

**500-M**

# **Human cytomegalovirus (HCMV)-specific CD4<sup>+</sup>- and CD8<sup>+</sup>-T cell mediated immunity in AIDS patients following HAART.**

G. Piccinini<sup>1\*</sup>, D. Lilleri<sup>1</sup>, G. Comolli<sup>1</sup>, E. Genini<sup>1</sup>, L. Minoli<sup>1</sup>, A. D'Arminio Monforte<sup>2</sup>, M. Catalina<sup>3</sup>, L. Gibson<sup>3</sup>, K. Luzuriaga<sup>3</sup>, M.G. Revello<sup>1</sup>, and G. Gerna<sup>1</sup>.

<sup>1</sup>IRCCS Policlinico San Matteo, Pavia; <sup>2</sup>University of Milan, Milan, Italy; <sup>3</sup>University of Massachusetts Medical School, Worcester, MA, USA.

Giuseppe Gerna  
g.gerna@smatteo.pv.it  
☎ +39-0382-502420  
📠 +39-0382-502599

## SUMMARY 1

**Objective.** To investigate the level of reconstitution of HCMV-specific T cell-mediated immunity in AIDS patients following HAART.

**Methods.** Two groups of HCMV-seropositive AIDS patients (26 patients without and 13 with previous HCMV disease), with pre-HAART CD4<sup>+</sup> T cell count <50/ $\mu$ l and no lymphoproliferative response (LPR) to HCMV were examined prospectively: i) first, after more than 3 years of successful HAART, and ii) one year later. In addition, a group of 11 HAART-*naive* patients was examined prospectively up to 13 months of HAART. The frequency of HCMV-specific CD4<sup>+</sup> was determined by cytokine flow cytometry (CFC) after short-term activation. The frequency of CD8<sup>+</sup> T cells specific for an immunodominant epitope of the HCMV pp65 was determined in a subset of patients by using HLA-A\*02 or -B\*07 tetramers.

## SUMMARY 2

**Results.** The percentages of HAART-treated patients with HCMV-specific CD4<sup>+</sup> T cells at the onset and at the end of follow-up were: 85% and 69% in the group without previous HCMV disease; 61% and 77% in the group with previous HCMV disease; and 0 and 36% in the *naive* group, respectively. Agreement of CFC and LPR was found in 78.5% of samples. The HCMV-specific-CD8<sup>+</sup> T cells frequency decreased in 75% HAART-*naive* patients after beginning of HAART. In long-term HAART-treated patients a positive CD8<sup>+</sup> response was found in 50% patients with HCMV-specific CD4<sup>+</sup> T cell response and in 75% patients with no CD4<sup>+</sup> response.

**Conclusions.** i) After more than 3 years of HAART a fair proportion of AIDS patients with pre-HAART CD4<sup>+</sup> T cells <50/ $\mu$ l had no detectable HCMV-specific CD4<sup>+</sup> response, while in the group without previous HCMV disease 16% patients lost HCMV-specific CD4<sup>+</sup> response after 1 year follow-up; ii) CFC and LPR agreed in about 80% of samples in detecting HCMV T-helper response; iii) in the majority of patients without CD4<sup>+</sup> response, HCMV-specific CD8<sup>+</sup> were detected in the absence of HCMV disease, thus suggesting that CD8<sup>+</sup> might protect HIV-infected patients against HCMV disease.

# INTRODUCTION 1

The extent of HCMV-specific T-helper immune response has been reported to correlate with control of HCMV replication (1, 2, 3) and therefore its evaluation can help the clinical decision to discontinue or maintain anti-HCMV therapy in patients with previous HCMV disease and intermediate CD4<sup>+</sup> T cell count. The role of HCMV-specific cytotoxic T cell response in controlling HCMV replication in HIV-infected patients is less defined (3, 4).

In this work, the recovery of cell-mediated immune response and its role in controlling HCMV replication were investigated by quantifying HCMV-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cell in HIV-infected patients, both HAART-*naive* and long-term HAART-treated.

HCMV-specific T-helper immune response was evaluated by determining HCMV-specific CD4<sup>+</sup> T cell count by flow cytometry detection of intracellular cytokines (Cytokine Flow Cytometry, CFC) after short-term antigen-specific activation of peripheral blood mononuclear cells (PBMC)(5).

## INTRODUCTION 2

HCMV-specific cytotoxic T cell response was evaluated by determining the frequency of CD8<sup>+</sup> T cell binding HLA Class I tetramer complexed to an immunodominant epitope of the HCMV-pp65 protein (6). The tetramer technique allows quantification of CD8<sup>+</sup> T cells that are specific for this epitope and are representative of the HCMV-specific CD8<sup>+</sup> T cell clonal expansion. In addition, the expression of CD27 molecule on the tetramer positive cell subset was evaluated. CD27 expression has been reported to inversely correlate with the perforin expression of CD8<sup>+</sup> T cells and therefore it would represent an index of functional maturity of these cells (7).

## OBJECTIVES

- ◆ To investigate whether determination of HCMV-specific CD4<sup>+</sup> T cell frequency can be considered a reliable alternative to LPR for the evaluation of HCMV-specific T-helper response.
- ◆ To investigate the effect of HAART on HCMV-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cell frequency in HIV-positive HAART-*naive* patients.
- ◆ To assess the recovery and stability of HCMV-specific CD4<sup>+</sup> T cell count in long-term HAART-treated (>3 years) HIV-positive patients with or without previous HCMV disease, with CD4<sup>+</sup> T cell count <50/μl before HAART and >100/μl at the time of first evaluation.
- ◆ To correlate the HCMV-specific CD4<sup>+</sup> T cell count with the frequency of HCMV-pp65-tetramer positive CD8<sup>+</sup> T cells and the expression of CD27 molecules on tetramer positive CD8<sup>+</sup> T cells.

# MATERIALS AND METHODS 1

## **PBMC preparation and HCMV-specific LPR.**

PBMC were isolated by standard Ficoll gradient centrifugation, resuspended in RPMI 1640 containing 5% human serum and plated in triplicate at  $1 \times 10^5$  cells/well in U-bottomed 96-well plates in the presence of HCMV or control antigen. After 6-day culture, 1  $\mu$ Ci of [ $^3$ H]-thymidine per well was added and cultures were incubated for further 18 hours. Stimulation index (SI) was the ratio of mean cpm of wells with HCMV antigen and the mean cpm of wells with control antigen. Positive LPR was defined by simultaneous presence of  $\geq 3,000$  net cpm and  $SI \geq 3$ .

## **HCMV viremia.**

HCMV viremia was determined in whole blood either as HCMV DNAemia by quantitative PCR (8) or as RNAemia by NASBA methodology (9).

