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Intracellular Production of Type I and Type II Cytokines During HIV-1 Progression in Thai Patients

Nattawat Onlamoon[1], Surada Lerdwana[1], Winai Ratanasuwan[2], Surapol Suwanagool[2], Pilaipan Puthavathana[3] and Kovit Pattanapanyasat[1]

SUMMARY A type I to type II cytokine switch on cells of the immune system has been suggested as a critical step in the etiology of HIV infection. In this study, type I and type II cytokine production of both CD4+ and CD8+ T cells activated by superantigen were investigated in 10 healthy donors and 39 HIV-1 infected patients. Patients were divided into 3 groups based on their CD4 count (< 200, 200-500, > 500 cells/μl). Whole blood from each subject was activated by staphylococcal enterotoxin B (SEB) and anti-CD28. Intracellular cytokine stainings for proinflamatory cytokine (TNF-α), type I cytokines (IFN-γ and IL-2) and type II cytokines (IL-4 and IL-5) in CD4+ and CD8+ T lymphocytes were determined by flow cytometer. Type I cytokine (IFN-γ) expression in CD4+ T cells co-expressing with CD69 were significantly increased in HIV infected patients, particularly in patients with CD4 counts < 200 and 200-500 cells/μl (means ± S.D. of 20.7 ± 18.7% and 10.5 ± 5.9%, respectively) when compared with 4.8 ± 1.8% in the normal group ($p < 0.05$). But IL-2 production in both groups of patients was significantly lower than the normal (3.8 ± 2.6% and 3.2 ± 1.4% in patients with <200, 200-500 cells/μl, and 5.9 ± 1.5% in the normal group) ($p < 0.05$). For type II cytokines, there was no difference in all groups of subjects when IL-4 was determined. However, IL-5 production was significantly higher in patients with a CD4 count < 200 cells/μl (0.6 ± 0.5%) than that in the normal group (0.1 ± 0.1%) ($p < 0.005$). CD8+ T cells also showed higher IFN-γ production in patients with a CD4 count < 200 cells/μl (11.9 ± 4.7%) and 200-500 cells/μl (12.0 ± 4.3%) than the normal group (5.3 ± 2.5%) ($p < 0.005$). In contrast, IL-2 production in CD8+ T cells was low in these HIV infected patients (0.3 ± 0.2%, 0.3 ± 0.2%, and 0.3 ± 0.4% in patients with < 200, 200-500, and > 500 cells/μl, respectively), which was significantly different compared to the control group (1.2 ± 0.8%) ($p < 0.05$). For type II cytokines, only IL-4 production in patients with a CD4 count < 200 cells/μl (0.1 ± 0.1%) was significantly reduced when compared to the other groups ($p < 0.05$). This study shows that although HIV infection alters the production of both type I and type II cytokines, it does not induce a polarized type I or type II state in the course of HIV-1 progression in Thai patients.

A definition of Th1 and Th2 cells was originally described in mouse CD4+ T cell clones based on their different profile of cytokine secretion. Th1 cells produce IFN-γ, IL-2 and TNF-β, and are primarily responsible for cell mediated immune responses including the production of opsonizing and complement fixing antibodies, macrophage activation, antibody dependent cell cytotoxicity and delayed type hypersensitivity. On the other hand, Th2 cells produce IL-4, IL-5, IL-6, IL-10 and IL-13 and are designated to promote humoral immune responses such as IgE and IgG_1 isotype switching, to enhance eosinophil function and to inhibit several macrophage functions. Both types of cytokines can cross-regulate the differentiation of the other cell type.[1] Some CD4+ T cells produce both types of cytokines. They are called Th0 cells. These cells may dominate in the earliest stage of some responses and mediate effector function. The same cytokine production pattern is also found in CD8+ T cells,[2] these subsets are termed Tc1 and Tc2. These cells play a role in the regulation of activation and differentiation of CD4+ T cells including alteration of the balance of Th1/Th2 responses, suppression of CD4 proliferative responses and the development of CD4 perforin mediated cytotoxicity. They also play an important role in the activation of macrophages and the regulation of antibody production of B cells. However, other cell types such as γδ T cells, NK cells, B cells and monocytes/macrophages also produce these cytokines. The term type

From the [1]Center of Excellence for Flow Cytometry, Division of Instruments for Research, Office for Research and Development, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand, [2]Dapartment of Preventive and Social Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University, [3]Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University.
Correspondence: Kovit Pattanapanyasat

type I and type II cytokine can be appropriately used to describe them based on the responses they modulate rather than on the cell types that produce cytokines.

