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# Poor recognition of HIV-1 Nef protein by CD8 T cells from HIV-1-infected children: Impact of age

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**Abstract**

Recognition of various HIV proteins by CD8 T cells from HIV-infected children was determined by two functional assays. First, using an Elispot assay, we show that 80% of patients recognized Gag, 77% recognized Pol, 61% recognized Env, 44% recognized Nef and 29% recognized Vif. Frequencies of Gag-, Pol-, and Env-specific IFN-γ producing CD8 T cells were higher than frequencies of Nef and Vif-specific CD8 T cells. The poor recognition of Nef by ex vivo CD8 T cells was confirmed by CTL assays performed in HAART naïve children: 25% of children had positive response against Nef versus 44, 63 and 62% for Env, Gag, and Pol, respectively. Memory Gag-specific CTL were positively correlated with age, whereas Nef-specific CTL were negatively correlated with age. The poor Nef-specific CD8 T cell response in HIV-infected children contrasts with dominance of Nef-specific responses in infected adults.


*Keywords:* HIV; HIV perinatal infection; Elispot; CTL; CD8 T cell; Children

## Introduction

HIV-specific CD8 T cells play a major role by suppressing HIV replication. However, characteristics and/or subsets of the HIV-specific CD8 T cell response that correlate with their in vivo antiviral potency are not completely defined. The diversity and specificity of HIV-specific CD8 cells may be important for the efficiency of this immune response. The diversity of the CD8 T cell response may help to control virus bearing immune escape mutations. The specificity is another important determinant of CD8 T cell's antiviral activity. As an example, earlier epitope expression increases the antiviral efficiency of CD8 T cells, probably through faster killing of productively infected cells (Yang et al., 2003b). Recent reports on immune recognition of the whole HIV genome highlight modification of the CD8 T cell repertoire during the course of HIV infection (Goulder et al., 2001; Alter et al., 2004). In particular, the HIV-specific CD8 repertoires from patients in acute and chronic phase of infection differ. Evolution of the CD8 repertoire is probably driven by continuous stimulation, as early antiretroviral treatment that suppresses viral replication allows persistence of early CD8 responses (Alter et al., 2003; Lichterfeld et al., 2004). Furthermore, in chronically infected untreated adults, diversity of HIV proteins recognition by CD8 T lymphocytes decreases faster in patients with high viral load (Kousignian et al., 2003). If the HIV-specific CD8 T cell repertoire is driven, at least in part, by the level of viral replication, one might expect differences between the CD8 repertoire of children and adults, as children have higher level of viral replication than adults (Mofenson et al., 1997; Rouzioux et al., 1997; Shearer et al., 1997; Palumbo et al., 1998).

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The present report is based on two independent sets of data. We first defined recognition of seven viral proteins by HIV-specific CD8 T cells from infected children using the highly sensitive ex vivo IFN-γ-based Elispot assay. This study revealed poor recognition of the nonstructural HIV-1 proteins. In particular, rate of children with positive Nef-specific responses was lower than rates of children responding to structural proteins Env, Gag, and Pol. The low response to Nef protein contrasts with other reports on the CD8 response from adults (Betts et al., 2001; Addo et al., 2003; Bansal et al., 2003; Cao et al., 2003; Currier et al., 2003; Draenert et al., 2004; Masemola et al., 2004). At time of assessment of CD8 response using the ex vivo Elispot assay, a significant fraction of children were receiving or previously received potent antiretroviral (ARV) therapy that alters HIV-specific CD8 T lymphocytes frequency. Therefore, we performed a retrospective analysis of memory cytotoxic T lymphocytes (CTL) from children that never received potent ARV combination therapy. This second study confirmed the poor recognition of Nef by CD8 T cells from infected children and showed that the HIV-specific CD8 T cell specificity varied according to age, with decreased Nef-specific CTL and increased Gag-specific CTL in the oldest children.

