7.6$ ) as compared to healthy donors ( $23.2 \pm 4.2$ , Table 1, Fig. 1).

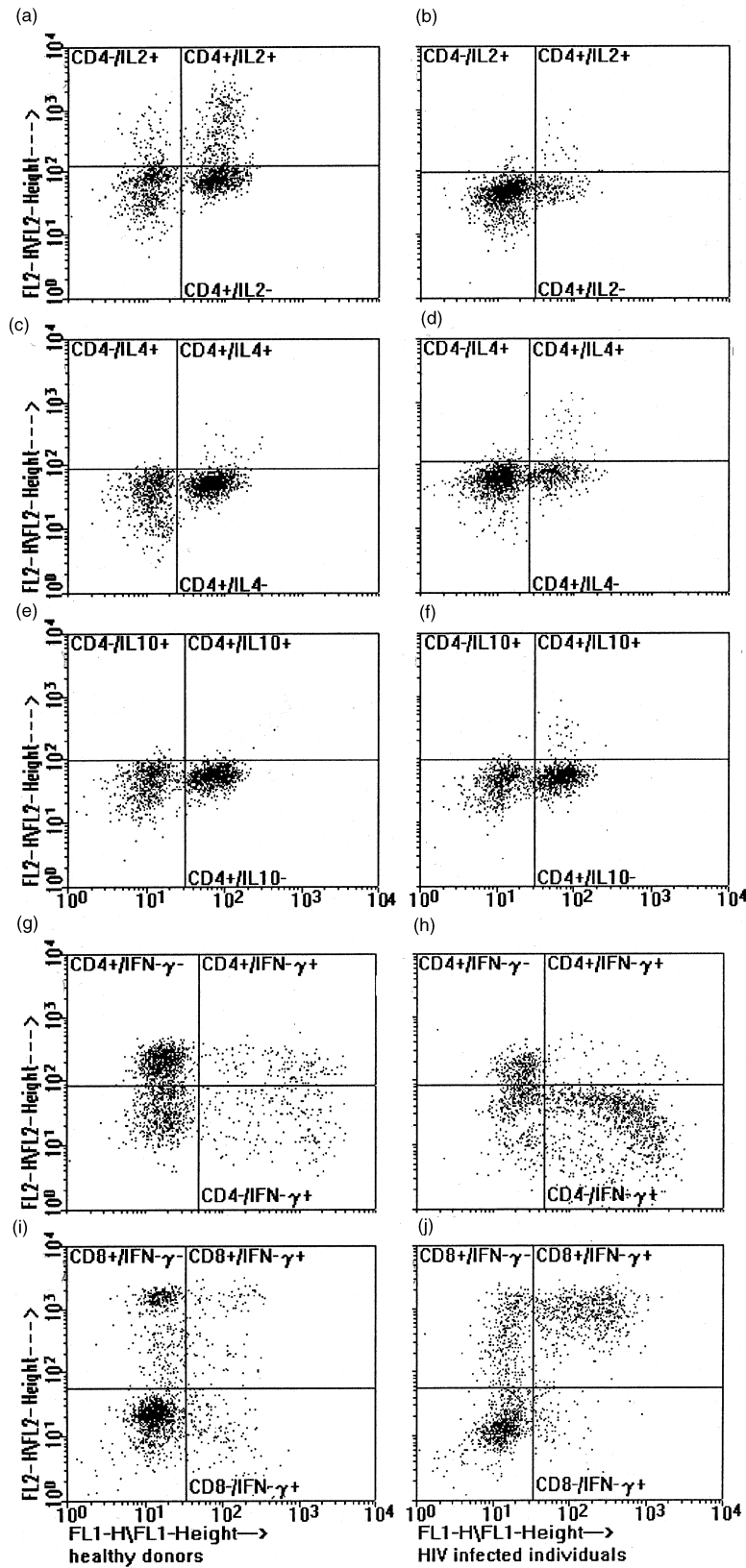
### Th2-type cytokines

The frequency of CD4+ T cells from healthy donors expressing IL-4 or IL-10 is significantly ( $P < 0.01$ ) lower than that of those expressing Th1-type cytokines: Only  $3.0 \pm 0.5\%$  of CD4+ T cells were positive for IL-4; IL-10 was almost undetectable. The percentage of both IL-4 and IL-10-producing cells among CD4+

**Table 1.** Percentage of interferon (IFN)- $\gamma$  or interleukin (IL)-2-expressing cells among CD4+ or CD8+ T cells from the indicated donor groups.

