HIV replication leads to skewed maturation of CD8-positive T-cell responses in infected children

Carla Montesano[1], Alessia Anselmi[1], Paolo Palma[2,3], Stefania Bernardi[2], Rosella Cicconi[1], Maurizio Mattei[1], Guido Castelli-Gattinara[2], Massimo Ciccozzi[4], Vittorio Colizzi[1], Massimo Amicosante[5]

[1]Department of Biology, University of Rome "Tor Vergata", Rome, Italy;
[2]Division of Immunology and Infectious Diseases, "Bambino Gesù" Children's Hospital, Rome, Italy;
[3]Chair of Pediatrics, Department of Public Health, University of Rome "Tor Vergata";
[4]Department of Infectious, Parasitic and Immunomediated Diseases, Istituto Superiore di Sanita', Rome, Italy
[5]Department of Internal Medicine, University of Rome "Tor Vergata", Rome, Italy

—————————————————— SUMMARY ——————————————————

HIV-1 infection causes a severe T-cell impairment with alteration of immune response. However, in children the natural decline of lymphocytes and CD4 cells in early life makes it more difficult to monitor immunocompetence and progression of HIV-infection.
Aim of this study was to characterize the CD8 response in non-vertically HIV-infected children exposed persistently to viremia and in HIV-infected children controlling efficiently viremia by ART, by analysing the effect of persistent viremia on CD4 and CD8 T-cells count, HIV-specific immune-response and naïve/memory pattern of CD8 T-cell. Whereas, no differences of CD4 count between viremic patients and viral controllers were observed (1046.9±472.1 cells/µl vs 1101.3±415.4 cells/µl; p>0.05), CD8 count was higher in the viremic patients (1080.6±652.1 cells/µl vs 747.5±389.9 cells/µl, p<0.05). In viremic patients, HIV-specific CD8 T-cells correlated with viral load. However, in this group a loss of HIV-specific CD8 response was associated with a 7 fold decrease of naïve and increase of pre-effector CD8 T-cells (62.8% ±10.21% vs 10.37% ±7.91%, p<0.03).
Persistent exposure to viremia alters HIV-specific CD8 response possibly through a persistent immune activation process leading to exhaustion of naïve CD8 T-cells and skewed maturation of memory subset. Therefore, memory CD8 T-cells might lose the ability to respond correctly and efficiently to HIV-antigen exposure.

KEY WORDS: Children, HIV-specific immune response, Naïve/memory lymphocytes

INTRODUCTION

Monitoring HIV-infected children is based on clinical symptoms, and assessment of the count or percentage of CD4 T-cells and HIV RNA viral load. (Gazzard, 2001; http://www.aidsinfo.nih.gov/guidelines/pediatric/PED_012004.pdf; Sharland et al., 2004). These parameters are evaluated to decide when to start antiretroviral ther-

apy and to monitor the response to antiretroviral therapy (ART), with particularly emphasis given to the CD4 cell counts (Dunn et al., 2003). However, the evaluation of the total count or the percentage of the CD4 T-cells, as well as the total lymphocytes count in children is complicated by the effect of age due to the natural decline of lymphocytes and CD4 cells in early life (Wade and Ades, 1998).
HIV-1 stimulates strong immune responses of CD4+ and CD8+ T lymphocytes in infected subjects (Walker et al., 1987) and diminishes cellular immunity by inducing a chronic state of T-cell activation (Chinen and Shearer, 2002; Douek, 2007). In particular, HIV-specific CD8 T-lymphocytes are important in the antiviral response (McMichael

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Corresponding author
Carla Montesano PhD
Department of Biology
University of Rome "Tor Vergata"
Via della Ricerca Scientifica, 1 - 00133 Rome, Italy
E-mail: montesano@uniroma2.it

and Rowland-Jones, 2001). In chronically HIV-infected patients, the expanded HIV-specific cytotoxic lymphocytes (CTL) persist at high frequencies (Ogg GS *et al.,* 1998) and decline after viral suppression induced by ART. Similarly, HIV-infected children responding to ART achieve suppression of viral load, an increase in CD4+ T lymphocytes (Borkowsky *et al.,* 2000) and decreased immune activation, with fewer effector CD8+ T cells (Resino *et al.*, 2003; Resino *et al.,* 2004).

