Journal of Clinical Immunology, Vol. 25, No. 1, January 2005 (© 2005)
DOI: 10.1007/s10875-005-0358-0

Not All Tetramer Binding CD8$^+$ T Cells Can Produce Cytokines and Chemokines Involved in the Effector Functions of Virus-Specific CD8$^+$ T Lymphocytes in HIV-1 Infected Children

DANIEL SCOTT-ALGARA,[1] FLORENCE BUSEYNE,[2] FRANÇOISE PORROT,[2] BEATRICE CORRE,[2] NASSIMA BELLAL,[2,4] CHRISTINE ROUZIOUX,[3] STEPHANE BLANCHE,[4] and YVES RIVIERE[2,5]

Accepted: September 24, 2004

In the pediatric human immunodeficiency virus type-1 (HIV-1) infection, the presence of cytotoxic T lymphocytes (CTL) is associated with a slow progression to AIDS. The secretion of cytokines by CTLs may be critical in the control of viral infection. We used the combination of cell surface and intracellular staining to study the functionality of tetramer binding CD8$^+$ T cells recognizing two HIV-1 immunodominant epitopes, in peripheral blood mononuclear cells from HIV-1-infected children. A fraction of tetramer positive CD8$^+$ T cells produce cytokines (IFN-γ, TNF-α) or chemokines (CCL4, CCL5) after *ex vivo* stimulation with the cognate peptide. There was a negative correlation between the plasma viral load and the percentage of CD8$^+$ Tetramer Gag$^+$ T cells secreting IFN-γ. This is the first report in the context of pediatric HIV-1 infection showing that only a fraction of HIV-1-specific CD8$^+$ T cells have the capacity to produce cytokines and chemokines implicated in their antiviral functions.

KEY WORDS: Pediatric AIDS; T lymphocytes; chemokines; cytokines; tetramer.

INTRODUCTION

Infection with human immunodeficiency virus type-1 (HIV-1) stimulates strong antiviral CD8$^+$ T-cell responses directed to most of viral proteins (1). In adult patients with primary HIV-1 infection, an inverse correlation between virus load and HIV-1 specific CTL precursor frequencies has been reported (2, 3). Moreover, in HIV-1 infected adult patients, a significant association between high frequencies of circulating CD8 $^+$T cells stained with HLA-A*02 HIV-1 Gag and Pol tetramers and disease progression has been documented in the absence of antiretroviral treatment (4). In the presence of highly active antiretroviral therapy (HAART), the frequency of HIV-1-specific tetramer-binding CD8$^+$ T lymphocytes declines (5). In the pediatric HIV-1 infection, the presence of cytotoxic T lymphocytes (CTL) is associated with a lack of rapid progression to AIDS during the first year of life (6), and in HAART naive children aged >5 years, Gag and Pol-specific CTL responses were positively correlated with CD4$^+$ T lymphocytes and negatively correlated with plasma virus load (7). However, in spite of their high frequency, CTLs are unable to eradicate virus, and a selective reduction of HIV-1 specific CTL frequency has been observed in the advanced stages of HIV-1 infection (8–10). These findings could be due to virus replication along with its antigenic variability or to the functional inability of CD8$^+$ T cells to lyse the virus-infected target cells.

Several methods have been used to measure the viral-specific CD8$^+$ responses in human viral diseases. During the past three decades, many laboratories have studied virus-specific CTL activities mainly with a chromium release assay that measures the degree of lysis of target cells presenting viral antigens. Alternative techniques

[1]Unité de Biologie des Rétrovirus, Institut Pasteur, Paris, France.
[2]Unité postulante d'Immunopathologie Virale URA CNRS 1930, Institut Pasteur, Paris, France.
[3]Laboratoire de Virologie, EA 3620 Université René Descartes, CHU Necker-Enfants Malades, Paris, France.
[4]Fédération de Pédiatrie, CHU Necker-Enfants Malades, Paris, France.
[5]To whom correspondence should be addressed at Département de Médecine Moléculaire, Institut Pasteur, Immunopathologie Virale, Bâtiment Lwoff, 28 rue du Dr Roux, 75724 Paris Cedex 15, France; e-mail: riviere@pasteur.fr.

Abbreviations used: HIV-1, human immunodeficiency virus type-1; CTL, cytotoxic T lymphocytes; HAART, highly active antiretroviral therapy; ART, antiretroviral treatment; FITC, fluorescein-isothiocyanate; PCy5, phycoerythrin-cyanin; PE, phycoerythrin.

have been developed. These are based on the detection of soluble factors secreted by $CD8^+$ T lymphocytes after specific activation (Elispot assay, intracellular staining) or of surface expression of a specific T-cell receptor (tetramers). The use of these alternative techniques has provided a major advance in the study of the frequency of $CD8^+$ T cells specific to the viral proteins. Moreover, the results obtained with these new techniques have shown that the chromium release assay can underestimate the frequency of specific $CD8^+$ T cells during viral infections (11–14).

We and others have shown that chronically infected children had frequencies of tetramers binding cells (15), *ex vivo* IFN-γ producing HIV-1-specific $CD8^+$ T cells detected by Elispot (16) and memory CTL (8, 10, 17) similar to those of chronically infected adults. However, during the first month or year of infection, *ex vivo* IFN-γ production by HIV-1-specific $CD8^+$ T cells was significantly lower than that of older children or adults (18, 19). In a comparative study of HIV-1-specific $CD8^+$ responses in HIV-1-infected children, we found that the frequencies of epitope-specific cells measured by Elispot assay were lower than those measured with the tetramer assay, suggesting that only a fraction of HIV-1-specific $CD8^+$ T cells were able to produce IFNγ (16). The presence of nonfunctional HIV-1-specific $CD8^+$ T cells is not specific to the pediatric infection, as it was reported in adults infected by HIV-1 (20, 21). A combination of tetramer staining and detection of cytokine production using intracellular cytokine staining directly showed that not all tetramer-positive cells produced cytokine in response to stimulation with the epitope (20, 22–27). These differences in the number of specific $CD8^+$ T cells detected with the tetramer binding assay or with an IFN-γ secretion based assay could be accounted by different mechanisms. The tetramer binding cells that do not secrete IFN-γ may be the central memory $CD8^+$ T lymphocyte subsets previously described by Sallusto *et al*. (28) or they may be anergic as described by Lieberman *et al*. (29).

The cytolytic activity and the secretion of the various soluble factors by $CD8^+$ T cells in response to specific stimulation are critical for the control of virus infection. These two distinct antiviral functions of $CD8^+$ Tlymphocytes could be impaired by a defect in the production of soluble factors (20, 23, 27, 30). In addition to IFN-γ $CD8^+$ T cells secrete cytokines (IL-2 and TNF-α), β-chemokines [MIP1-α (CCL3), MIP1-β (CCL4), and RANTES (CCL5)], and other molecules called cytotoxins (perforin and granzymes). The β-chemokines (31) are potent HIV-suppressive factors produced by CD8+ T cells that block the entry of R5 HIV-1 strains into CD4+ cells by competing for binding to the chemokine receptors CCR5 and CCR3 (32–34). Moreover, it has been shown that CCL5 enhances HIV-1-specific lytic activity *in vitro* (35). The contribution of β-chemokines in protection to disease progression in pediatric AIDS has been reported in a recent study, where children with slow disease progression produced higher levels of β-chemokines than those detected in patients with AIDS (36).