In the course of HIV infection, a severe depletion of CD4+ T cells occurs in the later stage of the disease. However, the loss of T helper cell function occurs before CD4+ T cell numbers are critically reduced and AIDS is diagnosed. Peripheral blood mononuclear cells from asymptomatic HIV-infected patients show a defective T cell response and loss of IL-2 production. In contrast, enhanced B cell responses occur including hypergammaglobulinemia and spontaneous IgG production *in vitro*. These findings suggest that a switch from the Th1 to the Th2 cytokine phenotype may be important in the pathogenesis of the disease progression in HIV infection.[3] However, this hypothesis remains controversial.[4,5] Some investigators could not demonstrate this switch from Th1 to Th2,[6] whilst others did show a Th1 to Th0 shift.[7,8]

By using a flow cytometric intracellular cytokine staining technique, specific cytokine producing cells can be determined at the single cell level.[9-11] The assay requires only a short incubation time (4-6 hours) in the presence of secretion inhibitor Brefeldin A to increase the sensitivity of detection. Following fixation and permeabilization, staining with anti-cytokine monoclonal antibodies (mAbs) is easily performed.[12] This method has been extensively used to study cytokine production patterns in HIV infection.[13-20]

In this study, we investigated type I and type II cytokine productions of T lymphocytes activated by superantigen in healthy individuals and HIV-infected patients who were divided into 3 groups based on their CD4 count. Using this method, we found an increased percentage of IFN-γ expression in CD4+ and CD8+ T cells with no evidence of type I to type II cytokine shift in HIV-infected patients.

MATERIALS AND METHODS

Study population

Sodium heparinized peripheral blood was collected from 10 healthy normal volunteers and 39 HIV-infected patients. Patients were divided into 3 groups based on their CD4 counts. Twenty patients had CD4 counts < 200 cells/μl, 14 had CD4 counts of 200 to 500 cells/μl and 5 had CD4 counts > 500 cells/μl. None of the patients received antiretroviral therapy prior to the study. Absolute lymphocyte counts and T cell subset analysis were determined routinely by flow cytometric analysis (Table 1). Written informed consent was obtained from all patients. The study protocol was approved by the Ethics Committee, Faculty of Medicine Siriraj Hospital.

Monoclonal antibodies and reagents

The following anti-human cytokine mAbs and their conjugated fluorochromes were used: anti-TNF-α, clone mAb11, mouse IgG1 fluorescein isothiocyanate (FITC); anti-IFNγ, clone B27, mouse IgG1 (FITC); anti-IL-2, clone MQ1-17H12, rat IgG2a (FITC); anti-IL-4, clone MP4-25D2, rat IgG1 (FITC); anti-IL-5, clone TRFK5, rat IgG1 (FITC). These reagents were purchased from Pharmingen (San Diego, CA). Anti-human CD4 conjugated with peridinin chlorophyll protein (PerCP), CD8 (PerCP), and anti-human CD69 phycoerythrin (PE) mAbs were obtained from Becton Dickinson Biosciences (BDB: San Jose, CA). These mAbs were used at optimal concentration recommended by the manufacturers.

Table 1 Mean values of CD4 and CD8 T-cell subsets in different HIV-1 infected groups

Subjects		N	CD4 count/μl	% CD4	CD8 count/μl	% CD8	Ratio
HIV+	CD4 < 200 cells/μl	20	70 (1-192)	5.0 (0.3-13.2)	791 (33-1590)	58.1 (35.2-80.3)	0.09 (0.01-0.29)
	CD4 200-500 cells/μl	14	318 (238-450)	16 (9.6-29.7)	1037 (502-1502)	49.4 (34.0-63.0)	0.34 (0.18-0.87)
	CD4 > 500 cells/μl	5	605 (524-699)	19.8 (13.4-25.9)	1451 (804-1046)	43.9 (33.4-51.4)	0.48 (0.25-0.67)
HIV-		10	720 (479-1,240)	33.0 (21.6-42.0)	659 (271-1240)	27.6 (17.1-41.6)	1.34 (0.66-2.38)

Anti-human CD28, clone CD28.2, mouse IgG1 (purified) was also purchased from Pharmingen and used at a final concentration of 3 μg/ml. Staphylococcal enterotoxin B (SEB) and Brefeldin A (BFA) were obtained from Sigma Chemical Co., USA, and used at 10 μg/ml.