## MATERIALS AND METHODS 2

### **HCMV-specific CD4<sup>+</sup> T cell frequency.**

Two millions of cryopreserved PBMC were resuspended in RPMI 1640 containing 10% fetal calf serum and dispensed in 15-ml polypropylene conical tubes. Anti-CD28 and anti-CD49d monoclonal antibodies (0.5 µg each, Caltag) and 40 µl of a commercially available HCMV antigen preparation (Biowhittaker) or its negative control were then added. After 1 hour at 37°C Brefeldin A was added (10 µg/ml final concentration); cells were incubated for additional 14 hours, washed once with cold PBS containing 2 mM EDTA, fixed, permeabilized (Fix & Perm™, Caltag) and then labelled with anti-CD4-FITC, anti-TNFα-PE and anti-CD69-PerCP monoclonal antibodies (Becton Dickinson). Cell suspensions were analyzed using a FACSCalibur flow cytometer (Becton Dickinson) equipped with CellQuest software. Viable lymphocytes were identified by scattering parameters. For each sample, 5x10<sup>4</sup> viable CD4<sup>+</sup> T lymphocytes were evaluated. CD4<sup>+</sup> cells expressing TNFα and CD69 were considered as activated cells. HCMV-specific CD4<sup>+</sup> T cell frequency was calculated by subtracting from the value of the test sample the value of the sample incubated with control antigen (consistently < 0.05 %). The absolute number of HCMV-specific CD4<sup>+</sup> T cells per ml of blood was calculated by multiplying the HCMV-specific CD4<sup>+</sup> T cell frequency by the number of CD4<sup>+</sup> T cells / ml blood (5).

## MATERIALS AND METHODS 3

### **HLA Class I HCMV-pp65 tetramer positive CD8<sup>+</sup> T cell frequency.**

HLA-A\*02 or -B\*07 patients were initially identified by the immunocytofluorimetric technique. HCMV-pp65 peptides 495-503 or 417-426 (specific for HLA-A\*02 or -B\*07, respectively) bound to tetrameric HLA Class I-complexes (tetramers) combined with APC-labeled streptavidin were used (6). One million of cryopreserved PBMC were incubated in presence of the relevant tetramer, anti CD8-FITC, CD27-PE and CD3-TC monoclonal antibodies (Caltag). HLA-unmatched tetramers were used as negative control. The percent of tetramer positive cells within the CD3<sup>+</sup>CD8<sup>+</sup> gates and the percent of tetramer positive CD8<sup>+</sup> T cells expressing CD27 were determined by FACS analysis. In patients identified as A\*02 or -B\*07 by the immunocytofluorimetric technique but without detectable tetramer binding to CD8<sup>+</sup> lymphocytes, HLA A\*02 or -B\*07 expression was confirmed by molecular techniques (PCR-SSP, Essemedical, Milan, Italy).

## RESULTS 1

### **HCMV-specific CD4<sup>+</sup> T cell count.**

HCMV-specific CD4<sup>+</sup> T cell count was first evaluated in normal HCMV seropositive (n=9) and seronegative (n=6) subjects. Based on results shown in Figure 1, the cut-off to define the presence of HCMV reactive CD4<sup>+</sup> T cells by CFC analysis was defined as the simultaneous presence of HCMV-specific CD4<sup>+</sup> T cell  $\geq 0.1\%$  and  $\geq 400/\text{ml}$  blood (dotted line in figure 1).

### **Comparison of HCMV-specific CD4<sup>+</sup> T cell count and LPR.**

To assess whether the HCMV-specific CD4<sup>+</sup> T cell count could be reliably used for the evaluation of HCMV-specific T-helper response, both HCMV-specific CD4<sup>+</sup> T cell count and LPR were determined in parallel in 130 PBMC samples from HCMV-seropositive HIV-infected patients. Results are shown in Figure 2, where dotted lines indicate the cut-offs for the HCMV-specific CD4<sup>+</sup> T cell presence (400 cells/ml blood) and for HCMV-specific LPR (3,000 net cpm). Overall, 78.5% of assayed samples gave concordant results with both techniques.

## RESULTS 2

### **HCMV-specific CD4<sup>+</sup> T cell count in HAART-*naive* HIV- HCMV-seropositive patients**

HCMV-specific CD4<sup>+</sup> T cell count was determined prospectively in a group of 11 HAART-*naive* patients before and after HAART (Figure 3). None of the HAART-*naive* patients had detectable HCMV-specific CD4<sup>+</sup> T cells at the beginning of the follow-up. However, after a median time of 13 months of HAART, 4 patients showed a clear HCMV-specific response whereas in most of the remaining patients a slight increase in HCMV-specific CD4<sup>+</sup> T cells could be detected. In parallel, the number of patients positive for HCMV viremia decreased from 8/11 (72%) to 1/11 (9%).

## RESULTS 3

### **HCMV-specific CD4<sup>+</sup> T cell count in long-term HAART-treated HIV- HCMV-positive patients**

HCMV-specific CD4<sup>+</sup> T cell count was determined prospectively in 2 groups of HIV-HCMV-seropositive patients treated with HAART for >3 years: one group without and one with HCMV disease before the first evaluation. All patients had CD4<sup>+</sup> T cell count <50/ $\mu$ l blood and no HCMV-specific T-helper response before HAART and >100/ $\mu$ l blood at the time of the first evaluation (Table 1). None of the long-term HAART-treated patients had active HCMV disease at the time of the analysis. The percentage of patients with detectable HCMV-specific CD4<sup>+</sup> T cells in the two groups were 85% and 61% at the beginning of follow-up, and 69% and 77% at time of the second evaluation, respectively (Table 1). HCMV-specific CD4<sup>+</sup> T cell counts in the two groups of patients are presented in detail in Figures 4 and 5.

## RESULTS 4

### **HLA Class I HCMV-pp65-tetramer positive CD8<sup>+</sup> T cell (Tetr<sup>+</sup> cells) frequency and CD27 expression in HIV- HCMV-positive HAART-*naive* patients**

Tetr<sup>+</sup> cell frequency was evaluated prospectively in 4 HAART-*naive* patients before and after HAART (see patient data in Table 2). Results are presented in Figure 6. Tetr<sup>+</sup> cells frequency in HAART-*naive* patients was consistently higher (1.0% to 5.2%) than in control subjects (0.12% to 0.77%, Figure 8). However, the frequency decreased in 3 of 4 patients after onset of HAART. In addition, HCMV viremia disappeared in 2 of 3 patients (Figure 6).

The percentage of Tetr<sup>+</sup> CD27<sup>+</sup> cells in 4 patients before and after HAART is shown in Figure 7. The trend of CD27 expression in response to HAART was not clear but, interestingly, the only examined patient with HCMV viremia after onset of HAART had almost undetectable expression of CD27 on Tetr<sup>+</sup>CD8<sup>+</sup> T cells during follow-up, suggesting in this patient a presence of HCMV-specific CD8<sup>+</sup> effector cell frequency very high yet unable to eliminate HCMV viremia.