	IL-2 [mean $\pm$ SD (range)]		IFN- $\gamma$ [mean $\pm$ SD (range)]	
	CD4+ T cells	CD8+ T cells	CD4+ T cells	CD8+ T cells
Uninfected (n = 16)	$35.2 \pm 5.5$ (24.3–64.0)	$17.1 \pm 3.5$ (11.2–23.6)	$19.6 \pm 3.0$ (16.0–24.2)	$23.2 \pm 4.2$ (9.2–29.1)
HIV-1-positive, no AIDS (CDC A1, B1, A2, B2, n = 18)	$30.4 \pm 5.1^*$ (19.6–64.3)	$12.5 \pm 2.5^*$ (5.2–16.0)	$14.0 \pm 1.8^\dagger$ (11.3–16.4)	$32.4 \pm 5.5^\dagger$ (27.1–41.6)
HIV-1-positive, AIDS (CDC C3; n = 14)	$9.6 \pm 1.5^\ddagger$ (5.2–13.3)	$7.5 \pm 1.4^\S$ (4.7–9.8)	$10.6 \pm 1.9^{  }$ (7.1–13.3)	$51.4 \pm 7.6^{  }$ (40.2–66.3)

\*Not significant in comparison with uninfected donors.  $^\dagger P < 0.05$  comparing uninfected with HIV-infected individuals of Centers for Disease Control and Prevention (CDC) 1993 criteria, stage A1, B1, A2 and B2.  $^\ddagger P < 0.01$  comparing uninfected with HIV-infected individuals staged CDC C3.  $^\S P < 0.05$  comparing HIV-infected CDC stage C3 with CDC stages A1, B1, A2 and B2.  $^{||} P < 0.01$  comparing uninfected with HIV-infected CDC stage C3.



**Fig. 1.** Fluorescence-activated cell sorter dot-plots of phorbol myristate acetate/ionomycin/monensine-stimulated cells in the lymphocyte gate of healthy donors (a, c, e, g and i) and of HIV-1-infected individuals (b, d, f, h and j). The following combinations of antibody staining were used: (a) and (b), CD4 and interleukin-2; (c) and (d), CD4 and interleukin-4; (e) and (f), CD4 and interleukin-10; (g) and (h), interferon- $\gamma$  and CD4; (i) and (j), interferon- $\gamma$  and CD8.

**Table 2.** Percentage of interleukin (IL)-4 or IL-10 expressing cells among CD4+ or CD8+ T cells from the indicated donor groups.

	IL-4 [mean $\pm$ SD (range)]		IFN-10 [mean $\pm$ SD (range)]	
	CD4+ T cells	CD8+ T cells	CD4+ T cells	CD8+ T cells
Uninfected (n = 16)	3.0 $\pm$ 0.5 (2.0–4.1)	< 1% (< 1.2)	< 1% (< 0.9)	< 1% (< 0.6)
HIV-1-positive, no AIDS (CDC A1, B1, A2, B2, n = 18)	6.2 $\pm$ 1.3* (4.0–10.2)	2.1 $\pm$ 1.4 <sup>†</sup> (0.9–3.8)	2.9 $\pm$ 1.1* (1.7–4.3)	< 1% (< 0.8)
HIV-1-positive, AIDS (CDC C3; n = 14)	2.8 $\pm$ 0.6 <sup>‡</sup> (1.5–4.0)	< 1% <sup>†</sup> (< 1.0)	5.3 $\pm$ 1.4 <sup>§§</sup> (4.0–7.8)	< 1% (< 0.8)