The goal of the present work was to identify in HIV-1-infected children the relative impairment of cytokines (IFN-γ, TNF-α) and of the three β-chemokines secretion by PBMC upon specific stimulation with the cognate epitope. We used the combination of intracellular and cell surface tetramer stainings to study the capacity of HIV-1 specific $CD8^+$ T cells to secrete cytokines/chemokines in response to a specific stimulation. We show that in addition to a defect in IFN-γ production, a variable proportion of tetramer positive $CD8^+$ T cells are unable to produce TNF-α and chemokines (CCL4, CCL5) after *ex vivo* restimulation with the cognate peptide. There was a negative correlation between the plasma viral load and the percentage of Tetramer Gag^+ $CD8^+$ T cells secreting IFN-γ

PATIENTS AND METHODS

Patients

We studied children previously included in the longitudinal follow-up program of HIV-1-specific CD8 activities (17). These children were followed-up prospectively at the Hôpital Necker, Paris, France. Consent was obtained from parents or legal guardians and the institutional review ethical board approved the study. Seventeen HIV-1 infected children were selected because they were HLA-A*02 positive. Fifteen children were born to HIV-1 infected mothers. Two were infected by blood transfusion (EM50, EM67). The mean of age was 11.8 years (range 2.2–17.7). Table I shows the biological data, the CDC disease stage (37), and the antiretroviral treatment (ART) at the time when patients were tested. Mean values for the patients were: CD3: 81% (range 63–90%), CD4: 27% (range 11–52%), CD8: 50% (range 23–71%), and $\log_{10}$ HIV-1 RNA (copies/mL): 3.9 (range $<$1.7–5.2).

Tetrameric Peptide/MHC Complexes

The HLA-A*02 tetrameric complexes used in this study were obtained from Beckman-Coulter-Immunotech (Marseille, France). The MHC–peptide complexes were formed with two HIV-1 immunodominant epitopes, one in the p17 region of Gag (SLYNTVATL referred to as GAG)

Table I. Patient Characteristics

Patient	Age[a]	ART[b]	CD3[c]	CD4[c]	CD8[c]	Viral load[d]	CDC stage[e]	Gag[f]	Pol[f]
EM031	17	0	86	13	65	4.8	*N*	0.3	0.1
EM040	14.4	0	88	41	44	2.6	*N*	1.7	0.1
EM078	9.2	0	82	24	53	4.5	*B*	1.3	0.2
EM089	4.5	0	88	21	63	4.8	*N*	2.5	5.5
EM096	14.2	0	75	24	51	4.5	*A*	3.2	0
EM102	3.0	0	88	35	50	5.0	*C*	0.2	0.1
EM103	2.2	0	65	38	23	4.3	*N*	2.4	0
EM045	9.9	1	83	19	56	4.2	*N*	3.9	0.45
EM067	16.1	1	90	52	36	2.7	*N*	1.4	0
EM086	14.4	1	89	42	43	4.1	*A*	2.1	1.1
EM018	12.9	2	71	30	39	<1.7	*B*	0.3	0.1
EM020	16.7	2	84	25	56	3.4	*C*	0.7	0
EM030	13.3	2	84	11	67	3.5	*A*	6.3	0.3
EM050	17.7	2	87	14	71	5.2	*B*	0.9	0.1
EM063	8.2	2	82	26	52	5.0	*C*	0.4	1.4
EM023	13.7	3	63	19	40	3.9	*B*	0.3	1.3
EM026	13.4	3	75	28	42	2.4	*A*	0.9	1

[a] Age in years.
[b] ART Group 0: no treatment; 1: two RT inhibitors; 2: tritherapy with a nonnucleoside RT inhibitor; 3: tritherapy with an HIV protease inhibitor.
[c] Percentage of total lymphocyte.
[d] $\log_{10}$ HIV–RNA copies/mL of plasma.
[e] Stage of HIV disease as defined by the Centers for Diseases Control and Prevention (37).
[f] Percentage of tetramer positive CD8$^+$ T cell among total CD8$^+$ T lymphocytes.

and the other in reverse transcriptase (ILKEPVHGV referred to as POL).

Flow Cytometry Analyses

Absolute CD4 and CD8 counts were performed on fresh whole blood samples by four-color flow cytometry analysis of cells positive for CD45, CD3, CD4, or CD8, with fluorescent beads as an internal standard (flow-count beads, Beckman-Coulter, Paris, France). CD8$^+$ T cell populations were studied in the same whole blood samples by four-color flow cytometry analysis and standard lysis procedure of red blood cells as described (15). After staining, cells were washed in PBS once, adjusted to 500 μL in PBS, and stored at 4°C until analysis. The following associations were used: 1—CD45-FITC, CD3-PCy5, CD4-RD1, CD8-ECD; 2—CD4-ECD, CD8-PCy5, Tetra-PE, CD16-FITC. Labeled cells were analyzed on EPICS-XL MCL flow cytometer (Coulter, Paris, France). Events accumulation was followed up until to reach 50,000 living cells on the lymphocyte gate that was set up using both forward and right angle scatters. All monoclonal antibodies were purchased from Beckman-Coulter-Immunotech (Paris, France).

Flow Cytometric Cytokine Detection Assays

PBMC from each patient were isolated by density centrifugation, washed 3 times in PBS and then incubated overnight at 37°C with 5% CO_2 in RPMI 1640 culture medium supplemented with 10% heat-inactivated fetal calf serum with or without the Gag or Pol peptides (0.5 μg/mL). After 2 h Brefeldin A (2–10 μg/mL, Sigma, St Louis, MI) was added to the culture. Cells were recovered after 6 h or overnight incubation, and aliquotes were washed and incubated for 30 min at 25°C with phycoerythrin-Texas Red (ECD)-, or phycoerythrin-cyanin (PCy5)-conjugated monoclonal antibodies (CD8, CD3) or phycoerythrin (PE) conjugated tetramers (Beckman-Coulter). At the end of incubation, cells were washed twice in PBS and fixed with 200 μL of 2% formaldehyde solution for 15 min at 25°C. Cells were washed twice and treated for 10 min at 25°C with 150 μL of permeabilization solution (0.5% of saponin, Sigma St Louis, MI, in PBS solution with 5% fetal calf serum). After incubation, PBMC were centrifuged for 5 min and the supernatants poured out. Fifty μL of fluorescein-isothiocyanate (FITC) conjugated anti-cytokine/chemokine monoclonal antibodies (IFN-γ, TNF-α, CCL3, CCL4, or CCL5 from Becton-Dickinson, Paris, France) diluted in permeabilization buffer were added and cells incubated for 30 min at 25°C. Cells were washed twice in PBS and 500 μL of PBS were added before analysis. Positive controls for cytokine production were PBMC stimulated with PMA/Ionomycin (Sigma, St Louis, MI), at 10 and 500 ng/mL, respectively, and incubated with 2 μg/mL of Brefeldin A overnight. Negative controls were incubated with irrelevant, isotype-matched antibodies in all experiments and with a background

fluorescence below 0.03%. Background secretion of different cytokines was determined with medium alone. Cell events were acquired by using gates set by forward and side scatter to determine the different proportions of positive cells.

Purification of Tetramer Binding Cells

Tetramer binding cells were purified after specific peptide stimulation and Brefeldin overnight incubation as described above using anti-PE magnetic beads from Miltenyi Biotech (Paris, France). Briefly, PBMC were incubated with the Gag tetramer. After incubation, cells were washed twice in PBS and incubated with anti-PE magnetic beads reagent for 10 min at 4°C. After washing, PBMC were separated by magnet (MiniMACS) using MS column (Miltenyi Biotech, Paris, France). The degree of purification was monitored by flow cytometry.