## Results

### *Most HIV-specific CD8 T cells detected with an IFN-γ-based ex vivo Elispot assay recognize HIV structural proteins*

Recognition of HIV proteins by CD8 T cells from infected children was systematically tested on blood samples from 51 HIV-1-infected children. Their freshly isolated PBMC were incubated with rVV encoding HIV-$1_{Lai}$ Env, Gag, Pol, Nef, Vif, Tat, or Rev, and IFN-γ production was measured by Elispot assay, as described (Buseyne et al., 2005a). The most frequently recognized proteins were Gag (80% of children had positive responses), Pol (77%), and Env (61%) (Fig. 1A). Among nonstructural proteins, Nef and Vif were recognized by 44 and 29% of children tested, respectively. None recognized either Tat or Rev. In patients with positive responses, the frequencies of IFN-γ producing cells varied according to the HIV protein recognized, with mean values ranging from 822±822 SFC/$10^6$ PBMC for Gag to 97±51 SFC/$10^6$ PBMC for Vif (Fig. 1B). Frequencies of IFN-γ producing cells were significantly different when positive responses against five HIV antigens were compared (ANOVA, $p<0.0001$). The Bonferroni/Dunn test indicated significant differences between the frequencies of IFN-γ producing cells in response to Nef and Gag ($p<0.0001$), Nef and Env ($p=0.004$), Nef and Pol ($p=0.003$), Vif and Gag ($p=0.0009$), and Vif and Pol ($p=0.003$). A significant number of patients was of non-European origin and infected by nonclade B HIV-1 isolates (see Patients and methods). We may have underestimated the CD8 response of these patients, as we used HIV-1 antigens derived from a clade B isolate. Therefore, the analysis was restricted to the 36 patients with documented HIV-1 clade B infection. Percent of patients with positive responses were 73, 83, 88, 57 and 33% and mean of positive responses were 526±543, 827±739, 739±665, 178±178 and 83±40 SFC/$10^6$ PBMC for Env, Gag, Pol, Nef and Vif respectively (ANOVA, $p<0.004$). Thus, the hierarchy of HIV-1 protein recognition was similar for HIV-1 clade B-infected children and the whole group, with slightly higher percentages of positive responses for the former. In conclusion, HIV-specific IFN-γ producing cells from infected children targeted structural HIV-1 proteins (Env, Gag, and Pol) more frequently than the four nonstructural proteins tested (Nef, Vif, Tat, and Rev). In addition, responder patients have higher frequencies of IFN-γ producing cells specific for structural proteins than for nonstructural proteins.

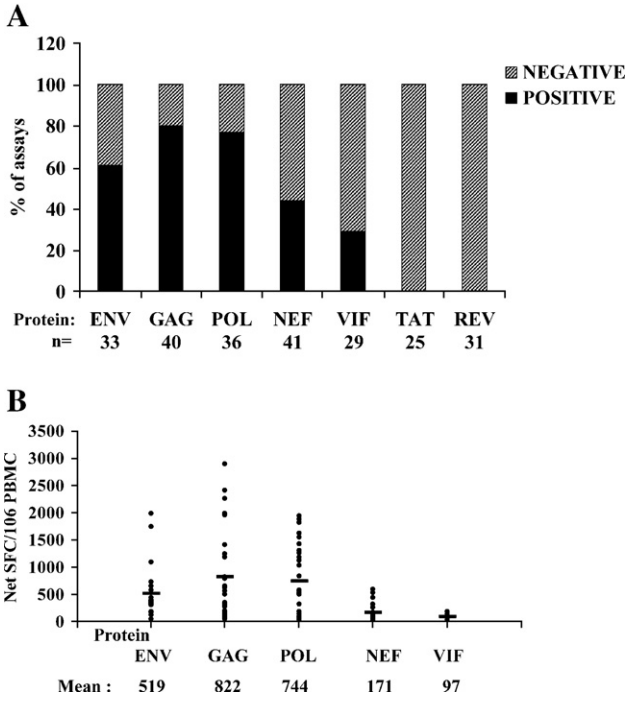


Fig. 1. The majority of HIV-specific IFN-γ producing cells from infected children recognize the structural proteins of the virus. Blood samples obtained from 51 HIV-1-infected children were tested for their IFN-γ production in response to rVV encoding HIV-$1_{Lai}$ Env, Gag, Pol, Nef, Vif, Tat, or Rev. For each HIV protein, percentages of positive and negative assays are presented in panel A. For positive assays, frequencies of antigen-specific IFN-γ producing cells, expressed as net SFC/$10^6$ PBMC, are presented in panel B. Black bars represent mean values.