Cell stimulation and immunofluorescence staining

Whole blood was stimulated in a 15 ml polypropylene tube with 10 μg/ml of SEB and 3 μg/ml of anti-human CD28 mAbs in the presence of BFA (10 μg/ml). Whole blood without SEB stimulation was used as control. Samples were incubated for 4 hours at 37 °C/5% CO_2. After incubation, stimulated and unstimulated samples were then mixed with 100 μl of 20 mM EDTA at a final concentration of 2 mM/ml and incubated for 15 minutes at room temperature (RT). The red blood cells were then lysed and fixed in 9 ml of 1x FACS™ Lysing solution (BDB) for 10 minutes at room temperature and centrifuged at 500 *g* for 5 minutes. Cells were washed in washing buffer (PBS with 2% fetal bovine serum) and permeabilized with 2.5 ml of 1x FACS™ Permeabilizing Solution (BDB) for 10 minutes at room temperature. After permeabilization, cells were washed by adding 8 ml of washing buffer and centrifuged at 500 *g* for 5 minutes. Immunofluorescence staining was performed by adding the staining antibody cocktail consisting of CD4 or CD8 PerCP, CD69 PE and anti-TNF-α, anti- IFN-γ, anti-IL-2, anti-IL-4 or anti-IL-5 FITC and incubating in the dark for 30 minutes at room temperature. After staining, cells were washed and fixed in 1% paraformaldehyde in PBS and stored at 4°C until flow cytometric analysis.

Flow cytometric analysis of intracellular cytokine production

Five-parameter analysis was performed on a FACSort flow cytometer (BDB) using CellQuest software. At least 3,000 cells gated on CD4 PerCP or CD8 PerCP were acquired and a lymphogate was done to include only viable lymphocytes. Data were displayed as two-color dot plot (FITC *vs* PE) to measure the percentage of the double positive (CD69+/cytokine+) cells (upper right quadrant, see Fig. 1 for details). An unstimulated control was used to set a quadrant gate.

Statistical analysis

Data were presented as mean ± S.D. The Mann-Whitney U test was used to determine the statistical significance of the difference observed between groups. P values < 0.05 were considered statistically significant.

RESULTS

CD69 expression increased in HIV-infected patients

The representative fluorescent expression of CD69 with proinflammatory cytokine (TNF-α) and the type I-type II cytokine production patterns in CD4+ T cells from healthy normal controls and HIV-infected patients after superantigen stimulation are shown in Fig. 1. The mean percentage of CD4+ T cells expressing CD69 was significantly increased in patients with CD4 counts < 200 cells/μl (52.5 ± 25.1%) and 200-500 cells/μl (39.2 ± 10.5%) when compared to the healthy control group (22.8 ± 3.5%) (Table 2). However, CD69 expression in CD8+ T cells was not dif-

Table 2 Percentage of CD4+ T cells positive for TNF-α, IFN-γ, IL-2, IL-4 and IL-5 in different HIV-1 infected groups

Subjects		CD69* (%)	Proinflammatory cytokine (%)	Type I cytokine (%)		Type II cytokine (%)	
			TNF-α	IFN-γ	IL - 2	IL - 4	IL-5
HIV+	CD4<200 cells/μl	52.5 ± 25.1[1]	19.8 ± 14.0	20.7 ± 18.7[1]	3.8 ± 2.6[1]	1.3 ± 11. 2	0.6 ± 0. 5[2]
	CD4 200-500 cells/μl	39.2 ± 10.5[1]	11.6 ± 4.1	10.5 ± 5.9[1]	3.2 ± 1.4[1]	1.0 ± 0.7	0.2 ± 0.4
	CD4 > 500 cells/μl	31.3 ± 16.4	9.2 ± 6.4	5.9 ± 4.3	3.6 ± 2.9	0.6 ± 0.4	0.2 ± 0.2
HIV-		28.2 ± 6.6	12.1 ± 3.6	4.8 ± 1.8	5.9 ± 1.5	0.8 ± 0.4	0.1 ± 0.1

Statistical significance in comparison with healthy donors: [1]$p < 0.05$; [2]$p < 0.005$.

Fig. 1 Flow cytometric three-colour analysis of CD4+ T cells from unstimulated normal control (A), SEB stimulated healthy control (B) and SEB stimulated HIV infected patients (C). Upper left quadrant (CD69+/cytokine-); upper right quadrant (CD69+/cytokine+); lower left quadrant (CD69-/cytokine-); lower right quadrant (CD69-/cytokine+). The percentage of CD4+ T cells expressing CD69 was defined by the summation of the percentage in the upper left quadrant and the percentage in the upper right quadrant.