Plasma HIV-1 RNA Monitoring

Amplicor HIV-1 Monitor test (Roche, Neuilly, France) was used to quantitate HIV-1 RNA in plasma. This technique is based on reverse transcription of the RNA corresponding to the HIV-1 *gag* gene, followed by the amplification of the cDNA by PCR in the presence of a quantitation standard (38). The cutoff value was 2.6 log copies/mL for the classical assay and 1.7 log copies/mL for the ultra sensitive assay.

HLA Class I Typing

HLA genotyping was performed at the Chelsea and Westminster Hospital, London, UK, or at the Necker Hospital, Paris, France, using amplification refractory mutation system polymerase chain reaction (39).

Statistical Analysis

The biological parameters, disease stage and treatment of the patients were analyzed by the two-tailed t-test and the Kruskal–Wallis test. The correlations between the frequency of tetramer-binding cells, plasma viral load and percentage of $CD3^+$, $CD4^+$, and $CD8^+$ T cells were determined using the Spearman rank test.

RESULTS

The Secretion of IFN-γ is only Induced After Stimulation with the Cognate Peptide

In order to validate our stimulation protocol, different concentrations of Brefeldin A and different periods of incubation were used after stimulation of PBMC with specific peptides. Results are expressed as percentage of IFN-γ negative or positive tetramer binding cells among $CD3^+CD8^+$ T cells. Incubation of PBMC for 6 or 18 h in the presence of 2 or 10 μg/mL of Brefeldin A induced a similar proportion of IFN-γ secretion by Pol- and Gag-specific $CD3^+$ $CD8^+$ tetramer binding cells (Fig. 1). Overnight incubation led to a reduction of the percentage of interferon-γ negative cells, in particular for the Gag peptide. Thus, for the following experiments we used overnight incubation with 2 μg/mL of Brefeldin A. Otherwise, the percentages of tetramer binding $CD8^+$ $CD3^+$ T cells detected after overnight stimulation of PBMC was similar to those obtained after whole blood staining.

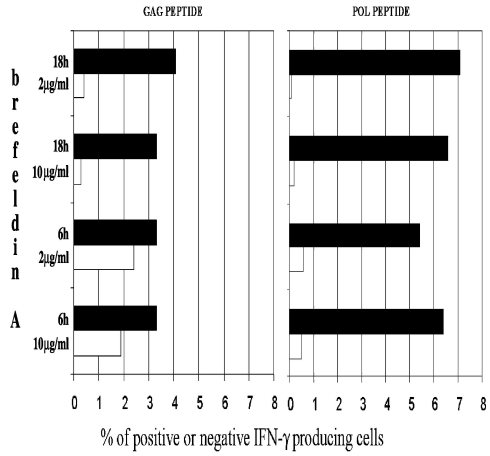

Fig. 1. Kinetics of IFN-γ production by $CD8^+$ T cells. Staining with either Gag (left) or Pol (right) tetramer was evaluated 6 or 18 h after specific peptide stimulation in the presence of different concentration of Brefeldin A (2 and 10 μg/mL). Results are expressed as percentage of IFN-γ positive (closed bars) or negative (open bars) tetramer binding cells among $CD3^+CD8^+$ T cells.

The next step was to investigate the specificity of IFN-γ production by peptide stimulation. Patients that had circulating $CD3^+CD8^+$ T cells specific for both tetramer (Gag and Pol) were studied. A representative result is presented in Fig. 2. PBMC were stimulated *in vitro* with Gag (Fig. 2A) or Pol peptide (Fig. 2B). As shown in Fig. 2A, the stimulation with the specific Gag peptide induced IFN-γ production by T cells binding tetramer folded with the same Gag peptide (63.1%) but not by the Pol specific tetramer binding cells (1.63%). Reciprocal results were obtained with Pol stimulation: the stimulation with the Pol peptide induced IFN-γ production by T cells binding tetramer folded with the same Pol peptide (50.6%) but not by the Gag specific tetramer binding cells (2.63%), (Fig. 2B). Thus, the secretion of IFN-γ by tetramer positive $CD8^+$ T cells was only induced by the cognate peptide.

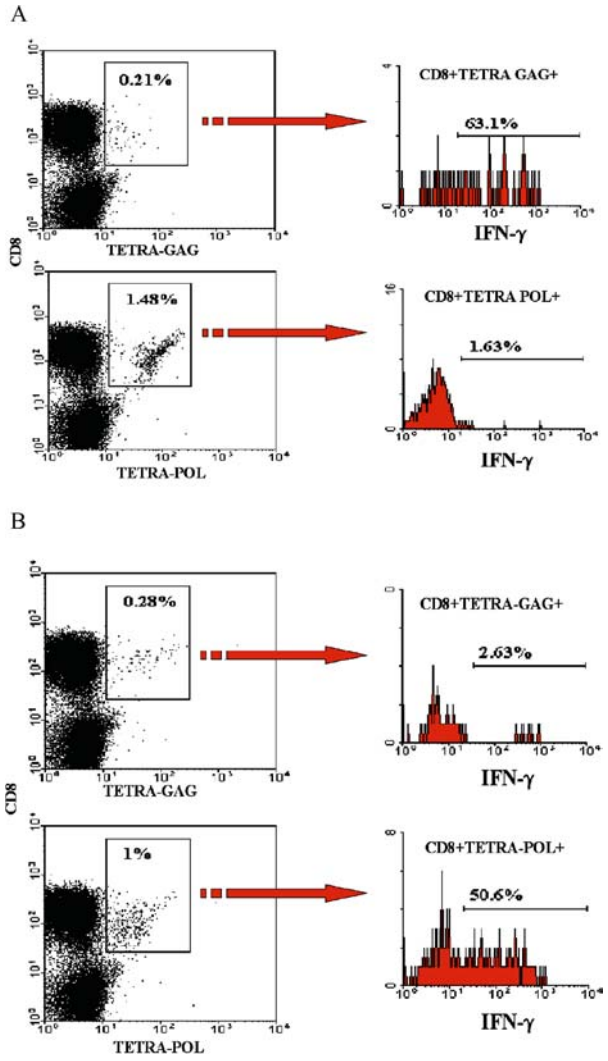

Fig. 2. Specific production of IFN-γ after peptide stimulation. IFN-γ production by CD3$^+$CD8$^+$ T cells after Gag peptide (A) or Pol peptide (B) stimulation was analyzed by cytometry. The percentage of CD8$^+$ T cells stained by Gag or Pol tetramers after overnight incubation is shown on the left panels (Gates). On the right panels, the percentage of tetramer Gag$^+$ (*Top*) or Pol$^+$ (*Bottom*) CD8$^+$ IFN-γ producing cells among tetramer positive CD8$^+$ cells are represented.