Potent ARV therapy significantly affects the frequency of HIV-specific CD8 T cell responses. Neither the rate of children with positive responses nor the frequencies of specific IFN-γ producing cells differed according to treatment status ($p>0.05$ for all $\chi^2$ and ANOVA tests). Of note, only 8 out of the 28 children treated with 3 or 4 ARV molecules had suppressed viral replication at time of assay. Using the same Elispot assay and a rVV encoding Env, Gag, and Pol antigens simultaneously, we recently reported that frequency of HIV-specific IFN-γ producing cells was strongly associated with age in untreated children, and was associated with both age and viremia in children receiving potent combination ARV (Buseyne et al., 2005b). Here, we observed a significant positive correlation between the frequency of Gag-specific CD8 T cells and age when both the whole group and untreated children were

analyzed (Pearson's $r=0.551$, $p=0.0002$, $n=39$; untreated children: Pearson's $r=0.557$, $p=0.03$, $n=15$). The frequency of CD8 T cells with other specificities was not related to age (data not shown).

*Differences in the rate of recognition of HIV proteins by CD8 T cells are not due to use of recombinant Vaccinia viruses*

We observed low-level recognition of the Nef protein compared to other publications (Addo et al., 2003; Bansal et al., 2003; Cao et al., 2003; Draenert et al., 2004; Masemola et al., 2004). In these reports, pools of peptides were used to induce IFN-γ production by PBMCs whereas we used rVV. Therefore, we compared peptide pools and rVV as source of antigens in the ex vivo Elispot assay for two HIV-infected children and one HIV-infected adult patient (Fig. 2A). Frequencies of Gag and Nef IFN-γ producing cells were of equivalent magnitude whether HIV proteins were encoded by rVV or presented as pools of peptides. Furthermore, IFN-γ production by PBMC from 13 HIV-infected children in the presence of Gag and Nef peptide pools was measured by intracellular cell cytometry (Fig. 2B). Frequencies of Gag-specific CD8 cells were significantly higher than those of Nef-specific CD8 cells (ANOVA, $p=0.02$). Ten out of the 13 children had positive response against Gag and 5 had positive response against Nef. Among responder patients, percents