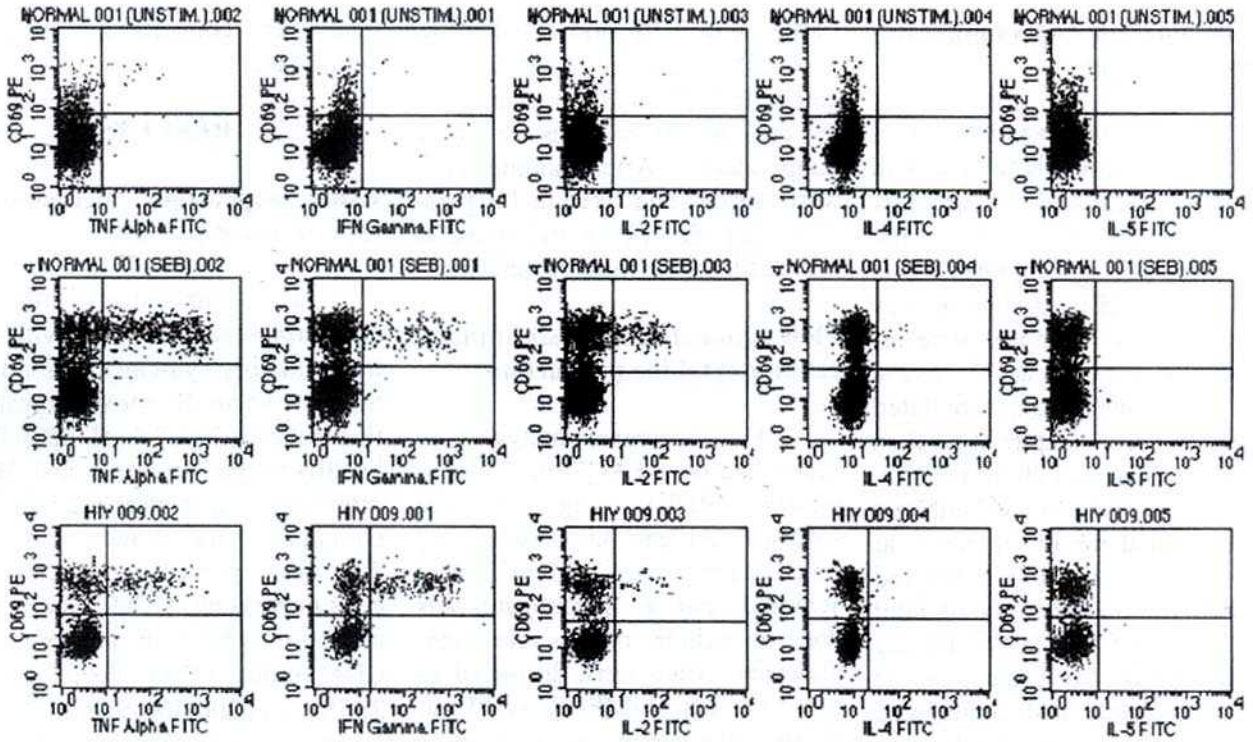

Table 3 Percentage of CD8+ T cells positive for TNF-α, IFN-γ, IL-2, IL-4 and IL-5 in different HIV-1 infected groups

Subjects		CD69⁺ (%)	Proinflamatory cytokine (%)	Type I cytokine (%)		Type II cytokine (%)	
			TNF-α	IFN-γ	IL-2	IL-4	IL-5
HIV+	CD4 < 200 cells/μl	22.9 ± 8.2	6.2 ± 3.3	11.9 ± 4.7[1]	0.3 ± 0.2[1]	0.1 ± 0.1[1]	0.1 ± 0.1
	CD4 200-500 cells/μl	24.2 ± 7.7	5.7 ± 2. 3	12.0 ± 4.3[1]	0.3 ± 0.2[1]	0.3 ± 0.4	0.1 ± 0.1
	CD4 > 500 cells/μl	20.6 ± 13.5	6.2 ± 6.0	10.4 ± 8.3	0.3 ± 0.4[1]	0.1 ± 0.1	0.1 ± 0.1
HIV-		28.2 ± 6.6	4.7 ± 1.8	5.3 ± 2.5	1.2 ± 0.8	0.3 ± 0.2	0.1 ± 0.2

Statistical significance in comparison with healthy donors: [1]$p < 0.05$.

ferent between patients and the control group (Table 3).