## RESULTS 5

### **HLA Class I HCMV-pp65-tetramer positive CD8<sup>+</sup> T cell (Tetr<sup>+</sup> cells) frequency in HIV-positive, HAART-treated patients with or without HCMV-specific CD4<sup>+</sup> response**

The Tetr<sup>+</sup> cell frequency was evaluated in two groups of HIV-positive patients, one group without and one group with detectable HCMV-specific CD4<sup>+</sup> T cells. All patients except one had been treated with HAART for >1 year (Table 2). The results are presented in Figure 8, together with the values obtained in 8 normal HCMV-seropositive subjects. Both HIV-infected patients and normal control subjects showed a Tetr<sup>+</sup> T cell frequency markedly lower (Figure 8) than HAART-*naive* patients (Figure 6). Median Tetr<sup>+</sup> T cell frequency in HIV patients without HCMV-specific CD4<sup>+</sup> response and in control subjects was comparable, and markedly higher than in HIV patients with HCMV-specific CD4<sup>+</sup> response (Figure 8).

## RESULTS 6

### **CD27 expression on HLA Class I HCMV-pp65-tetramer positive CD8<sup>+</sup> T cells in HIV-positive, HAART-treated patients with or without HCMV-specific CD4<sup>+</sup> T cell response**

CD27 expressing cells within the HCMV Tetr<sup>+</sup> T cell subset were determined in HIV-positive HAART-treated patients with or without detectable HCMV-specific CD4<sup>+</sup> T cells as well as in healthy subjects (Figure 9). CD27 expression was comparable in the three groups examined.

## CONCLUSIONS 1

- ◆ CFC-based HCMV-specific CD4<sup>+</sup> T cell count can be considered a valuable alternative to LPR for the detection of HCMV-specific T-helper response in HIV-infected patients. CFC offers several advantages over LPR: rapidity, no use of radioactive compounds, applicability to frozen samples. Thus, CFC could facilitate wider screening of anti-HCMV T-helper activity in HIV-infected patients, with potential benefits for clinicians in deciding strategies of discontinuation or maintenance of anti-HCMV therapy.
- ◆ In most HIV-infected, HAART-*naive* patients the onset of HAART causes a slight but clear-cut increase in HCMV-specific CD4<sup>+</sup> T cell count during the first year of treatment, correlating with the nearly complete disappearance of HCMV viremia.

## CONCLUSIONS 2

- ◆ In the two groups of HIV-infected, long-term HAART-treated patients (with or without previous HCMV disease), a variable proportion of patients did not reconstitute the HCMV-specific CD4<sup>+</sup> T cell response despite a marked increase in total CD4<sup>+</sup> T cell count. In addition, few patients with positive CD4<sup>+</sup> T cell response showed declining levels of HCMV-specific immune reconstitution during follow-up. Median HCMV-specific CD4<sup>+</sup> T cell counts were lower in HIV-infected, long-term HAART-treated patients than in healthy HCMV-positive subjects.
- ◆ HCMV-pp65-tetramer positive CD8<sup>+</sup> T cell frequency was high in HIV-infected, HAART-*naive* patients examined, and showed a trend towards decrease after onset of HAART. The percentage of tetramer-positive CD8<sup>+</sup> T cells expressing CD27 did not vary significantly after onset of HAART. The frequency of tetramer positive CD8<sup>+</sup> T cells and their expression of CD27 did not correlate with the presence of HCMV-specific CD4<sup>+</sup> T cells.
- ◆ HCMV-specific CD8<sup>+</sup> T cells were detected in the majority of HAART-treated patients without HCMV-specific CD4<sup>+</sup> response and in the absence of HCMV disease, thus suggesting a potential key role of anti-HCMV CD8<sup>+</sup> T cells in protecting HIV-infected patients against HCMV disease.

## REFERENCES

1. Schrier, R.D. et al., 1995, *J. Clin. Invest.*, 95:1741-1746.
2. Jacobson , M.A. et al., 2001, *J. Infect. Dis.*, 183:1399-1404.
3. Hsieh, S-M et al., 2001, *J. Infect. Dis.*, 184:1386-1391.
4. Villacres, M.C. et al., 2001, *J. Infect. Dis.*, 184:256-267.
5. Pitcher, C.J. et al., 1999, *Nat. Med.*, 5:518-525.
6. Kern, F. et al., 1998, *Nat. Med.*, 4:975-978.
7. Appay, V. et al., 2000, *J. Exp. Med.*, 192:63-75.
8. Gerna, G. et al., 1998, *Transplantation*, 65:1378-1385.
9. Gerna, G. et al., 2000, *J. Clin. Microb.*, 38:1845-1853.

**Table 1**  
**Characteristics of the three groups of HIV-infected HCMV-seropositive patients  
examined for HCMV-specific T-helper response**

Evaluation	HAART- <i>naive</i> patients (n=11)		HAART-treated patients (no HCMV disease, n=26)		HAART-treated patients (previous HCMV disease, n=13)	
	1 <sup>st</sup>	2 <sup>nd</sup>	1 <sup>st</sup>	2 <sup>nd</sup>	1 <sup>st</sup>	2 <sup>nd</sup>
HAART (months) <sup>a</sup>	0	13 (6-21)	44 (32-51)	56 (45-73)	38 (36-48)	52 (47-62)
CD4 <sup>+</sup> T cells (count/ $\mu$ l) <sup>a</sup>	45 (1-577)	328 (96-492)	359 (135-762)	279 (22-848)	432 (160-914)	407 (175-1,108)
HIV RNA (copies/ml x 10 <sup>3</sup> ) <sup>a</sup>	265 (0.59-930)	< 0.05 (< 0.05-0.4)	2.36 (< 0.05-1,200)	0.13 (< 0.05-63.6)	< 0.05 (< 0.05-12.0)	< 0.05 (< 0.05-10.9)
HCMV specific CD4 <sup>+</sup> response <sup>b</sup>	0/11 (0%)	4/11 (36%)	22/26 (85%)	18/26 (69%)	8/13 (61%)	10/13 (77%)
HCMV viremia <sup>c</sup>	8/11(72%)	1/11 (9%)	1/25 (4%)	1/25 (4%)	1/13 (8%)	0/13 (0%)

<sup>a</sup> Median value (range). <sup>b</sup> HCMV-specific CD4<sup>+</sup> T cell  $\geq$  0.1% and  $\geq$  400/ml blood. <sup>c</sup> Determined as HCMV DNAemia and / or RNAemia.