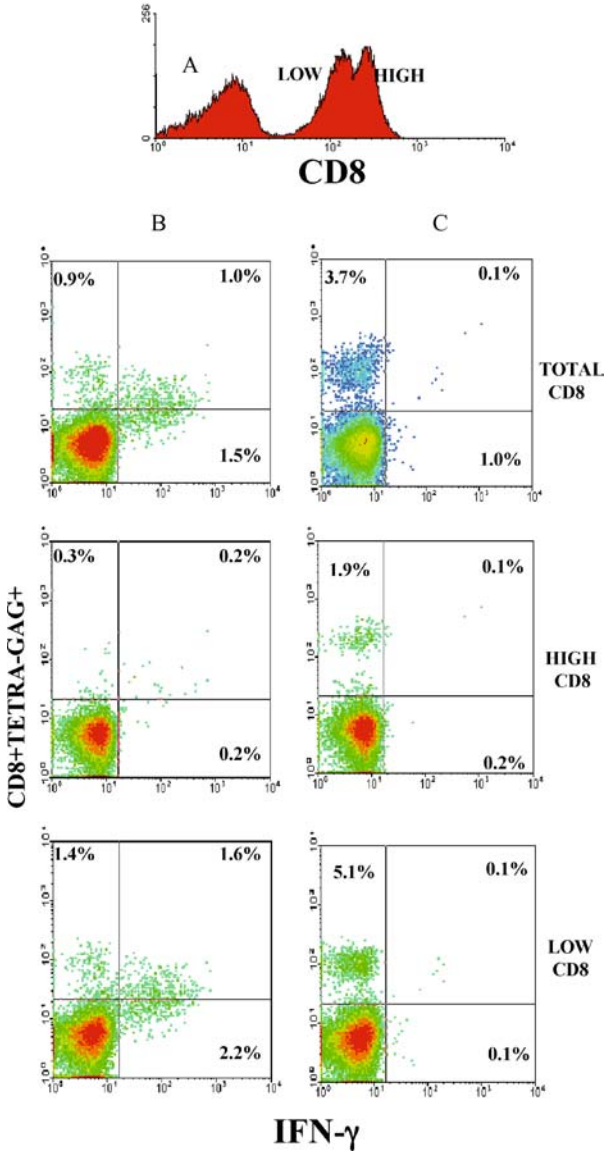

Fig. 3. Analysis of IFN-γ production among CD8$^+$ T cells. The diagram of CD8 expression after specific peptide stimulation is shown on the top panel A. CD8$^+$ populations were gated by the expression of CD8 marker: total CD8$^+$, CD8^{+high} or CD8^{+low} T cells. The results are expressed as percentage among gated population after stimulation with the cognate peptide (panel B) or a control peptide (panel C).

IFN-γ is Secreted by a Fraction but not all Tetramer Binding Cells

As shown in Fig. 2, a fraction of tetramer binding cells did not secrete IFN-γ after specific peptide stimulation. In order to better understand the significance of IFN-γ negative tetramer-positive T cells, we analyzed the production of IFN-γ according to the level of CD8 expression after peptide stimulation of fresh PBMC (Fig. 3A). The production of IFN-γ was observed only upon stimulation with the cognate peptide, mainly in tetramer binding cells that downregulated CD8 molecule (Fig. 3B). Moreover, we observed a proportion of tetramer positive CD8^{+low} low T cells that were unable to produce IFN-γ (Fig. 3B). The stimulation with a control peptide did not induced IFN-γ production by CD8$^+$ T cells regardless of CD8 intensity (Fig. 3C).

In order to confirm previous results obtained with whole PBMC, Gag-specific tetramer positive cells (1.8%

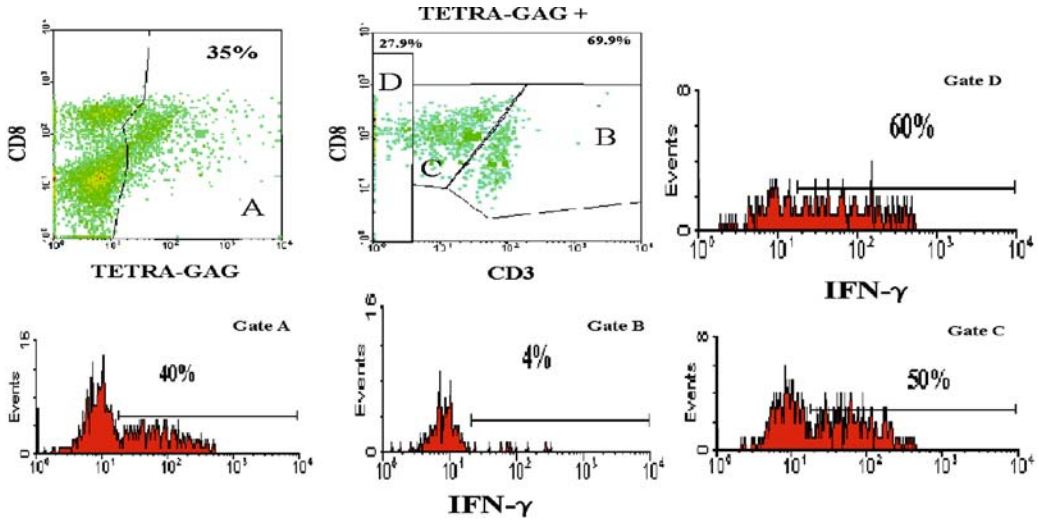

Fig. 4. Analysis of IFN-γ production among enriched tetramer Gag$^+$CD3$^+$CD8$^+$ T cells. Dot-blots show the percentage of CD8$^+$ tetramer Gag$^+$ cells on the top left panel and the level of CD3 expression among tetramer Gag$^+$CD8$^+$on the top middle panel. The histograms represent the percentage of IFN-γ positive producing cells among total tetramer Gag$^+$CD8$^+$ (gate A), CD3^{high} tetramer Gag$^+$CD8$^+$ (gate B), CD3^{low} tetramer Gag$^+$CD8$^+$ (gate C) or CD3^{negative} tetramer Gag$^+$CD8$^+$ cells (gate D). The percentages of cells in B, C, and D are 24.2, 42.5, and 27.3% respectively.

of CD8$^+$ T cells) were enriched after *in vitro* culture with Gag peptide as described in the methods section (35% of the cells were tetramer positive after culture and magnetic beads separation, Fig. 4 top left panel). Thereafter, Gag-specific tetramer positive cells were analyzed for the surface expression of CD8, CD3 molecules, and for IFN-γ production. As shown in Fig. 4, among the 35% of Gag-specific tetramer positive cells, only 40% were producing IFN-γ Gate A, bottom left panel). When Gag-specific tetramer positive cells were analyzed for the expression of CD3 marker, three different levels of fluorescence were detected: high (B), low (C), and negative (D) (Fig. 4, top middle panel). All of these cells expressed the CD8 marker at high or low levels (*Y* axis). The production of IFN-γ was analyzed according to the expression of CD3; IFN-γ positive cells were observed only in the CD3^{+low} or negative population (Gates C and D) but not in the CD3^{+high} population (Gate B).

The results obtained in the whole cohort (mean, standard error, and confidence interval, CI 95) and expressed as percentage of Tetramer$^+$ CD8$^+$ cells (Gag or Pol) producing IFN-γ after stimulation by specific peptides are summarized in Fig. 5. About 45% of Tetramer-Gag$^+$ CD8$^+$ and 50% of the Tetra-Pol$^+$CD8$^+$ cells were unable to produce IFN-γ and significant variations were observed between the patients. The inability to produce IFN-γ by CD8$^+$ T cells was HIV-1(antigen)-specific because most (>95%) Gag-tetramer binding CD3$^+$ CD8$^+$ T cells were able to produce interferon-γ after PMA stimulation (Fig. 5).