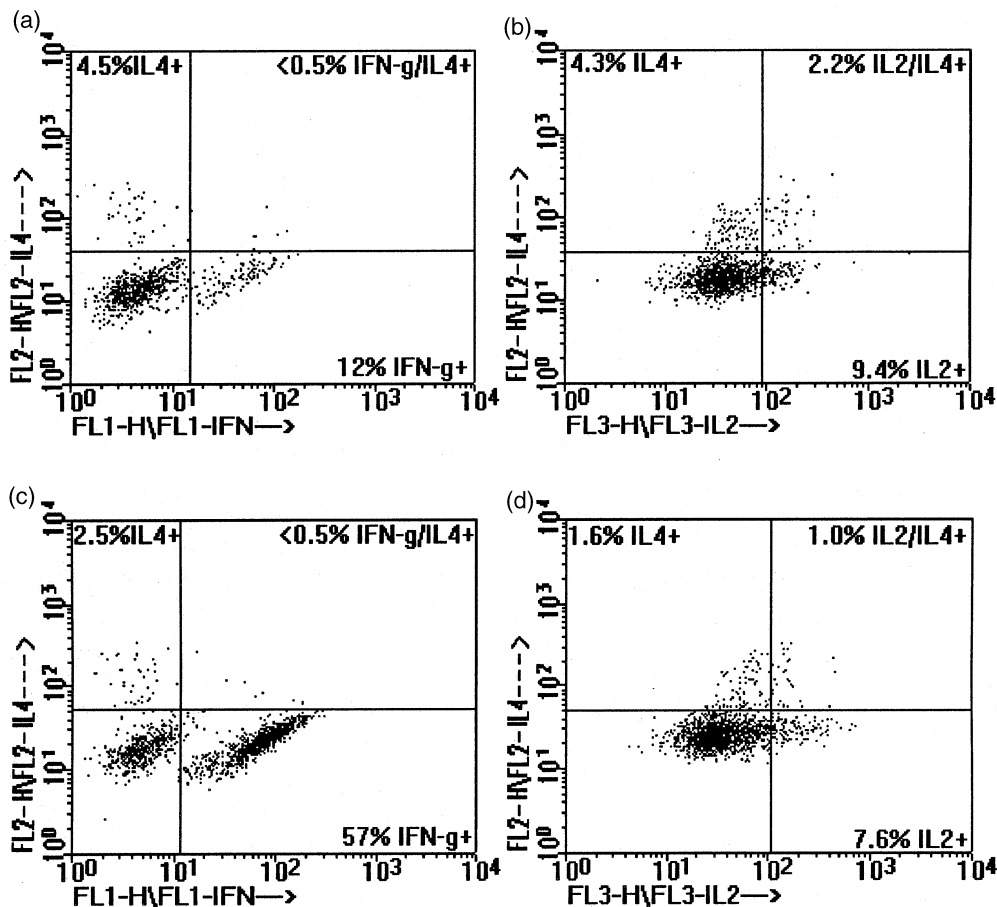
\* $P < 0.05$  comparing uninfected with HIV-infected individuals CDC stage A1, B1, A2 and B2; <sup>†</sup>Not significant in comparison with uninfected donors. <sup>‡</sup> $P < 0.05$  comparing HIV-infected individuals CDC stage C3 with those CDC stage A1, B1, A2 and B2; <sup>§§</sup> $P < 0.01$  comparing uninfected with HIV-infected individuals staged CDC C3; CDC, Centers for Disease Control and Prevention 1993 criteria.

T cells from HIV-1-infected individuals was increased in comparison to healthy donors. The proportion of CD4+ T cells expressing IL-4 was significantly ( $P < 0.05$ ) higher in cells from HIV-1-infected individuals without AIDS (6.2  $\pm$  1.3%). Whereas the frequency of IL-4 expression was reduced among CD4+ T cells from patients with AIDS, the percentage of IL-10-producing cells increased significantly ( $P < 0.01$ ) during the course of infection, reaching 5.3  $\pm$  1.4 among CD4+ T cells obtained from patients with AIDS (Table 2, Fig. 1). Both IL-4 and IL-10 were almost

undetectable in CD8+ T cells from healthy donors and expressed weakly in a minor fraction of cells from HIV-infected individuals (< 2.5%).