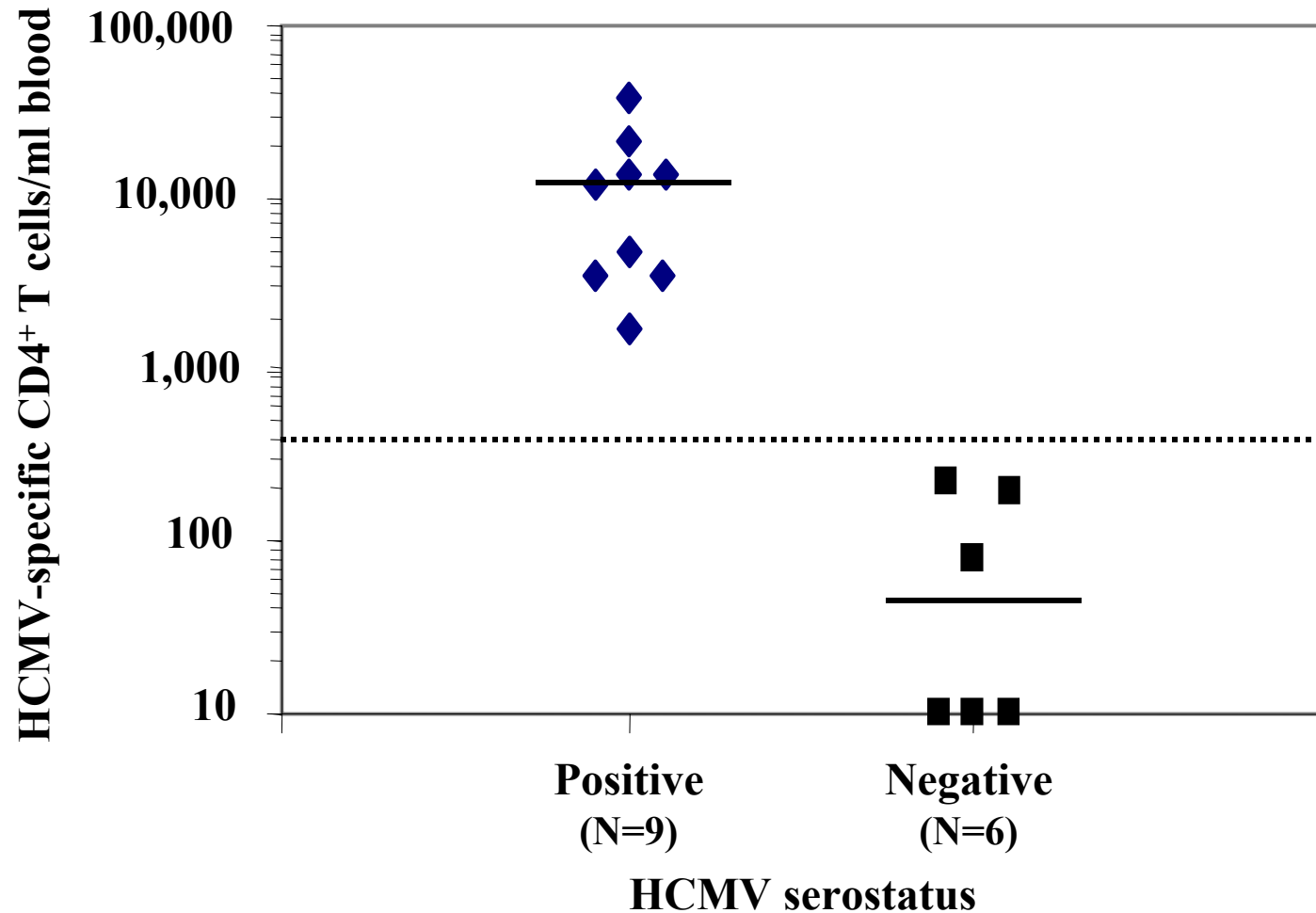
**Table 2****Characteristics of the three groups of HIV-infected patients examined for HCMV-pp65 tetramer positive CD8<sup>+</sup> T cell frequency**

	HAART <i>naive</i> patients (n=4)		HAART patients w/o HCMV T- helper response (n=8)	HAART patients with HCMV T- helper response (n=8)
	First evaluation	Last evaluation		
HAART (months) <sup>a</sup>	0	11.5 (8-17)	49 (3-65)	46 (12-62)
CD4 <sup>+</sup> T cells (count/ $\mu$ l) <sup>a</sup>	45 (37-98)	367 (136-423)	332 (32-563)	219 (50-848)
HIV RNA (copies/ml x 10 <sup>3</sup> ) <sup>a</sup>	390 (0.51-830)	< 0.05 (< 0.05 -0.10)	18.3 (< 0.05 -105)	2.22 (< 0.05 -20.27)
HCMV specific CD8 <sup>+</sup> T cell response <sup>b</sup>	4/4 (100%)	4/4 (100%)	6/8 (75%)	4/8 (50%)
HCMV viremia <sup>c</sup>	3/4 (75%)	1/4 (25%)	2/8 (25%)	1/8 (13%)

<sup>a</sup> Median values (range). <sup>b</sup> Tetramer positive CD8<sup>+</sup> T cells  $\geq$ 0.1 %. <sup>c</sup> Determined as HCMV DNAemia and / or RNAemia.