Then, we addressed whether the frequencies of Gag-specific tetramer positive cells secreting IFN-γ were associated with the parameters of HIV infection. There was a negative correlation between the viral load (plasma

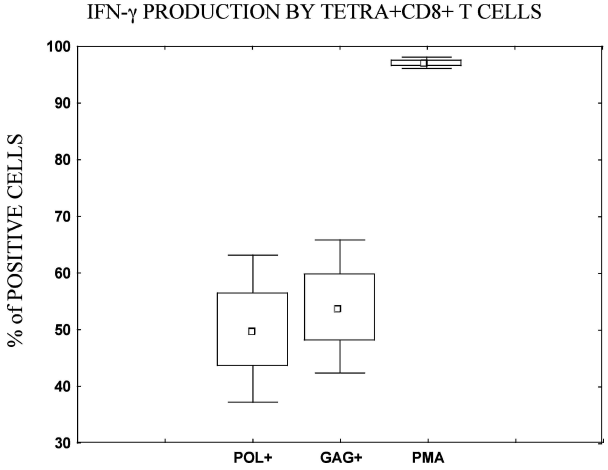

Fig. 5. IFN-γ production by tetramer Gag$^+$ or Pol$^+$CD8$^+$ cells. IFN-γ production after specific peptide stimulation was studied in 17 HIV-1 infected patients. Results are expressed as percentage (mean; +/– SE; +/– Confidence Interval, 0.95) of positive IFN-γ stained cells among tetramer positive CD8$^+$ cells. IFN-γ production by tetramer Gag$^+$ CD8$^+$ cells after PMA stimulation is shown as a positive control (PMA).

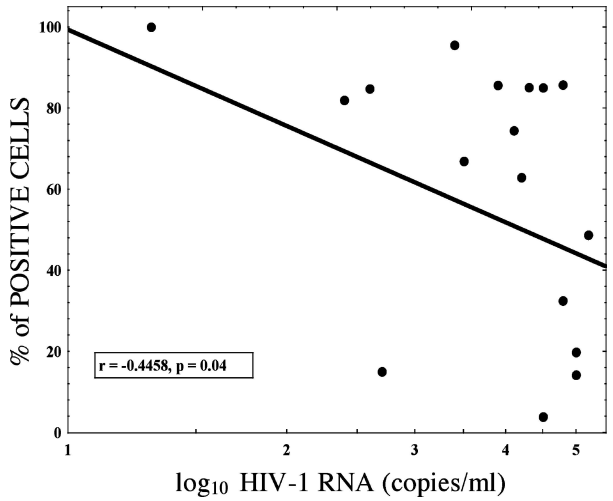

Fig. 6. Correlation between viral load and the percentage of tetramer Gag$^+$ CD3$^+$CD8$^+$ cells secreting IFN-γ. The percentage of IFN-γ producing cells among tetramer Gag$^+$CD3$^+$CD8$^+$ cells is presented as a function of plasma viremia expressed as $\log_{10}$ HIV–RNA copies/mL.

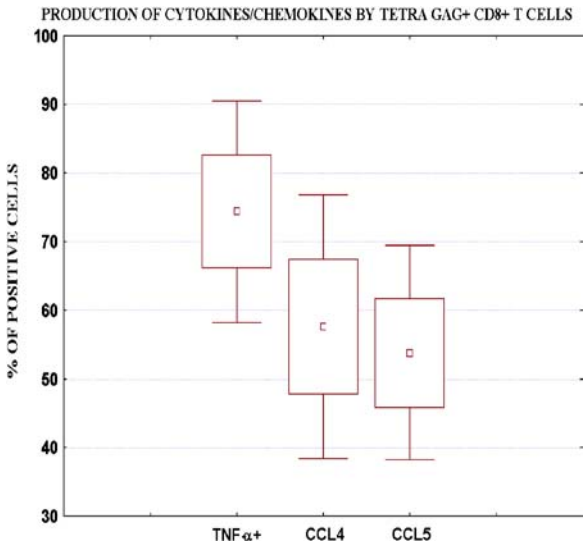

Fig. 7. TNF-α, CCL4, and CCL5 production among tetramer Gag$^+$CD8$^+$cells. TNF-α, CCL4, and CCL5 production after specific Gag peptide stimulation was studied in 17 HIV-1 infected patients. Results are expressed as percentage (mean; +/− SE; +/− Confidence Interval, 0.95) of positive cytokine stained cells among tetramer Gag$^+$CD8$^+$ cells.

HIV-1 RNA) and the percentage of CD8$^+$ Tetramer Gag$^+$ secreting IFN-γ (Spearman rank test, $r = 0.4458$, $p = 0.04$, Fig. 6). No significant difference was found between the percentage of tetramer positive cells secreting IFN-γ detected in children according to antiretroviral treatment (two-tailed t-test) or disease stages (Kruskal–Wallis test). We observed no correlation between the frequency of Gag-tetramer positive cells secreting IFN-γ and CD3$^+$, CD4$^+$, or CD8$^+$T cell number or percentage (Spearman rank test).

Overall, these results showed that the nonresponding cells were found in the CD3^{+high}, CD8^{+high} population after stimulation, indicating that they were not able to downregulate these surface markers. In contrast, all IFN-γ producing cells were found to downregulate CD3 and /or CD8 molecules.

A Proportion of Gag-Tetramer Positive CD8$^+$ T Cells are also Unable to Produce TNF-α, CCL4, and CCL5 after Specific Peptide Stimulation

Tetramer positive T cells that did not produce IFN-γ may secrete other cytokines after specific peptide stimulation. Thus, we investigated whether the incapacity to secrete IFN-γ by tetramer positive T cells was only restricted to this cytokine or could be extended to other lymphokines. In order to answer this question, we studied the secretion of TNF-α, CCL3, CCL4, and CCL5 after specific peptide stimulation. Results from the whole cohort are presented in Fig. 7. As previously shown for IFN-γ production, a fraction of Gag tetramer positive CD8$^+$ T cells were unable to secrete TNF-α, CCL4, or CCL5 (26, 42, and 46% respectively). Concerning CCL3, we never observed its production after specific stimulation of Gag tetramer-positive T cells. The percentages of Gag tetramer-positive CD8$^+$ T cells secreting individual cytokine/chemokine were not correlated with each other. There was no correlation between the viral load and the percentage of Gag-tetramer positive CD8$^+$ T cells secreting TNF-α, CCL4, or CCL5 (Spearman rank test). No correlation was found between the percentage of CD3$^+$, CD4$^+$, or CD8$^+$ T lymphocytes and the frequency of tetramer positive cells secreting cytokines except for CCL5. For this chemokine, a positive correlation was found between the frequency of Gag tetramer-positive cells secreting CCL5, and CD3$^+$ or CD8$^+$ T cell percentages (Spearman rank test, $p < 0.05$). These results showed that the inability of the tetramer positive CD8$^+$ T cells to secrete IFN-γ is also observed for TNF-α CCL4, and CCL5.

DISCUSSION

Despite the increasing evidence about the role of CD8$^+$ T cells in the suppression of HIV-1 viral replication and in the control of disease progression, dysfunction of cytotoxic T cells seems to be important during the evolution of HIV-1 infection. In this study, we investigate the

functionality of tetramer binding T cells by combining surface staining and intracellular cytokine detection after specific stimulation with the cognate peptide. Our results show that in HIV-1-infected children, a fraction of tetramer binding T cells are unable to secrete TNF-α, IFN-γ, CCL4, and CCL5. Another consistent finding in our study is the downregulation of CD8 and CD3 molecules by activated $CD8^+$ T cells, as attested by the production of IFN-γ found in T cells that have downregulated the two molecules after specific stimulation.