### Cytokine expression in non-T-cells

In order to evaluate the frequency of non-T-cells expressing Th1 or Th2 cytokines B cells, NK cells and monocytes were also investigated regarding their cytokine production. Following PMA/ionomycin or LPS stimulation no IL-2, IL-4, IL-10 or IFN- $\gamma$  production could be detected in either monocytes (CD14+ cells) or B cells (CD20+ cells; data not shown).



**Fig. 2.** Fluorescence-activated cell sorter dot-plots of phorbol myristate acetate/ionomycin/monensine stimulated cells from an HIV-infected individual (CDC B2) gated for CD4+ T cells (a and b) or CD8+ T cells (c and d). Cells were stained intracellularly with anti-interferon- $\gamma$  and anti-interleukin-4 (a and c) or anti-interleukin-2 and anti-interleukin-4 (b and d).

Whereas IL-4 was undetectable in NK cells from any donor group, 5–24% of NK cells (CD8+/CD56+) obtained from healthy donors as well as from HIV-infected individuals expressed the Th1-type cytokines IL-2 or IFN- $\gamma$ . The expression of these cytokines did not correlate with the presence or the stage of HIV infection (data not shown).

### Double staining of Th1- and Th2-cytokines

In order to determine whether cells expressing Th-2 cytokines are Th-2 or Th-0 type T cells, cells were stained simultaneously with MAbs against Th1- and Th2-cytokines. Double-staining of IFN- $\gamma$  and IL-4 revealed that nearly all CD4+ T cells ( $98.6 \pm 2.3\%$ ) from HIV-infected individuals expressing IL-4 do not produce IFN- $\gamma$  (Fig. 2). Moreover, the majority ( $64.3 \pm 5.4$ ) of IL-4 expressing CD4+ T cells from HIV-1-infected individuals did not co-express IL-2. The frequency of IL-4 expressing cells among CD8+ T cells was lower than among CD4+ T cells. IL-4 and IFN- $\gamma$  were not co-expressed in CD8+ T cells. Concerning the double staining of IL-2 and IL-4, fewer than 40% of the CD8+ T cells that expressed IL-4 produced IL-2.

### Quantification of cytokine production

In order to compare the average cytokine expression per CD4+ T cell obtained from the three donor-groups, a ratio of the mean fluorescence intensity per cytokine expressing cell and the fluorescence intensity at the cut-off point was calculated. The intensity of Th1-cytokine staining per CD4+ T cell was reduced significantly in patients with AIDS. Concerning IL-4, there was a peak of cytokine expression per cell in HIV-infected individuals without AIDS compared with both the patients with AIDS and the healthy controls. The level of IL-10 expression per cell was highest in CD4+ T cells from patients with AIDS (Table 3).

## Discussion

The depletion of CD4+ T cells during the course of HIV infection is accompanied by a progressive loss of cell-mediated immunity [2,3]. This loss of T-cell function occurs with a reduced production of IL-2 and

IFN- $\gamma$  by PBMC [6,7,9,12]. Observations such as polyclonal B-cell activation resulting in hypergammaglobulinaemia or increased IgE-serum-levels led to the suggestion of an increased Th2-type cytokine expression resulting in a Th1 to Th2 shift during the course of HIV infection [6,7]. Moreover, dysregulation of Th1/Th2 cytokine expression had been proposed as a mechanism for T cell loss via induction of apoptosis in HIV infection [27].

Our data showing a reduced frequency of IL-2 or IFN- $\gamma$ -producing cells among CD4+ T cells and an increased percentage of IL-4 or IL-10 positive cells among CD4+ T cells confirm for the first time on a single cell level the proposed Th1 to Th2 shift of cytokine production in CD4+ T cells of HIV-1-infected individuals.

As it is shown in Table 1 the percentage of IL-2-expressing cells among CD4+ T cells from HIV-infected individuals classified CDC A1, B1, A2 and B2 was reduced significantly in comparison with patients with AIDS (CDC C3). Thus, the loss of IL-2-expressing CD4+ T cells may be a critical step towards the development of AIDS.