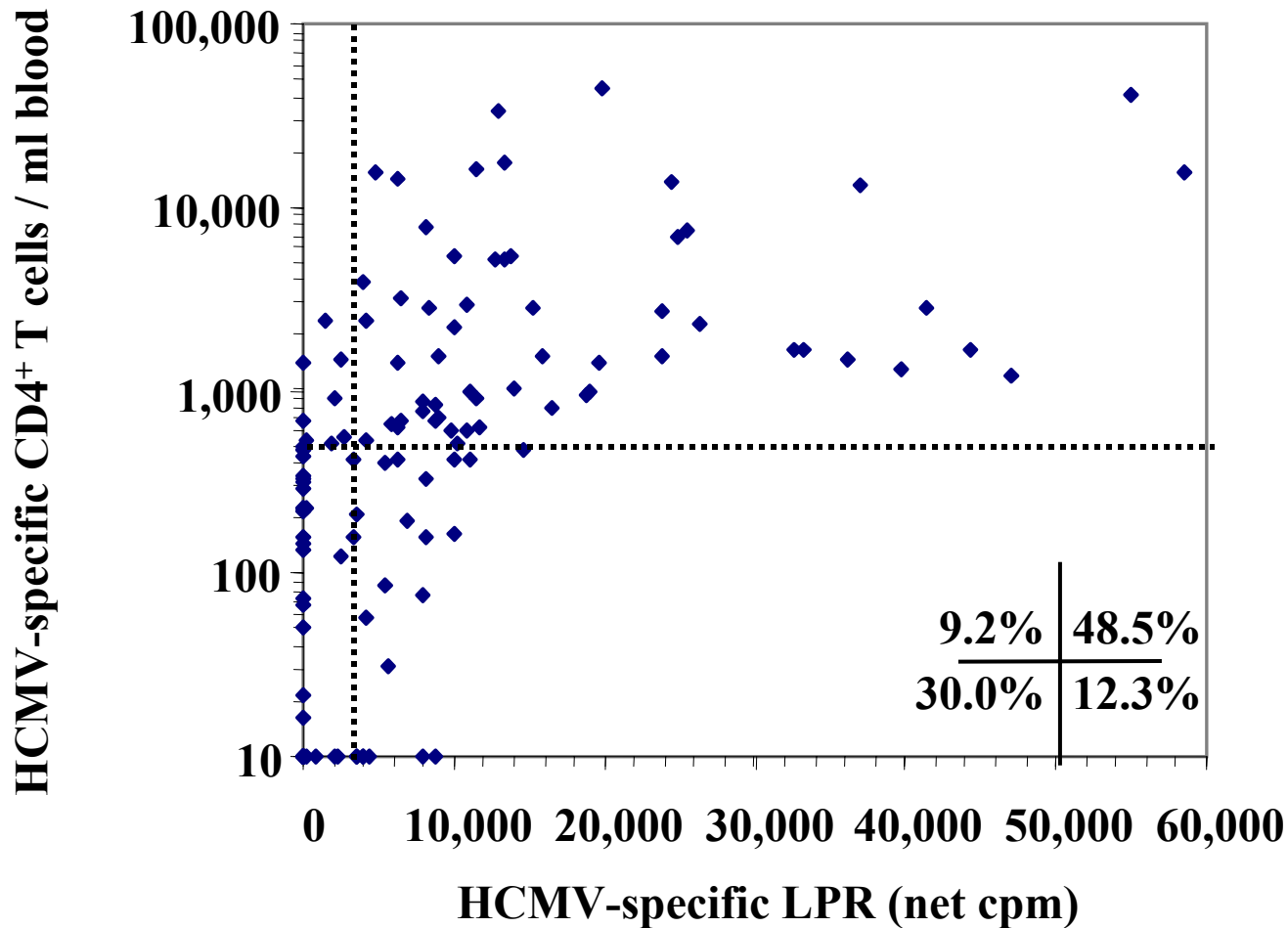
# FIGURE 1

## HCMV-specific CD4<sup>+</sup> T cell count in normal subjects



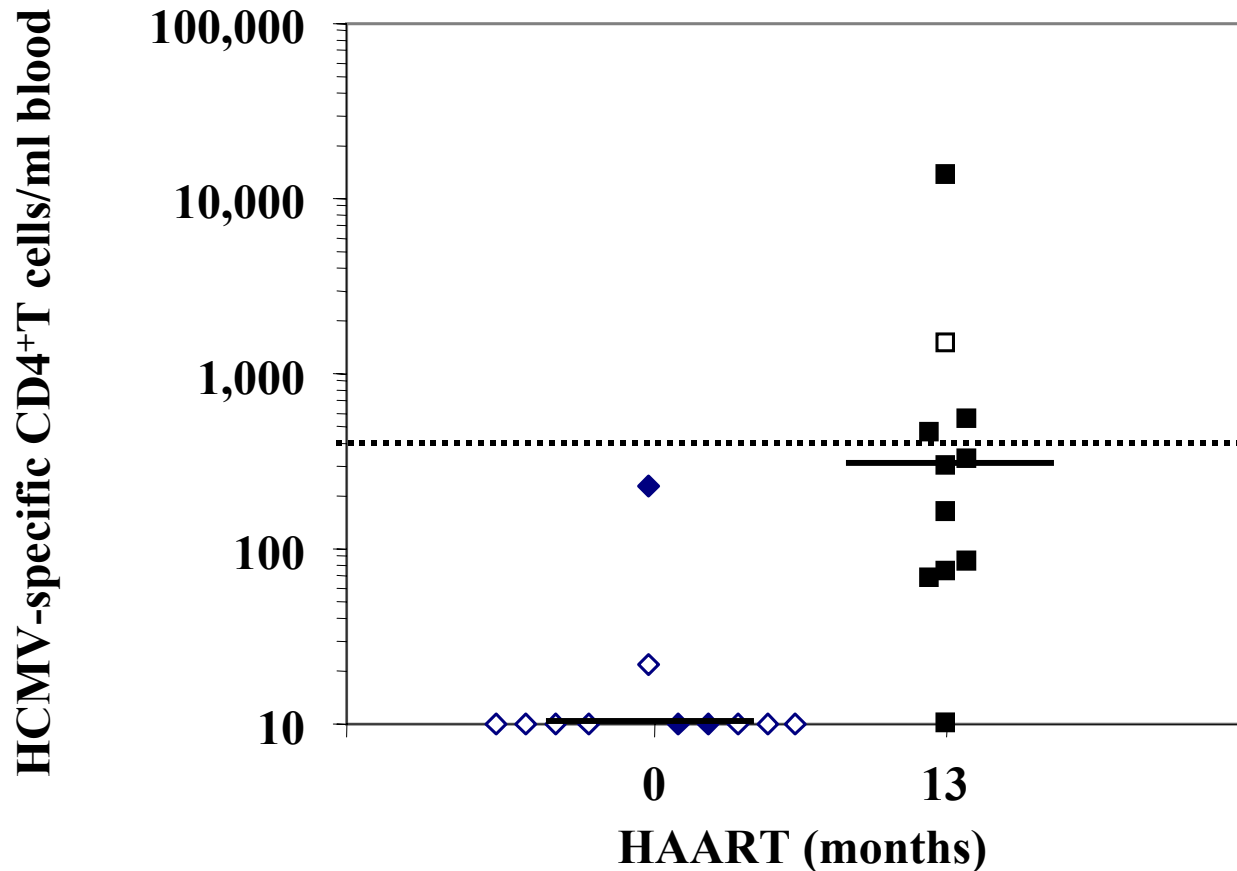
## FIGURE 2

**Comparison of HCMV-specific CD4<sup>+</sup> T-cell count and lymphoproliferative response in 130 sample from HIV-infected HCMV-seropositive patients**