CD8 T-cells exist in the peripheral circulation either as naive or as antigen-experienced cells. The latter consist of a heterogeneous population that may be classified according to immediate or delayed effector function, expression of homing receptors, cytokine secretion profile, proliferation potential and longevity or survival characteristics (Sallusto *et al.,* 2004; Seder and Ahmed, 2003). CCR7-negative effector T-cells have lost the ability for autocrine proliferation, display specialized functions such as IFN-γ production, and acquire mature cytolytic function after CD45RA expression (Campbell and Butcher, 2000; Sallusto *et al.,* 1999). Moreover, T-cell activation induces a transient increase in CD27 expression that gradually switches on effector cells off (Hintzen *et al.*, 1993).

In adults, a skewed maturation of HIV-specific CTL has been demonstrated, indicating that the HIV-specific CTL pool is largely composed of pre-terminally differentiated CCR7-negative CD45RA-negative and CD27-negative CD45RA-negative CD8 T-cells with poor cytotoxic activities (Champagne *et al.,* 2001). Similarly, systemic changes in CD8 T-cell population have been described in adults, children and infants with loss of naive CD45RA-positice CD27-positive CD8 T-cells and expansion of CD27-negative subset due to an increased activation state of T-cells occurs during HIV-1 infection altering T-cell differentiation (D'Offizi *et al.,* 2002; McCloskey *et al.,* 2001; Resino *et al.,* 2003)**.**

Monitoring both CD45RA and CD27 surface expression will discriminate among different stages of differentiation of T cell subsets, and the fine characterization of CD8 responses against HIV could be useful in monitoring the patient's immune status during HIV infection. Integration of extended immune phenotyping of T-cell activation and differentiation markers can provide precise and global means to measure viral effects on

T-cell development and activation and establish the risk of disease progression (Appay and Rowland-Jones, 2004).

This study compared the immunological status of HIV-infected children exposed to viremia and children in ART treatment controlling viremia. The characterization was performed through the analysis of HIV-specific CD8+ T cells response and the analysis of phenotype of naïve/memory T cell subsets.

We show that the frequency of HIV-specific CD8 T-cells from viremic patients is higher in children with high viral load but it decreases when a deep loss of naïve subset is present and associated with a strong skewed maturation towards a pre-effector phenotype. The alteration of the maturative pattern of CD8 T lymphocytes is not restricted to HIV-specific cells but it is extended to the entire CD8 T cells population and it is associated with both normal CD4 count and CD4 frequency.

MATERIALS AND METHODS

Study population

The present study was performed in 27 horizontally HIV-infected children and in an age-matched group of 17 vertically HIV-infected children treated at the "Bambino Gesù" Children's Hospital. Antiretroviral therapy was prescribed to both groups of HIV-infected children according to the international guidelines (Sharland *et al.*, 2004) but for several reasons the group of horizontally HIV-infected children was not under antiviral treatment due to poor adherence or since they do not meet guideline criteria to start the treatment. The analysis was carried out on available residual blood samples from HIV-infected children collected at the "Bambino Gesù" Children's Hospital. Written informed consent was obtained from each patient's legal guardian before enrolment. This study was approved by the Ethical Commission of "Bambino Gesù" Children's Hospital (Palma *et al.,* 2007).

Samples were stored at -80°C until analyzed. Data from these cohorts were completely anonymous and patients identified by code.

Total CD4 and CD8 cell counts and HIV-1 RNA viral load

The total number and percentage of CD4+ and

CD8+ T-cell counts and the quantification of plasma HIV-1 RNA viral load were carried out as previously reported (Palma *et al.,* 2007).