The data presented herein provide evidence for defective functional capacity in regards to cytokine/chemokine secretion of circulating virus-specific tetramer binding $CD8^+$ cells in HIV-1-infected children. Our observations are consistent with those reported in adult patients that progress to AIDS with functionally defective HIV-1-specific $CD8^+$T cells (20, 21, 40). In other human viral persistent infection, impaired effector functions of virus-specific $CD8^+$ T cells has been documented (41). However a functional heterogeneity of cytokine production has been reported in PBMC from CMV seropositive healthy individuals (42, 43). Thus, the detection of tetramer positive/cytokine negative cells may simply reflect the heterogeneity of possibly normal virus-specific $CD8^+$ T cells.

The levels of HIV-1-specific CTL activities are dependent on the presence of virus-specific helper cell functions (44). In pediatric AIDS, association between HIV-1-specific T helper responses and CTL activities has been reported (36, 45). In this cohort of infected children, CD4 help of CD8 response should be still present because all patients produced at different levels all the cytokines/chemokines that were tested. As shown in Table I, children in our study were more than 2-year-old. Most of them (10 out of 17) had more than 25% CD4 while a minority (2 out of 17) presented a severe immunosuppression (<15% CD4). The CD4 percentage ranged from 12 to 52% (mean 27%), and neither CD4 percentage nor the absolute CD4 counts were correlated to the fraction of tetramer binding $CD8^+$ T cells that were able to produce cytokines/chemokines. We cannot exclude that our results are biased by the number and/or by the selection of these HLA-A2 patients. All the children studied were selected for the presence of tetramer binding $CD8^+$ T cells in blood. Therefore, this study was restricted to two HIV-1 HLA-A2*01 epitopes. Thus, additional studies are required to show the relationship between the dysfunction of tetramer binding $CD8^+$ T cells and the presence of a specific $CD4^+$ T cell response in HIV-1-infected infants.

The majority of children were under ART at the time of testing. There was no significant difference between the percentages of cytokine secreting tetramer positive $CD8^+$ T cells of ART- or untreated patients. No significant difference was found between the frequency of Gag-tetramer positive cells secreting IFN-γ and disease stages. In HIV-1 infected patients the percentage of $CD8^+$ T lymphocytes is increased with a high level of T-cell activation markers (5). We observed no correlation between the frequency of Gag-tetramer positive $CD8^+$ T cells secreting cytokines and the percentage or absolute value of $CD8^+$T cell, except for CCL5. Association between markers of disease progression and increased frequency of CCL5 positive $CD8^+$ T cells has been reported in HIV-infected female sex workers (46). However, although beta chemokines can block virus-entry into target cells (32–34), their correlation with HIV-1 disease progression is still controversial.

The proportions of tetramer binding $CD8^+$ T cells that produce IFN-γ, CCL4, and CCL5 were very similar. This result suggests that T cells, which secreted IFN-γ, also produced CCL4 and CCL5. In a study of HIV-1-infected adult patients, all tetramer-positive cells that secreted CCL4 were shown to secrete IFNγ and vice versa (20), and recently CCL4 and IFN-γ have been shown to be synthetized congruently in most $CD8^+$ T cells (47). The β-chemokines CCL3, CCL4, and CCL5 were first identified as the major HIV-suppressive factors produced by $CD8^+$ T cells (32), and it as been shown that HIV-1-specific CTL clones release significant amounts of these three β-chemokines complexed to proteoglycans following epitope-specific stimulation (48). In this study, we did not detect the production of CCL3 after specific stimulation of tetramer positive $CD8^+$ T cells. This latter might be due to the threshold of the detection of the cytometric analysis and/or to a poor efficiency of CCL3-specific antibodies for intracellular studies as reported by others (20).

The proportions of tetramer binding $CD8^+$ T cells stained with TNF-α were higher compared to other soluble factors tested. This finding was not reported in HIV-1-infected adult patients (20). Functional heterogeneity of specific $CD8^+$ T cells has been described (42). Cytokine production by virus-specific $CD8^+$ T cells can segregate into different subsets. TNF-α single or IFN-γ single expressing cells were predominant in CMV infected population whereas double positive cells were uncommon. Thus, the $CD8^+$ T cell repertoire could be committed to have preferential effector mechanisms according to infectious pathogen as well as the site of infection and clinical parameters (42). Altogether, the results concerning cytokine secretion suggested that different functional HIV-1-specific $CD8^+$ T populations could co-exist in HIV-1 infected children.

Secretion of IFN-γ has an important role in the effector functions of cytotoxic T cells and in the control of viral infections. The production of IFN-γ has been correlated to

cytotoxic function (32). Moreover, the secretion of IFN-γ is an important factor in the control of HIV-1 replication (49, 50). In this group of 17 children, the viral load was detectable for most of them (log mean value: 3.9, range <1.7–5.2) (Table I). There was a negative correlation between the viral load and the percentage of CD8$^+$ Tetramer Gag$^+$ secreting IFN-γ Spearman rank test, $p = 0.04$). These results are in agreement with data reported by Kostense *et al*. showing a negative association between the proportion of tetramer positive T cells that produced IFN-γ and the viral load, although in their work the correlation was not statistically significant (27). Our observation is also consistent with a recent report by Oxenius *et al*. (51) showing the proportion of IFN-γ negative HIV-1-specific CD8$^+$ T lymphocytes detected by tetramer staining increased in relation to the level of virus rebound during therapeutic intervention. The HIV-1-specific CD8$^+$ T cells that are unable to secrete IFN-γ may have a defect in specific TCR stimulation as most tetramer positive cells (>95%) were able to produce IFN-γ after polyclonal stimulation with PMA and Ionomycin. We (16) and others (52) have reported a positive correlation between the frequency of HIV-specific IFN-γ producing CD8$^+$ T cells measured by IFN-γ Elispot assays and plasma viral load, suggesting that the magnitude of *ex vivo* activated HIV-specific CD8$^+$ T cell mediated IFN-γ production is dependent upon continuous restimulation with viral load. Thus increased viral replication appears to exert two antagonistic effects on the CD8$^+$ T cell frequency: an enhancing effect probably reflecting enhanced antigen dose and a direct or indirect negative effect on the functional status of HIV-specific CD8$^+$ T cells. These two opposite effects may explain why some investigators did not found correlation between the frequency of *ex vivo* IFN-γ producing HIV-specific T cells and plasma viral load (22, 53).

The impaired cytokine/chemokine secretion might be explained by a skewed maturation of tetramer binding CD8$^+$ T cells as it has been suggested in HIV-1 infected adult patients (20). Moreover, an impaired development and inhibition of T-cell differentiation in HIV-1 infected infants has been described (36). We have previously reported that HIV-1-specific CD8$^+$ T cells detected in peripheral lymphocytes from whole blood of HIV-1-infected children using HLA tetramers combined to HIV-1 peptides express a phenotype of mature memory activated CD8$^+$ T cells (CD28$^-$, CD45RA$^-$,CD45R0$^+$, CD69$^-$, and HLA-DR$^+$) (15). The HIV-1-specific CD8$^+$ T cells that do not produce cytokines upon specific stimulation in our study may also have impaired cytolytic activity. It is likely that a defective production of cytokines/chemokines by a fraction of tetramer binding cells could render them less effective to kill HIV-1 infected cells.