# FIGURE 3

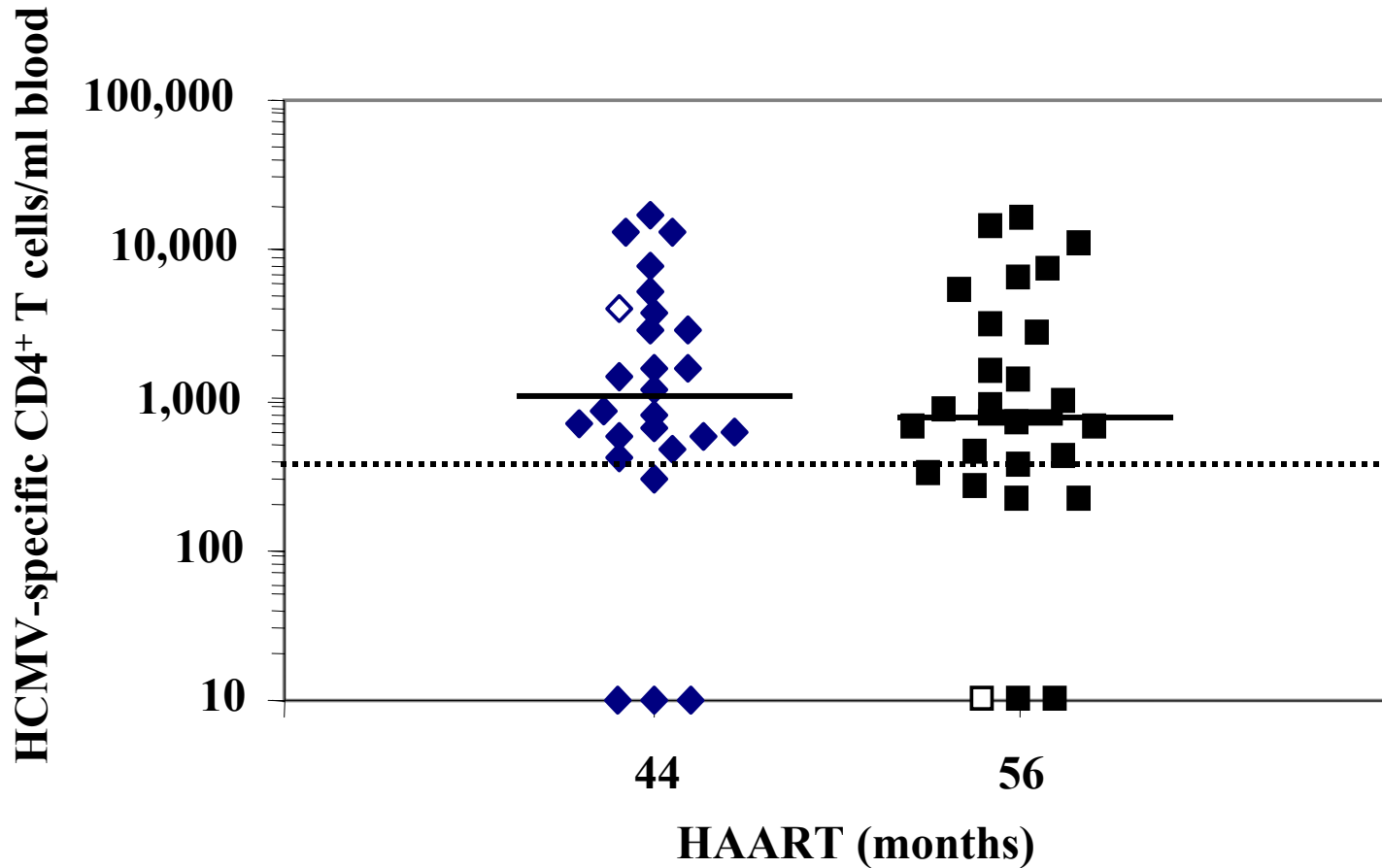
## HCMV-specific CD4<sup>+</sup> T cell count in 11 HIV-HCMV-seropositive HAART-naïve patients before and after HAART



(Open symbols represent samples with positive HCMV viremia)

**FIGURE 4**

**HCMV-specific CD4<sup>+</sup> T cell count in 26 HAART-treated HIV-infected patients with no previous HCMV disease**

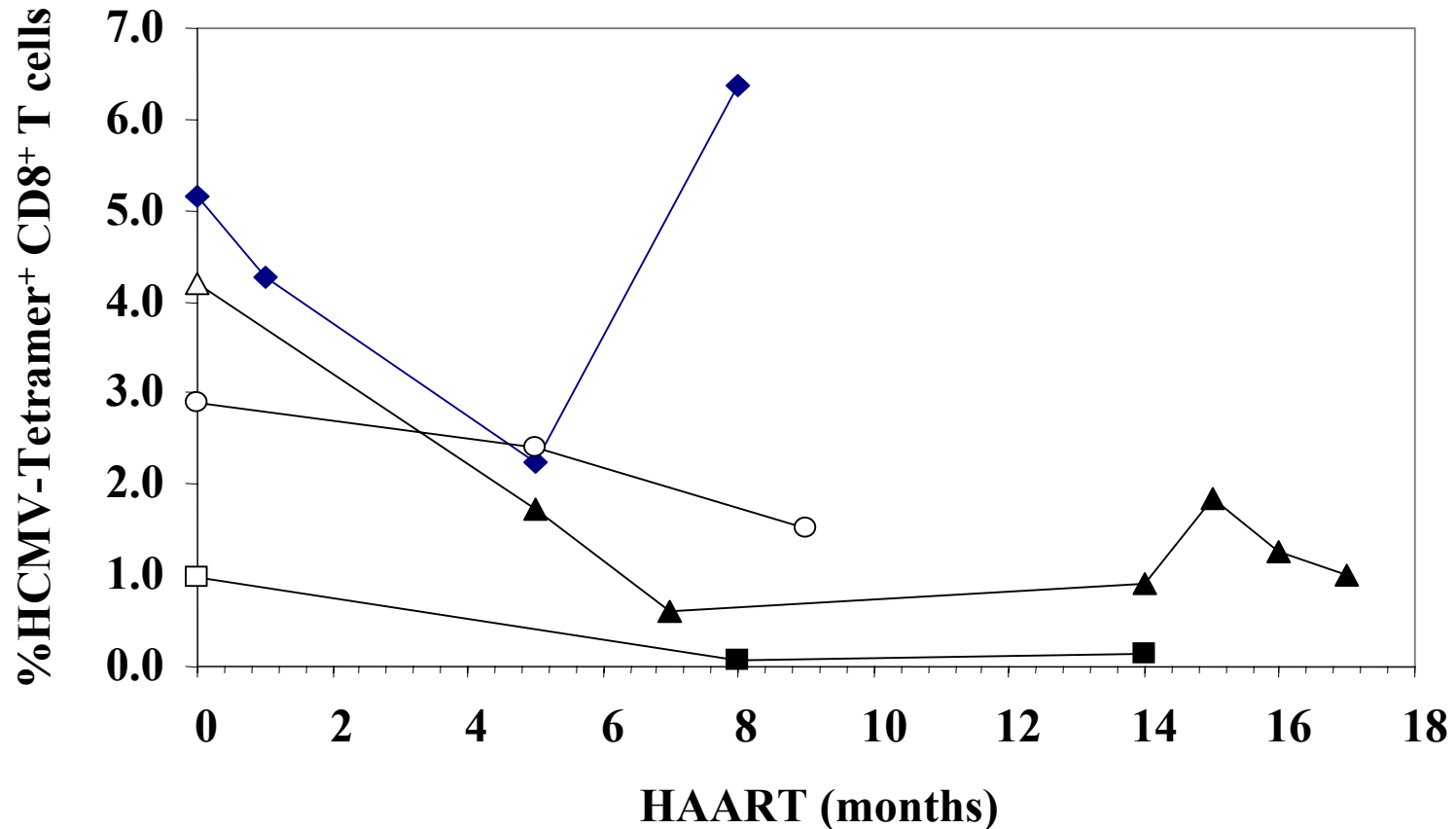


(Open symbols represent samples with positive HCMV viremia)