Cell isolation and stimulation

Peripheral blood mononuclear cells were obtained using standard Ficoll–Hypaque (Pharmacia, Uppsala, Sweden) density centrifugation and frozen in dimethyl sulphoxide 10% and fetal calf serum 90% at -80°C. Briefly, 1×10^6 thawed cells in 1 ml of complete RPMI 1640, 10% v/v heat-inactivated fetal calf serum, 2 mM L-glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin were incubated with 1 µg each of anti-CD28 and CD49d mAb (IgG1 clone CD28.2, and IgG1 clone 9F10, Becton Dickinson) and pooled peptides (1 µg of each peptide) or with phorbol myristate acetate (50 ng/ml) and ionomycin (10 µg/ml). The pool of Gag, Tat and Nef peptides included 29, 10 and 13 different 15-mers, respectively, designed on the most immunogenic and conserved area of Gag, Tat and Nef gene product (Amicosante *et al.,* 2002; Palma *et al.,* 2008). The peptides were purchased from Sigma-Genosys Cambridge, UK. Cells incubated with only anti-CD28 and CD49d were included as control samples. The cultures were finally incubated at 37°C in a 5% carbon dioxide incubator for 1 hour, followed by an additional 5 hours incubation in the presence of 10 µg/ml brefeldin-A to inhibit cellular exocytosis (Sigma, St Louis, MO, USA).

Monocolonal antibodies and flow cytometry

Monoclonal antibodies (mAb) coupled with fluorescein, phycoerythrin, phycoerythrin-cyanin 5.1 (PE-Cy5) and allophycocyanin were combined for simultaneous staining. The anti-human antibodies used in this study were: anti-CD4 (IgG1, clone RPA-T4), anti-CD8 (IgG1, clone RPA-T8), anti-CD27 (IgG1, clone MT271), anti-CD45RA (IgG2b, clone HI100), anti-IFN-γ mAb (IgG1, clone B27). All mAb were obtained from Becton Dickinson, Mountain View, CA, USA.
Stainings for membrane or intracellular antigens were performed as described (Amicosante *et al.,* 2002). Control for non-specific staining was monitored with isotype-matched mAb and non-specific staining was always subtracted from the specific results and the result was considered positive when the difference was ≥0.02%. Flow cytometric analysis was performed on a Facscalibur flow cytometer (Becton Dickinson). At least 100.000 live events were acquired, gated on small viable lymphocytes. Data files were analysed using CellQuest software (Becton Dickinson).

STATISTICS

Data are presented as means ± standard deviation (S.D.) of the mean. Comparisons among groups were performed by using *t*-test. The correlation between viremia and percentage of HIV-specific CD8 T-cells was analyzed by the non-parametric Spearman rank test. Data were analyzed by using GraphPad Prism Software version 4.00.

RESULTS

Correlation between HIV-RNA viral load, CD4 and CD8 T-cells

The immunological and virological characteristics of HIV-infected patients are described in Table 1.
Vertically infected patients were under therapy for 4.36±0.93 years and the therapy was effective in all of them as demonstrated by plasma HIV-RNA viral load below 50 copies/ml and they could be define as viral controllers. The HIV-RNA viral load was significantly higher in patients from horizontally infected group (22.870±40.995 copies/mL) compared to the viral controller group (p<0.001), from now on defined as viremic patients.
CD4 T-lymphocytes were not statistically different between viremic patients and viral controllers both in terms of frequency (34.66%±8.57% *vs* 34.35%±6.61%) and cell count (1046.9±472.1 cells/µl *vs* 1101.3±415.4 cells/µl; p>0.05 all comparisons).
On the contrary, the CD8 T-cells count was significantly higher in the viremic patients, compared to viral controllers (1080.6±652.1 cells/µl *vs* 747.5±389.9 cells/µl, p<0.05).

HIV-specific CD8 T-cell response

HIV-specific response was analysed by a multi-parametric analysis of IFN-γ released during HIV-peptides stimulation. The mean percentage of IFNγ-positive CD8 T-cells in viremic patients and viral controllers was similar (Figure 1 panel A).

TABLE 1 - *Main characteristic of viraemic patients and viral controllers HIV-infected children.*

	Age	HIV-RNA copies/mL	CD4%	CD4/μl	CD8/μl	Antiretroviral Therapy
Viraemic patients (n=27)	9.00±3.5	22.870±40.995	34.66±8.57	1046.9±472.1	1080.6±652.1	Untreated or low adherence
Viral controllers (n=17)	8.14±3.51	<50	34.35±6.61	1101.3±415.4	747.5±389.9	4.36±0.93[1]
P	ns	<0.001	ns	ns	<0.05	

Data are expressed as mean ± standard deviation. [1]Time on therapy (years).