In conclusion, the results presented herein show that a proportion of tetramer binding CD8$^+$ T cells detected in the circulating PBMC of infected infants are able to produce cytokines (IFN-γ, TNF-α) or chemokines (CCL4, CCL5) involved in the effector function of cytotoxic T cells and in the suppression of HIV-1 replication. However, a fraction of tetramer positive CD8$^+$ T cells is unable to produce these cytokines/chemokines after specific peptide stimulation. Further studies are necessary to determine the clinical significance of this fraction of tetramer binding CD8$^+$ T cells with impaired secretion of cytokines/chemokines. This defect in lymphokine production may minimize the cytolytic and the antiviral ability of the HIV-1-specific CD8 responses. These results could have implications in the design of novel therapeutic approaches in HIV-1-infected infants.

ACKNOWLEDGMENTS

We thank A. Hovanessian and C. Retière for discussions. This work was supported by grants from Agence Nationale de la Recherche sur le SIDA, Aventis Pasteur, Sidaction, Institut Pasteur, and the Elisabeth Glaser Pediatric AIDS foundation.

REFERENCES

1. Chouquet C, Autran B, Gomard E, Bouley JM, Calvez V, Katlama C, *et al*.: Correlation between breadth of memory HIV-specific cytotoxic T cells, viral load and disease progression in HIV infection. AIDS 16:2399–2407, 2002
2. Borrow P, Lewicki H, Hahn BH, Shaw GM, Oldstone MB: Virus-specific CD8$^+$ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. J Virol 68:6103–6110, 1994
3. Koup RA, Safrit JT, Cao Y, Andrews CA, McLeod G, Borkowsky W, *et al*.: Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. J Virol 68:4650–4655, 1994
4. Ogg GS, Kostense S, Klein MR, Jurriaans S, Hamann D, McMichael AJ, *et al*.: Longitudinal phenotypic analysis of human immunodeficiency virus type 1- specific cytotoxic T lymphocytes: Correlation with disease progression. J Virol 73:9153–9160, 1999
5. McMichael AJ, Rowland-Jones SL: Cellular immune responses to HIV. Nature 410:980–987, 2001
6. Buseyne F, Burgard M, Teglas JP, Bui E, Rouzioux C, Mayaux MJ, *et al*.: Early HIV-specific cytotoxic T lymphocytes and disease progression in children born to HIV-infected mothers. AIDS Res Hum Retroviruses 14:1435–1444, 1998
7. Buseyne F, Le Chenadec J, Corre B, Porrot F, Burgard M, Rouzioux C, *et al*.: Inverse correlation between memory gag-specific cytotoxic T lymphocytes and viral replication in human immunodeficiency virus-infected children. J Infect Dis 186:1589–1596, 2002

8. Carmichael A, Jin X, Sissons P, Borysiewicz L: Quantitative analysis of the human immunodeficiency virus type 1 (HIV-1)-specific cytotoxic T lymphocyte (CTL) response at different stages of HIV-1 infection: Differential CTL responses to HIV-1 and Epstein-Barr virus in late disease. J Exp Med 177:249–256, 1993
9. Greenough TC, Brettler DB, Somasundaran M, Panicali DL, Sullivan JL: Human immunodeficiency virus type 1-specific cytotoxic T lymphocytes (CTL), virus load, and CD4 T cell loss: Evidence supporting a protective role for CTL *in vivo*. J Infect Dis 176:118–125, 1997
10. Klein MR, van Baalen CA, Holwerda AM, Kerkhof Garde SR, Bende RJ, Keet IP, *et al*.: Kinetics of Gag-specific cytotoxic T lymphocyte responses during the clinical course of HIV-1 infection: A longitudinal analysis of rapid progressors and long-term asymptomatics. J Exp Med 181:1365–1372, 1995
11. Herr W, Protzer U, Lohse AW, Gerken G, Meyer zum Buschenfelde KH, Wolfel T: Quantification of $CD8^+$ T lymphocytes responsive to human immunodeficiency virus (HIV) peptide antigens in HIV-infected patients and seronegative persons at high risk for recent HIV exposure. J Infect Dis 178:260–265, 1998
12. Larsson M, Jin X, Ramratnam B, Ogg GS, Engelmayer J, Demoitie MA, *et al*.: A recombinant vaccinia virus based ELISPOT assay detects high frequencies of Pol-specific CD8 T cells in HIV-1-positive individuals. AIDS 13:767–777, 1999
13. Rininsland FH, Helms T, Asaad RJ, Boehm BO, Tary-Lehmann: Granzyme B ELISPOT assay for *ex vivo* measurements of T cell immunity. J Immunol Methods 240:143–155, 2000
14. Wilson JD, Ogg GS, Allen RL, Davis C, Shaunak S, Downie J, *et al*.: Direct visualization of HIV-1-specific cytotoxic T lymphocytes during primary infection. Aids 14:225–233, 2000
15. Scott-Algara D, Buseyne F, Blanche S, Rouzioux C, Jouanne C, Romagne F, *et al*.: Frequency and phenotyping of human immunodeficiency virus (HIV)-specific CD8+ T cells in HIV-infected children, using major histocompatibility complex class I peptide tetramers. J Infect Dis 183:1565–1573, 2001
16. Buseyne F, Scott-Algara D, Porrot F, Corre B, Bellal N, Burgard M, *et al*.: Frequencies of *ex vivo*-activated human immunodeficiency virus type 1-specific gamma-interferon-producing CD8(+) T cells in infected children correlate positively with plasma viral load. J Virol 76:12414–12422, 2002
17. Buseyne F, Blanche S, Schmitt D, Griscelli C, Riviere Y: Detection of HIV-specific cell-mediated cytotoxicity in the peripheral blood from infected children. J Immunol 150:3569–3581, 1993
18. Scott ZA, Chadwick EG, Gibson LL, Catalina MD, McManus MM, Yogev R, *et al*.: Infrequent detection of HIV-1-specific, but not cytomegalovirus-specific, CD8(+) T cell responses in young HIV-1-infected infants. J Immunol 167:7134–7140, 2001
19. Spiegel HM, Chandwani R, Sheehy ME, Dobroszycki J, Fennelly G, Wiznia A, *et al*.: The impact of early initiation of highly active antiretroviral therapy on the human immunodeficiency virus type 1-specific CD8 T cell response in children. J Infect Dis 182:88–95, 2000
20. Appay V, Nixon DF, Donahoe SM, Gillespie GM, Dong T, King A, *et al*.: HIV-specific CD8(+) T cells produce antiviral cytokines but are impaired in cytolytic function. J Exp Med 192:63–75, 2000
21. Goepfert PA, Bansal A, Edwards BH, Ritter GD Jr, Tellez I, McPherson SA, *et al*.: A significant number of human immunodeficiency virus epitope-specific cytotoxic T lymphocytes detected by tetramer binding do not produce gamma interferon. J Virol 74:10249–10255, 2000
22. Gea-Banacloche JC, Migueles SA, Martino L, Shupert WL, McNeil AC, Sabbaghian MS, *et al*.: Maintenance of large numbers of virus-specific $CD8^+$ T cells in HIV-infected progressors and long-term nonprogressors. J Immunol 165:1082–1092, 2000
23. Goulder PJ, Tang Y, Brander C, Betts MR, Altfeld M, Annamalai K, *et al*.: Functionally inert HIV-specific cytotoxic T lymphocytes do not play a major role in chronically infected adults and children. J Exp Med 192:1819–1832, 2000
24. Hel Z, Nacsa J, Kelsall B, Tsai WP, Letvin N, Parks RW, *et al*.: Impairment of Gag-specific CD8(+) T-cell function in mucosal and systemic compartments of simian immunodeficiency virus mac251- and simian-human immunodeficiency virus KU2-infected macaques. J Virol 75:11483–11495, 2001
25. Kern F, Khatamzas E, Surel I, Frommel C, Reinke P, Waldrop SL, *et al*.: Distribution of human CMV-specific memory T cells among the CD8pos subsets defined by CD57, CD27, and CD45 isoforms. Eur J Immunol 29:2908–2915, 1999
26. Shankar P, Russo M, Harnisch B, Patterson M, Skolnik P, Lieberman J: Impaired function of circulating HIV-specific CD8(+) T cells in chronic human immunodeficiency virus infection. Blood 96:3094–3101, 2000
27. Kostense S, Ogg GS, Manting EH, Gillespie G, Joling J, Vandenberghe K, *et al*.: High viral burden in the presence of major HIV-specific CD8(+) T cell expansions: Evidence for impaired CTL effector function. Eur J Immunol 31:677–686, 2001
28. Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A: Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature 401:708–712, 1999
29. Lieberman J, Shankar P, Manjunath N, Andersson J: Dressed to kill? A review of why antiviral CD8 T lymphocytes fail to prevent progressive immunodeficiency in HIV-1 infection. Blood 98:1667–1677, 2001
30. Zanovello P, Vallerani E, Biasi G, Landolfo S, Collavo D: Monoclonal antibody against IFN-gamma inhibits moloney murine sarcoma virus-specific cytotoxic T lymphocyte differentiation. J Immunol 140:1341–1344, 1998
31. Alkhatib G, Combadiere C, Broder CC, Feng Y, Kennedy PE, Murphy PM, *et al*.: CC CKR5: A RANTES, MIP-1alpha, MIP-1beta receptor as a fusion cofactor for macrophage-tropic HIV-1. Science 272:1955–1958, 1996
32. Cocchi F, DeVico AL, Garzino-Demo A, Arya SK, Gallo RC, Lusso P: Identification of RANTES, MIP-1 alpha, and MIP-1 beta as the major HIV-suppressive factors produced by $CD8^+$ T cells. Science 270:1811–1815, 1995
33. Feng Y, Broder CC, Kennedy PE, Berger EA: HIV-1 entry cofactor: Functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. Science 272:872–877, 1996
34. Deng H, Liu R, Ellmeier W, Choe S, Unutmaz D, Burkhart M, *et al*.: Identification of a major co-receptor for primary isolates of HIV-1. Nature 381:661–666, 1996
35. Hadida F, Vieillard V, Autran B, Clark-Lewis I, Baggiolini M, Debre P: HIV-specific T cell cytotoxicity mediated by RANTES via the chemokine receptor CCR3. J Exp Med 188:609–614, 1998
36. Wasik TJ, Wierzbicki A, Whiteman VE, Trinchieri G, Lischner HW, Kozbor D: Association between HIV-specific T helper responses and CTL activities in pediatric AIDS. Eur J Immunol 30:117–127, 2000
37. CDC 1994: Revised classification system for human immunodeficiency virus infection in children less than 13 years of age. MMWR Recomm Rep 43:1–10, 1994
38. Mulder J, Resnick R, Saget B, Scheibel S, Herman S, Payne H, *et al*.: A rapid and simple method for extracting human immunodeficiency virus type 1 RNA from plasma: Enhanced sensitivity. J Clin Microbiol 35:1278–1280, 1997