# FIGURE 6

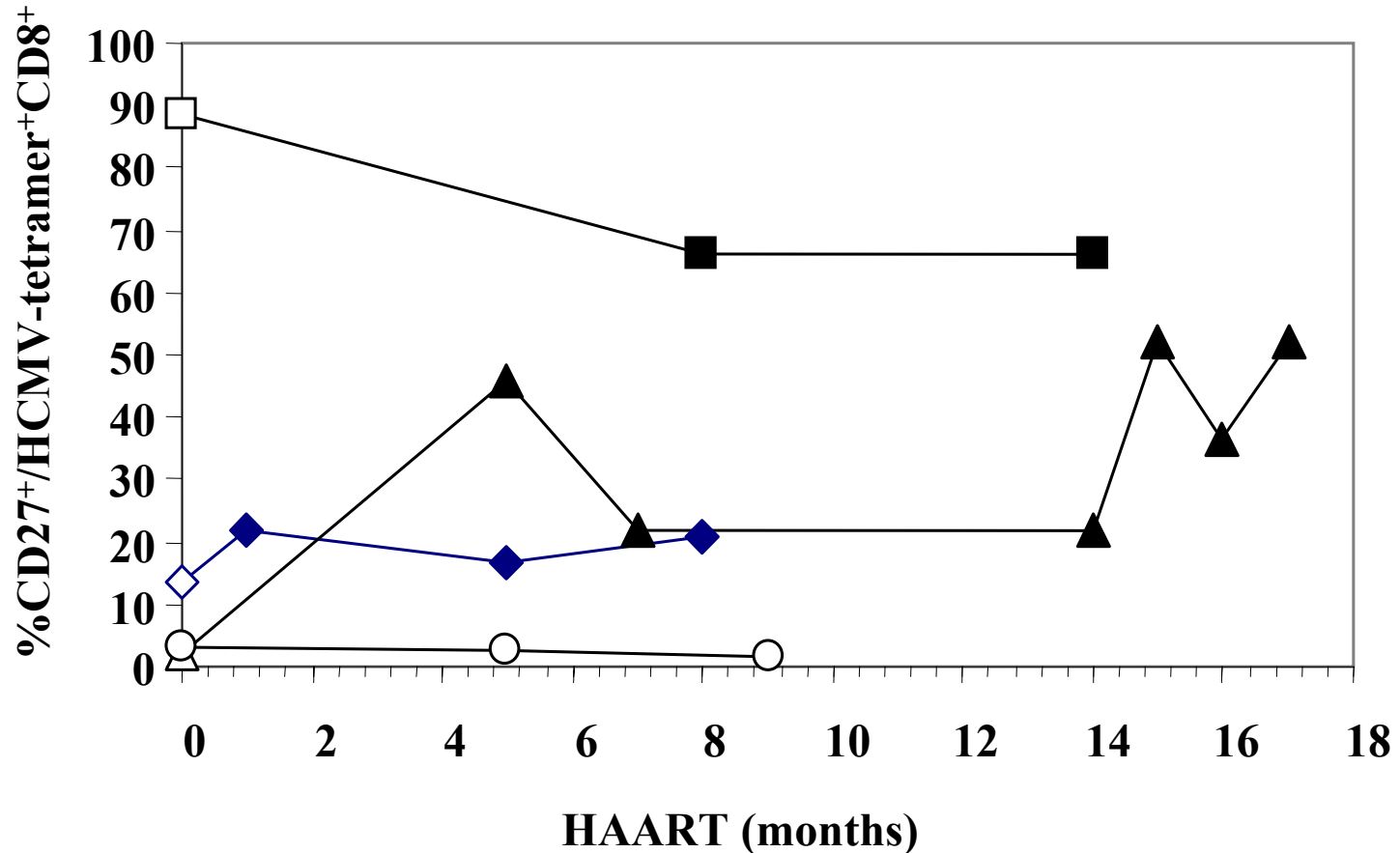
## HCMV-pp65-tetramer positive CD8<sup>+</sup> T cell frequency in 4 HAART-*naive* HIV-infected patients before and after HAART



(Open symbols represent samples with positive HCMV viremia)