Specifically, 0.09%±0.01% HIV-specific IFNγ-positive CD8 T-cells in viremic patients and 0.06%±0.09%, in viral controls. Fifteen out of 27 viremic patients showed a frequency of HIV-specific CD8+ lymphocytes higher than 0.02% and seven of them had a frequency higher than 0.1%. When we analysed separately the reactivity to HIV peptides in viremic patients showing a low plasma viral load below 1000 copies/mL (n=8) that is associated with transient episodes of viremia (Palma *et al.,* 2007; van Sighem *et al.,* 2008), or high plasma viremia above 1000 copies/mL (n=19) (Figure 1 panel B), the percentage of HIV-specific CD8 T cells in the group with high viral load was higher than in the group with low viremia as previously described by Buseyne (Buseyne *et al.,* 2002). However, six out

of 19 patients with high viral load were not able to produce IFN-γ to HIV-peptides stimuli, as also suggested by a weak negative correlation between viremia and percentage of HIV-specific CD8 T-cells (R^2=0.015, p<0.03).

Naïve memory CD8 T-cells phenotype
To investigate the memory phenotype of CD8 T-cells, the expression of CD45RA and CD27 on CD8 T lymphocytes was analysed by flow cytometry. Figure 2 shows data of viremic patients with undetectable HIV-specific CD8 T-cells, viral controllers and three adult healthy donors used as reference control.
The frequency of naïve CD45RA-positive CD27-positive CD8 T-cells was approximately 7-fold lower in viremic children compared to viral con-
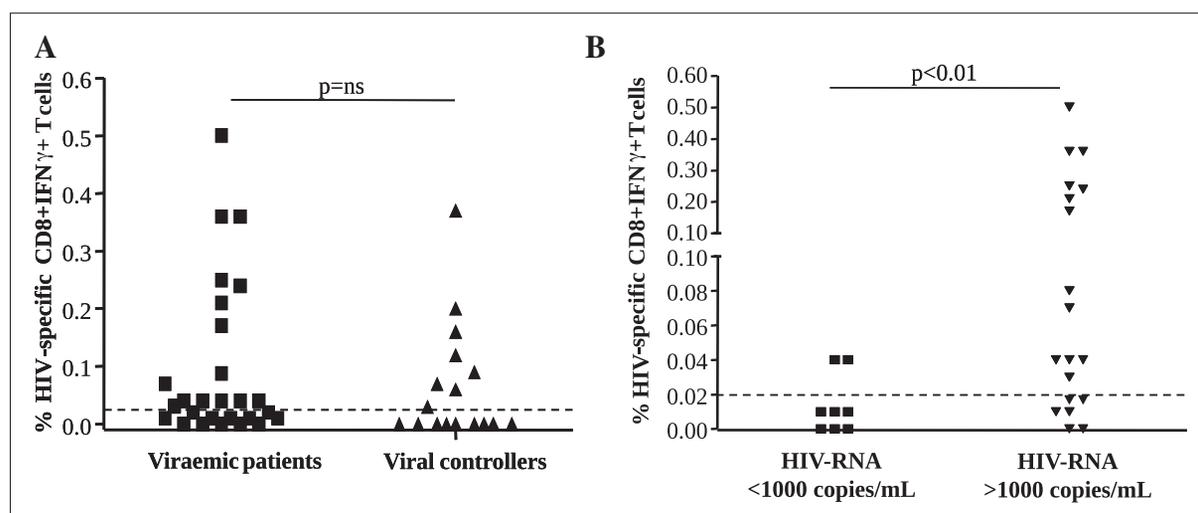
FIGURE 1 - *Frequencies of HIV-specific CD8 T-cells in peripheral blood of a. viremic patients and viral controllers and b. viremic patients with plasma viral load below or above 1000 HIV-RNA copies/ml. The frequency of IFN-γ-producing CD8 T-cells were measured after HIV-specific (Gag, Tat and Nef peptides) stimulation by intracellular flow cytometry. Dashed lines represent the threshold. Differences between groups were evaluated by t-test.*
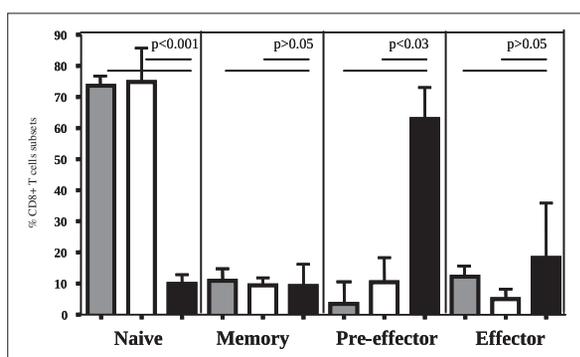
FIGURE 2 - *Frequencies of CD8 T-cell naive/memory subsets in peripheral blood of viremic patients with high viral load and undetectable HIV-specific CD8 T-cells (black bars), viral controllers (white bars) and adult healthy controls (dotted bars). The naive/memory phenotype of CD8 T-cells was defined by CD45RA and CD27 expression as naive (CD45RA+CD27+), memory (CD45RA-CD27+), pre-effector (CD45RA-CD27-) and effector (CD45RA+CD27-) CD8 T-cells. Bars represents mean value ± standard deviation. Differences between groups were evaluated by t-test.*