Type 1 cytokine production increased in CD4+ T cells

The mean percentages of IFN-γ producing CD4+ T cells were increased in HIV-infected patients with CD4 counts < 200 and 200-500 cells/μl. The mean percentage of IFN-γ producing CD4+ T cells was highest in patients with CD4 counts < 200 cells/μl (20.7 ± 18.7%). In contrast, a lower production of IL-2 in CD4+ T cells was found when compared to the control group (Table 2).

For type II cytokine production, both IL-4 and IL-5 showed no difference between patients and healthy controls except in patients with the CD4 counts < 200 cells/μl, in whom an increase in the percentage of IL-5 producing CD4+ T cells was found (0.6 ± 0.5%; Table 2).

IFN-γ producing CD8+ T cells increased in HIV-infected patients

The mean percentage of IFN-γ producing CD8+ T cells in patients with CD4 counts < 200 cells/μl was 11.9 ± 4.7% and 12.00 ± 4.3% in patients with CD4 counts of 200-500 cells/μl. The values were significantly higher than in the controls (5.3 ± 2.5%). As for CD4+ T cells, low percentages of IL-2 producing CD8+ T cells were observed in all 3 groups of patients (Table 3).

No evidence of increased type II cytokine production in CD8+ T cells was found in patients. Only IL-4 production in patients with CD4 counts < 200 cells/μl was significantly reduced when compared to the control group (Table 2).

DISCUSSION

In this study, we demonstrated a reduced ability of CD4+ and CD8+ T cells to produce IL-2 in HIV-infected patients. In contrast to an increase of IFN-γ producing cells in both CD4+ and CD8+ T cells, no type II cytokine was observed except an increased IL-5 production in CD4+ T cells in HIV-infected patients with CD4 counts < 200 cells/μl.

The reduced production of IL-2 observed in this study is in agreement with previous studies using the flow cytometric intracellular cytokine detection technique.[17,20] An impaired function of CD4+ T cells in HIV infection leading to a reduced production of IL-2 was well documented. We found an increased percentage of IFN-γ producing CD8+ T cells in HIV-infected patients which is comparable to other studies using the same technique.[14,16] The significance of increasing IFN-γ production in CD8+ T cells, though to date unclear, is believed to be associated with CD28.[16] In contrast to other studies, we found an increase in IFN-γ producing CD4+ T cells in HIV-infected patients, with the highest production in patients with CD4 counts < 200 cells/μl. An increased percentage of this cell type may play an important role in protecting against disease progression in patients with very low CD4 counts. However, a notable trend between increasing IFN-γ producing and decreasing IL-2 producing CD4+ T cells suggests that further assessment of these parameters may be warranted in a larger cohort of patients.

Some studies showed evidence that type I cytokines decreased and type II cytokines increased in HIV-infected patients in the progression toward AIDS.[5-8] Type I cytokines can prevent spontaneous or antigen driven programmed cell death, whereas type II cytokines do the opposite. At the same time HIV appears to infect and replicate in Th2 clones more efficiently than Th1 clones. Combining all these findings suggest that many more Th1 cells would die by programmed cell death upon release of type II cytokines relative to the infection induced death of Th2 cells. This would lead to a progressive loss in the proportion of Th1 cells and a progressive enrichment of Th2 cells.[21]

However, it is very difficult to demonstrate a switch from a type I to type II state in many studies.[5-8] Short term cultures with polyclonal activators showed a decreased IL-4 and IL-10 production with unaffected IFN-γ production.[7] A study on patients with full-blown AIDS showed an increased production of IL-4 mRNA but it appeared to be associated with an increase of IFN-γ mRNA.[6] Other studies failed to show expression of IL-4 mRNA in lymph node cell suspensions obtained from patients. Taken together, these results show no evidence of a type I/type II switch associated with the disease progression in HIV-infected patients. Like other studies, we could not demonstrate an increased production of type II cytokines in HIV-infected patients even with very low CD4 counts.[16,17,20] The absence of polarized T cell responses may be due to the small sample size of this study that is reflected in the wide variation of these results. Other biological variations among the HIV-infected patients and types of opportunistic organisms, together with other immunological factors may be warranted for better defining the prognostic values for these patients. It is possible that the use of SEB may not be a suitable stimulus to

assess cytokine production in these patients. Specific antigens, such as HIV antigens, cytomegalovirus or even recalled antigens such as tetanus toxoids, or other antigens that are commonly found in the patients may provide a better comparison of type I and type II cytokine phenotypes.

In summary, we could not demonstrate a type I to type II cytokine shift in the course of HIV infection except an increased percentage of IFN-γ producing CD4+ and CD8+ T cells and a reduction of IL-2 production in HIV-infected patients.

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