# FIGURE 7

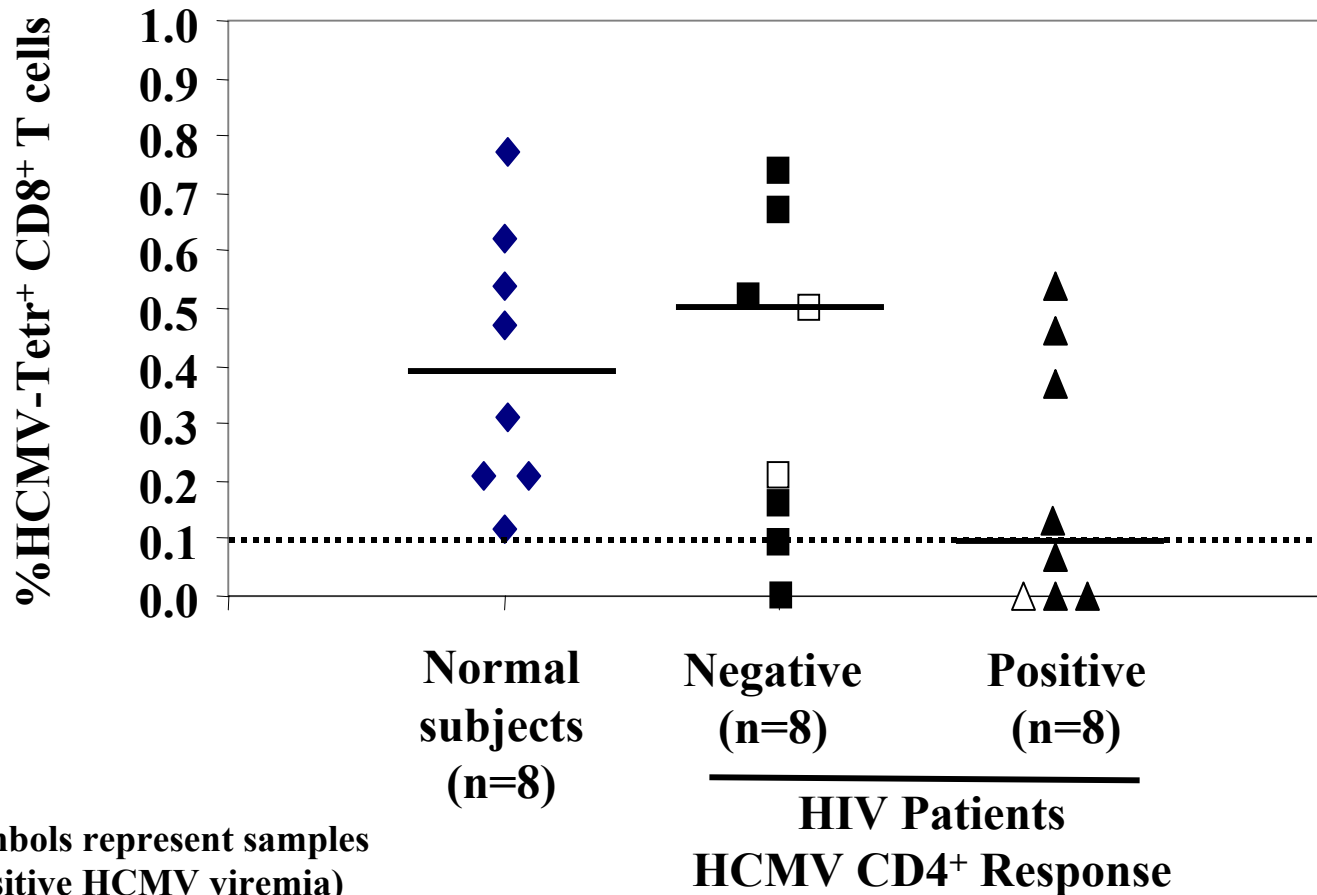
**CD27 expression on HCMV-pp65-tetramer positive CD8<sup>+</sup> cells in 4 HAART *naive* HIV-infected patients before and after HAART**



(Open symbols represent samples with positive HCMV viremia.  
Same symbols were used for patients in Figure 6)

**FIGURE 8**

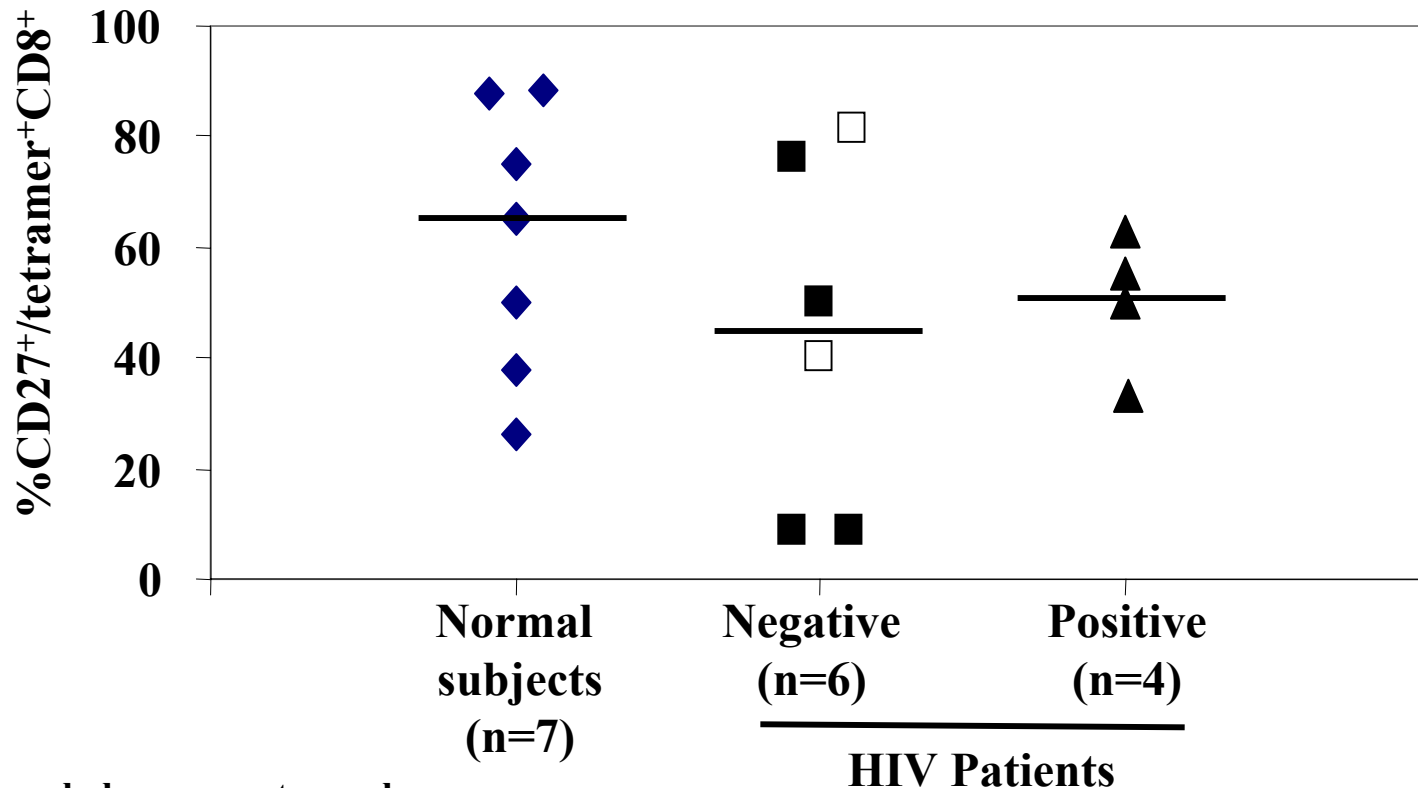
**HCMV-pp65-tetramer positive CD8<sup>+</sup> frequency in normal subjects and in HIV-positive, HAART-treated patients with or without HCMV-specific CD4<sup>+</sup> response**



(Open symbols represent samples with positive HCMV viremia)

## FIGURE 9

**CD27 expression on HCMV-pp65-tetramer positive CD8<sup>+</sup> T cells in normal subjects and in HIV-positive, HAART-treated patients with or without HCMV-specific CD4<sup>+</sup> T cell response**



(Open symbols represent samples with positive HCMV viremia)

HIV Patients  
HCMV CD4<sup>+</sup> response