trollers (p<0.001). Moreover, viremic patients present an expanded pre-effector CD45RA-negative CD27-negative subset of CD8 T-cells (62.8%±10.21%) compared with viral controllers (10.37%±7.91%, p<0.03) and adult healthy controls (3.55%±7.22%, p<0.03). No differences were observed in central memory and effector CD8+ T cell subsets between groups.

DISCUSSION

HIV-infected children who respond to ART achieve suppression of viral load and an increase in CD4+ T lymphocytes (Borkowsky *et al.*, 2000). These events are paralleled by a decreased immune activation, with lower effector CD8+ T cells and an increase in activated CD4+ T cells (Resino *et al.*, 2003; Resino *et al.*, 2004).

Firstly we observed that although viremic patients and viral controllers showed significantly different viral loads, they had similar CD4 count and similar HIV-specific CD8 response. Moreover, both analysed groups showed a normal value of CD4 T-cell count demonstrating that the different route of infection does not affect the immune response of HIV-positive children. In spite of, vertical HIV infection is related to a faster disease progression and decrease in CD4 count compared

to the horizontally infected patients (Goulder *et al.*, 2001).

Secondly, the viremic patients with plasma HIV-RNA >1000 copies/ml showed a higher percentage of HIV-specific CD8 lymphocytes than children with viremia <1000copies/mL confirming a positive association between HIV-specific response and viral load (Buseyne *et al.*, 2002). However, some children with long exposure to high viremia showed no HIV-specific CD8 lymphocytes suggesting an inability to respond to HIV stimuli. By performing a correlation analysis of viremia and HIV-specific CD8 lymphocytes we observed a negative association between them, in agreement with data by Buseyne (Buseyne *et al.*, 2005) that showed a positive correlation between viremia and HIV-specific CD8 lymphocytes was found only in children receiving ART, while a negative correlation was observed in children not receiving ART.

Finally, children with high viral load and without HIV-specific CD8 lymphocytes showed a massive alteration of naïve/memory/effector phenotype pattern of CD8 T lymphocytes regardless of antigen specificity, with a major loss of naïve cells and a massive expansion of not fully differentiated and functional CD8+ T lymphocytes.

These data are consistent with exhaustion of HIV-specific CD8 T cells, naïve CD8 T cells regardless antigen specificity and skewed maturation of CD8 memory subset due to chronic immune activation induced by persistent exposure to viremia (Hazenberg *et al.*, 2003).

All this information confirms that although viremic patients show a normal number of CD4 T cells, they are deeply immunocompromised as demonstrated by the low frequency or absence of HIV-specific CD8 T lymphocytes and by loss of naïve CD8 T cells. The over-stimulation of immune system leads to loss of HIV-specific CD4 and CD8 clones (McMichael and Rowland-Jones, 2001) and to a general immunosuppression with impaired antigen-specific CD4 and CD8 maturation. These data imply exposure of children to opportunistic infections and increased risk for developing tumors (Hazenberg *et al.*, 2003; Aoki and Tosato, 2004).

In conclusion, since the monitoring of CD4-cell count and CD4-cell percentage and total lymphocytes count in children are complicated by the effect of age due to large natural decline of

lymphocytes and CD4 cells in early life, monitoring the immunocompetence of HIV-infected children by the evaluation of naïve/memory subsets might help better define the progression of HIV-infection.

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