39. Krausa P, Brywka M III, Savage D, Hui KM, Bunce M, Ngai JL, *et al*.: Genetic polymorphism within HLA-A*02: significant allelic variation revealed in different populations. Tissue Antigens 45:223–231, 1995
40. Kostense S, Vandenberghe K, Joling J, Van Baarle D, Nanlohy N, Manting E, *et al*.: Persistent numbers of tetramer+ CD8(+) T cells, but loss of interferon-gamma+ HIV-specific T cells during progression to AIDS. Blood 99:2505–2511, 2002
41. Wedemeyer H, He XS, Nascimbeni M, Davis AR, Greenberg HB, Hoofnagle JH, *et al*.: Impaired effector function of hepatitis C virus-specific CD8$^+$ T cells in chronic hepatitis C virus infection. J Immunol 169:3447–3458, 2002
42. Sandberg JK, Fast NM, Nixon DF: Functional heterogeneity of cytokines and cytolytic effector molecules in human CD8$^+$ T lymphocytes. J Immunol 167:181–187, 2001
43. Gillespie GM, Wills MR, Appay V, O'Callaghan C, Murphy M, Smith N, *et al*.: Functional heterogeneity and high frequencies of cytomegalovirus-specific CD8(+) T lymphocytes in healthy seropositive donors. J Virol 74:8140–8150, 2000
44. Kalams SA, Buchbinder SP, Rosenberg ES, Billingsley JM, Colbert DS, Jones NG, *et al*.: Association between virus-specific cytotoxic T-lymphocyte and helper responses in human immunodeficiency virus type 1 infection. J Virol 73:6715–6720, 1999
45. Sandberg JK, Fast NM, Jordan KA, Furlan SN, Barbour JD, Fennelly G, *et al*.: HIV-specific CD8$^+$ T cell function in children with vertically acquired HIV-1 infection is critically influenced by age and the state of the CD4$^+$ T cell compartment. J Immunol 170:4403–4410, 2003
46. Jennes W, Sawadogo S, Koblavi-Deme S, Vuylsteke B, Maurice C, Roels TH, *et al*.: Positive association between beta-chemokine-producing T cells and HIV type 1 viral load in HIV-infected subjects in Abidjan, Cote d'Ivoire. AIDS Res Hum Retroviruses 18:171–177, 2002
47. Kamin-Lewis R, Abdelwahab SF, Trang C, Baker A, DeVico AL, Gallo RC, *et al*.: Perforin-low memory CD8$^+$ cells are the predominant T cells in normal humans that synthesize the beta-chemokine macrophage inflammatory protein-1 beta. Proc Natl Acad Sci USA 98:9283–9288, 2001
48. Wagner L, Yang OO, Garcia-Zepeda EA, Ge Y, Kalams SA, Walker BD, *et al*.: Beta-chemokines are released from HIV-1-specific cytolytic T-cell granules complexed to proteoglycans. Nature 391:908–911, 1998
49. Emilie D, Maillot MC, Nicolas JF, Fior R, Galanaud P: Antagonistic effect of interferon-gamma on tat-induced transactivation of HIV long terminal repeat. J Biol Chem 267:20565–20570, 1992
50. Kornbluth RS, Oh PS, Munis JR, Cleveland PH, Richman DD: Interferons and bacterial lipopolysaccharide protect macrophages from productive infection by human immunodeficiency virus *in vitro*. J Exp Med 169:1137–1151, 1989
51. Oxenius A, Sewell AK, Dawson SJ, Gunthard HF, Fischer M, Gillespie GM, *et al*.: Functional discrepancies in HIV-specific CD8$^+$ T-lymphocyte populations are related to plasma virus load. J Clin Immunol 22:363–374, 2002
52. Betts MR, Ambrozak DR, Douek DC, Bonhoeffer S, Brenchley JM, Casazza JP, *et al*.: Analysis of total human immunodeficiency virus (HIV)-specific CD4(+) and CD8(+) T-cell responses: Relationship to viral load in untreated HIV infection. J Virol 75:11983–11991, 2001
53. Addo MM, Yu XG, Rathod A, Cohen D, Eldridge RL, Strick D, *et al*.: Comprehensive epitope analysis of human immunodeficiency virus type 1 (HIV-1)-specific T-cell responses directed against the entire expressed HIV-1 genome demonstrate broadly directed responses, but no correlation to viral load. J Virol 77:2081–2092, 2003