The percentage of CD4+ T cells capable of expression of Th-2 cytokines is very low in uninfected donors (IL-4, 2.9%; IL-10, < 1%). As it has been shown earlier, a significant increase of IL-4 expression is associated with early stages of the disease [8,28]. More than twice as many CD4+ T cells were positive for IL-4 in HIV-infected donors classified CDC A1, B1, A2 and B2 compared with healthy donors. Moreover, the average IL-4 production per CD4+ T cell was enhanced significantly in HIV-infected individuals indicating a strong Th2-type response. Surprisingly IL-4 expression peaked in early stages of HIV infection and decreased again with the development of AIDS. Although these results are consistent with earlier results [6,8] the cause and significance of this phenomenon still have to be identified. Whereas IL-4 expression had its maximum in early stages of the disease, IL-10 was expressed particularly in patients with AIDS: both, the percentage of CD4+ T cells producing IL-10 and the average IL-10 expression per cell peaked in individuals suffering from AIDS.

**Table 3.** Ratio of the mean of fluorescence intensity per cytokine expressing CD4+ T cell and fluorescence intensity at the cut-off point of cytokine expression.

	IFN- $\gamma$ (mean $\pm$ SD)	IL-2 (mean $\pm$ SD)	IL-4 (mean $\pm$ SD)	IL-10 (mean $\pm$ SD)
Uninfected (n = 16)	8.3 $\pm$ 2.5	7.9 $\pm$ 3.0	2.8 $\pm$ 1.0	NE
HIV-1-positive, no AIDS (CDC A1, B1, A2, B2, n = 18)	6.9 $\pm$ 1.8*	7.0 $\pm$ 2.8*	4.3 $\pm$ 1.6 <sup>†</sup>	3.9 $\pm$ 1.4 <sup>†</sup>
HIV-1-positive, AIDS (CDC C3; n = 14)	4.2 $\pm$ 1.2 <sup>†</sup>	2.6 $\pm$ 0.7 <sup>‡§</sup>	3.0 $\pm$ 1.0*	4.6 $\pm$ 1.4 <sup>‡</sup>

\*Not significant. <sup>†</sup> $P < 0.05$  comparing uninfected with HIV-infected individuals CDC stage A1, B1, A2 and B2. <sup>‡</sup> $P < 0.01$  comparing uninfected with HIV-infected individuals CDC stage C3. <sup>§</sup> $P < 0.05$  comparing HIV-infected individuals CDC stage C3 with those staged CDC A1, B1, A2 and B2. NE, Cytokine was not expressed. CDC, Centers for Disease Control and Prevention 1993 criteria.

The increased Th2-type cytokine expression in CD4+ T cells from HIV-infected individuals could be demonstrated not to be a Th0-like response since we were able to show by double staining of IFN- $\gamma$  versus IL-4 and IL-2 versus IL-4 that the Th2 cytokine IL-4 is rarely co-expressed with Th-2 cytokines. Thus, we suggest that there is a Th1 to Th2 shift of cytokine expression in HIV-infected individuals. Unfortunately we could not generate similar data for IL-10 because IL-10 has its maximal expression after a 10 h incubation whereas an optimal staining for IL-2 and IFN- $\gamma$  is achieved after 5 h.

As demonstrated in Table 1 and Fig. 1, an increase of the percentage of IFN- $\gamma$ -expressing cells among CD8+ T cells was observed during the course of HIV infection. These results confirm similar earlier reported data [18,29]. The increased IFN- $\gamma$  expression by CD8+ T cells may reflect an enhanced activation of cytotoxic T cells in course of HIV infection. This gives support to the theory of an increasing population of anti-HIV-specific CD8+ T cells [30,31], or an enhanced activation of CD8+ T cells in HIV infection.

Although the frequency of CD8+ T cells expressing the Th1-type cytokine IFN- $\gamma$  is increased in HIV-1-infected patients the total amount of IFN- $\gamma$  production is not enhanced as it has been shown earlier [32,33]. Thus, the enhanced IFN- $\gamma$  expression in CD8+ T cells does not contradict the suggested Th1 to Th2 shift.

However, examination of Th1/Th2-cytokine expression in non-T-cell lymphocytes demonstrated no significant cytokine production in B cells. The percentage of Th1-type cytokine expressing cells among NK cells obtained from HIV-infected individuals was neither increased nor decreased compared with healthy donors: between 5% and 24% of NK cells obtained from healthy donors and HIV-infected individuals expressed IL-2 or IFN- $\gamma$ . Th2-type cytokines were not detectable in NK cells from all donor groups. Thus, neither B cells nor NK cells appear to contribute to a change of Th1/Th2 cytokine expression during the course of HIV infection.

Monocytes could be important producers of Th1 (IL-12) or Th2 (IL-10) cytokines. In this study we could not characterize monocytic expression of these cytokines by cytoplasmic cytokine staining. Thus, stimulation, culture and intracellular cytokine staining of monocytes have to be optimized to characterize the contribution of this cell type to changes of Th1/Th2 cytokine expression in HIV infection.

One may criticize the fact that the low frequency of Th2-type cytokine-expressing cells among CD4+ T cells, which represent also only a minority of cells in HIV infection, could hardly contribute significantly to the change of cytokine pattern: the Th1/Th2 cytokine

heterogeneity could be demonstrated for CD4+ T cells and for CD4 negative lymphocytes [20]. As described above, even monocytes are producers of Th1- or Th2-type cytokines. However, a Th1 to Th2 shift could be demonstrated on a single cell level only in CD4+ T cells in early (IL-4) and in late (IL-10) stages of HIV infection. Therefore the Th1 to Th2 shift in HIV infection, which was already demonstrated in culture supernatants or by RT-PCR [6–12] should be due to the Th1 to Th2 shift in the CD4+ T-cell compartment. Moreover, for the induction of a cell mediated immunity by Th1-type or a humoral immune response by Th2-type cytokines CD4+ T cells are playing a central role. Therefore, even in HIV-infected individuals with a low count of CD4+ T cells the cytokine expression of this cell type should be pivotal for the Th1/Th2-cytokine pattern.

For stimulation of PBMC, a combination of PMA and ionomycin was used. This maximal activation gives the opportunity to define the capability of lymphocytes to produce cytokines. Other more physiological stimuli such as anti-CD3-MAb (OKT3) or anti-CD28 MAb did not result in a cell activation sufficient for cytoplasmic cytokine staining. After such a stimulation even IL-2 or IFN- $\gamma$  were expressed in fewer than 5% of CD4+ T cells from healthy donors. Th2-type cytokines were almost undetectable in cells cultured for 4–12 h under these suboptimal stimulation conditions. Only after several restimulations with OKT3 and a culture time of 4–7 days were more than 20% of CD4+ T cells positive for IL-2 or IFN- $\gamma$  (data not shown). Data obtained after such a long *in-vitro* culture time can be extremely biased in relation to the culture conditions and are therefore hardly reliable or valid to characterize or to represent any *in vivo* situation. Therefore we used a maximal stimulation in a short term culture as the best indicator for the *in vivo* capacity of cells to express Th1/Th2-cytokines.

In addition to the characterization of cells by their cytokine production, the method of cytoplasmic cytokine staining permits quantification of cytokine production per cell. Due to the inhibition of cytokine release by monensine, the intensity of cytokine staining per cell reflects the amount of cytokines produced during monensine incubation. Thus, we were able to compare the amount of cytokine production per cell between different donors by creating a ratio between the mean of the fluorescence intensity and the staining intensity at the threshold of cytokine expression. In contrast to the absolute value of fluorescence intensity this ratio is not influenced by instrument settings and measurement-to-measurement variability of fluorescence intensity. As it has been shown earlier by Jung *et al.*, quantification of cytokine expression by the method of cytoplasmic cytokine staining is valid and reliable [25,34].

Thus, cytoplasmic cytokine staining gives the unique opportunity to quantify cytokine expression within surface-marker-defined single cells. It can be a tool to characterize cytokine expression and to clarify the role of cytokines for pathogenesis of AIDS. Moreover, the question of whether cytoplasmic cytokine staining can be used to monitor the immune function of HIV-infected individuals has still to be investigated. Particularly for monitoring of disease progression under antiretroviral therapy, cytoplasmic cytokine detection would certainly add important information concerning the quality of the T-cell function to a monitoring based only on quantitative parameters such as the count of CD4+ T cells and virus burden. Moreover, compared with alternative techniques to analyse cytokine expression (e.g. RT-PCR or ELISA) cytoplasmic cytokine detection is performed more easily and cheaply and it also provides additional information.

The data presented in this paper demonstrate a shift of Th1-type to Th2-type cytokine expression by CD4+ T cells in the course of HIV infection in the setting of a cross-sectional study. In order to definitively prove the Th1 to Th2 cytokine shift during the course of HIV infection a longitudinal study will be performed.

## References

1. Shearer GM, Clerici M: **Early T-helper cell defects in HIV infection.** *AIDS* 1991, **5**:245–253.
2. Clerici M: **Cell-mediated immunity in HIV infection.** *AIDS* 1993, **7**:135–40.
3. Meyaard L, Schuitemaker H, Miedema F: **T-cell dysfunction in HIV infection: anergy due to defective antigen-presenting cell function?** *Immunol Today* 1993, **14**:161–164.
4. Klein SA, Dohmeyer JM, Dohmeyer TS, et al.: **TNF- $\alpha$  mediated apoptosis of CD4 positive T-lymphocytes; a model of T-cell depletion in HIV infected individuals.** *Eur J Med Res* 1996, **1**:249–258.
5. Klein SA, Dohmeyer TS, Helm EB, Hoelzer D, Rossol R: **Production of Th1 cytokines in lymphocytes of HIV infected individuals; detection of cytokines on single cell level by flow cytometry.** *Blood* 1994, **84** (suppl 1):247a.
6. Clerici M, Shearer GM: **A Th1 $\rightarrow$ Th2 switch is a critical step in the etiology of HIV infection.** *Immunol Today* 1993, **14**:107–111.
7. Clerici M, Shearer GM: **The Th1 $\rightarrow$ Th2 hypothesis of HIV infection: new insights.** *Immunol Today* 1994, **15**:575–581.
8. Meroni L, Trabattini D, Balotta C, et al.: **Evidence for type 2 cytokine production and lymphocyte activation in the early phases of HIV-1 infection.** *AIDS* 1996, **10**:23–30.
9. Barcellini W, Rizzardi GP, Orietta Borghi M, Fain C, Lazzarin A, Meroni PL: **TH1 and TH2 cytokine production by peripheral blood mononuclear cells from HIV-infected patients.** *AIDS* 1994, **8**:757–762.
10. Clerici M, Wynn TA, Berzofsky JA, et al.: **Role of interleukin-10 in T helper cell dysfunction in asymptomatic individuals infected with the human immunodeficiency virus.** *J Clin Invest* 1994, **93**:768–775.
11. Meyaard L, Otto SA, Keet IP, van Lier RA, Miedema F: **Changes in cytokine secretion patterns of CD4+ T-cell clones in human immunodeficiency virus infection.** *Blood* 1994, **84**:4262–4268.
12. Meyaard L, Miedema F: **Changes in cytokine release by CD4+ T cells in HIV-1 infection.** *Res Immunol* 1994, **145**:607–610.
13. Romagnani S, Del-Prete G, Manetti R, et al.: **Role of TH1/TH2 cytokines in HIV infection.** *Immunol Rev* 1994, **140**:73–92.
14. Maggi E, Mazelli M, Ravina A, et al.: **Ability of HIV to promote a Th1 to Th0 shift and to replicate preferentially in Th2 and Th0 cells.** *Science* 1994, **265**:244–248.
15. Romagnani S, Maggi E, Del-Prete G: **HIV can induce a TH1 to TH0 shift, and preferentially replicates in CD4+ T-cell clones producing TH2-type cytokines.** *Res Immunol* 1994, **145**:611–617.
16. Romagnani S, Maggi E, Del Prete G: **An alternative view of the Th1/Th2 switch hypothesis in HIV infection.** *AIDS Res Hum Retroviruses* 1994, **10**:3–9.
17. Romagnani S, Maggi E: **Th1 versus Th2 responses in AIDS.** *Curr Opin Immunol* 1994, **6**:616–622.
18. Graziosi C, Pantaleo G, Gant KR, et al.: **Lack of evidence for the dicotomy of Th1 and Th2 predominance in HIV infected individuals.** *Science* 1994, **265**:248–252.
19. Mosman TR, Cherwinski H, Bond MW, Gledin MA, Coffman RC: **Two types of murine helper T-cell clone. Definition according to two profiles of lymphokine activities and secreted proteins.** *J Immunol* 1986, **136**:2348–2354.
20. Romagnani S: **Biology of human TH1 and TH2 cells.** *J Clin Immunol* 1995, **15**:121–129.
21. Del Prete GF, De Carli M, Mastromauro C, et al.: **Purified protein derivative of *Mycobacterium tuberculosis* and excretory/secretory antigens of *Toxocara canis* expand in vitro T-cells with stable and opposite (type 1 T helper and type 3 T helper) profile of cytokine production.** *J Clin Invest* 1991, **88**:346–350.
22. Wierenga EA, Snoek M, Jansen HM, Bos JD, van Lier RAW, Kapsenberg ML: **Human atopen-specific types 1 and 2 T helper cell clones.** *J Immunol* 1991, **147**:2942–2949.
23. Elson LH, Nutman TB, Metcalfe DD, Prussin C: **Flow cytometric analysis for cytokine production identifies T helper 1, T helper 2, and T helper 0 cells within the human CD4+CD27- lymphocyte subpopulation.** *J Immunol* 1995, **155**:4294–4301.
24. Jung T, Schauer U, Heusser C, Neumann C, Rieger C: **Detection of intracellular cytokines by flow cytometry.** *J Immunol Methods* 1993, **159**:197–207.
25. Jung T, Schauer U, Rieger C, et al.: **Interleukin 4 and interleukin 5 are rarely coexpressed by human T-cells.** *Eur J Immunol* 1995, **25**:2413–2416.
26. Picker LJ, Singh MK, Zdravski Z, et al.: **Direct demonstration of cytokine synthesis heterogeneity among human memory/effector T cells by flow cytometry.** *Blood* 1995, **86**:1408–1419.
27. Clerici M, Sarin A, Coffman RL, et al.: **Type 1/type 2 cytokine modulation of T-cell programmed cell death as a model for human immunodeficiency virus pathogenesis.** *Proc Natl Acad Sci USA* 1994, **91**:11811–11815.
28. Clerici M, Hakim FT, Venzon DJ, et al.: **Changes in interleukin-2 and interleukin-4 production in asymptomatic human immunodeficiency virus-seropositive individuals.** *J Clin Invest* 1993, **91**:759–765.
29. Caruso A, Canaris AD, Licenziati S, et al.: **CD4+ and CD8+ lymphocytes of patients with AIDS synthesize increased amounts of interferon-gamma.** *J Acquir Immune Defic Syndr Hum Retrovirol* 1995, **10**:462–470.
30. Zinkernagel RM, Hengartner H: **T-cell mediated immunopathology versus direct cytolysis by virus.** *Immunol Today* 1994, **15**:262–268.
31. Poli G, Biswas P, Fauci AS: **Interferons in the pathogenesis and treatment of human immunodeficiency virus infection.** *Antiviral Res* 1994, **24**:221–233.
32. Rossol S, Rossol-Voth R, Laubenstein HP, et al.: **Interferon production in patients with HIV-1.** *J Infect Dis* 1989, **159**:815–821.
33. Rossol-Voth R, Rossol S, Klein K, et al.: **Differential gene expression of IFN and TNF- $\alpha$  in peripheral blood mononuclear cells from patients with AIDS related complex and AIDS.** *J Immunol* 1990, **144**:970–978.
34. Vikingsson A, Pederson K, Muller D: **Enumeration of IFN- $\gamma$  producing lymphocytes by flow cytometry and correlation with quantitative measurement of IFN- $\gamma$ .** *J Immunol Methods* 1994, **173**:219–228.