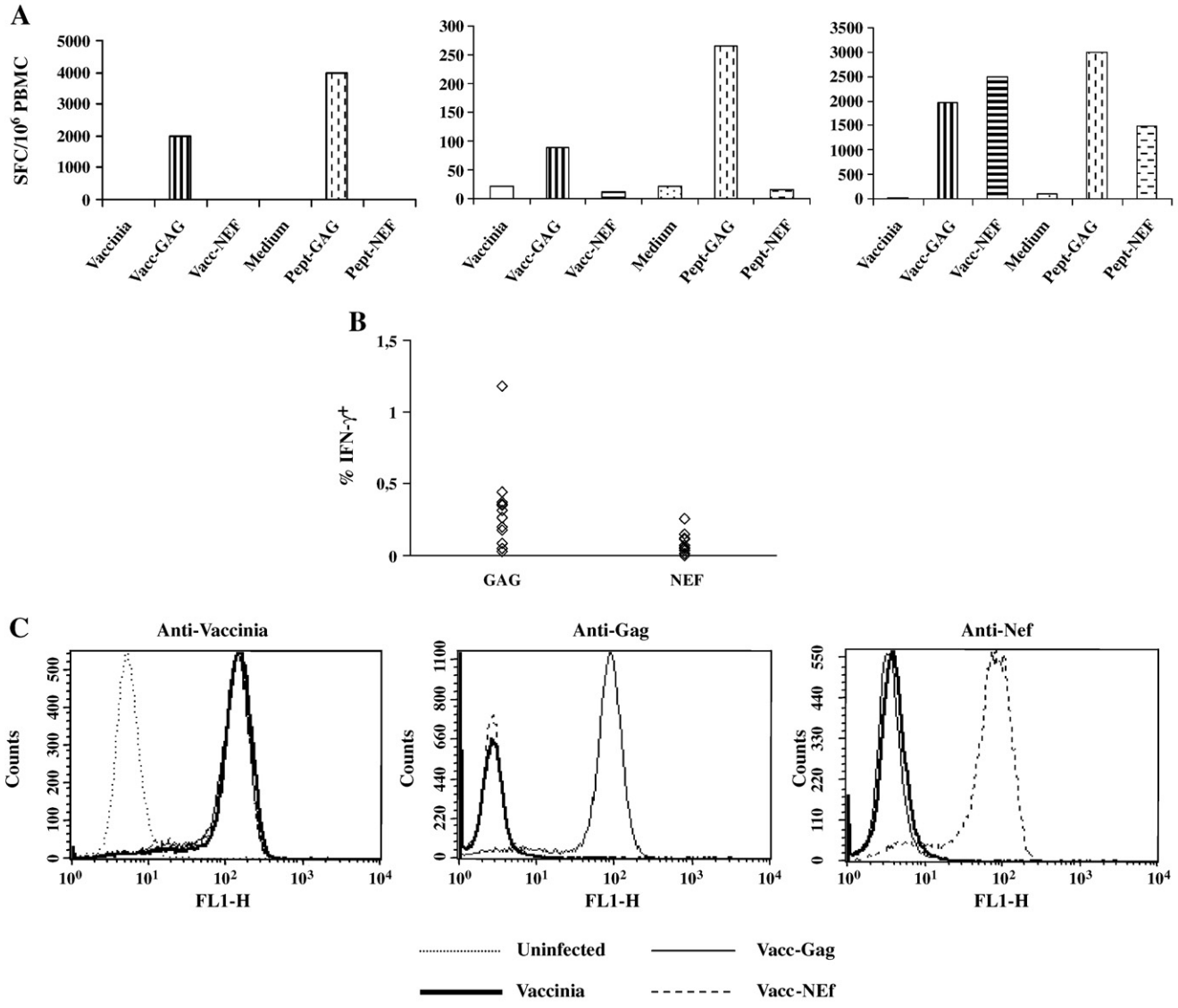


Fig. 2. Differences in the rate of recognition of HIV proteins by CD8 T cells are not explained by the level of antigen expression by recombinant Vaccinia Viruses. (A) PBMC from two children (left panels) and one adult (right panel) were tested for IFN-γ production using the Elispot assay in response to HIV-1 Gag and Nef proteins, using rVV or pools of overlapping peptides. (B) Freshly isolated PBMC from 13 HIV-1-infected children were stimulated overnight with pools of peptides spanning the whole Gag and Nef sequence in the presence of brefeldin A. After 16 h, PBMC were stained with an anti-IFN-γ-PC7 monoclonal antibody and cells were analyzed by flow cytometry. Percent IFN-γ specific lymphocytes among total lymphocytes are presented. (C) Epstein-Barr transformed B lymphocytes were either left uninfected or infected with wild-type Vaccinia, rVV encoding HIV-1 Gag protein (Vacc-Gag), or rVV encoding HIV-1 Nef protein (Vacc-Nef). After 16 h of infection, cells were intracellularly labeled with antibodies specific for Vaccinia, HIV-1 Gag, or HIV-1 Nef (as indicated above the panels) and a FITC-labeled anti-mouse IgG serum. Fluorescence intensity is represented in histograms.

IFN-γ producing HIV-specific lymphocytes were 0.37±0.28 for Gag and 0.16±0.05 for Nef.

Protein expression by all rVV was measured by Western blotting at time of vector construction, was regularly controlled by immunolabeling, and these rVV allowed detection of HIV-specific CTL of all specificities (Buseyne et al., 2002a; Chouquet et al., 2002). Difference in the rate of recognition of HIV proteins by infected patients could be related to the level of antigen expression. In order to compare the expression of Nef and Gag proteins in rVV-infected cells, Epstein-Barr transformed B lymphocytes were infected by rVV and then labeled with antibodies specific for Vaccinia antigens, HIV-1 Gag or HIV-1 Nef. Intensity of Vaccinia protein expression was similar for wild-type Vaccinia and both rVV (Fig. 2C, left panel). HIV-Gag and HIV-Nef expression were detectable, with similar fluorescence intensity, in Vacc-Gag and Vacc-Nef-infected cells, respectively (Fig. 2C, right panels). Altogether, these experiments exclude that low-level recognition of the HIV Nef protein was due to low or absent antigen expression by rVV-infected cells.

*Recognition of HIV Env, Gag, Pol and Nef proteins by in vitro stimulated CTL*

We were interested in the low levels of responses against regulatory proteins. In particular, the Nef-specific CD8 T cell response from our group of HIV-infected children was strikingly lower than the Nef-specific responses reported in adult patients using ex vivo IFN-γ production assays (Addo et al., 2003; Bansal et al., 2003; Cao et al., 2003; Draenert et al., 2004; Masemola et al., 2004). The Elispot assays presented in Fig. 1 were performed during the 1999–2001 period, when a significant proportion of children were receiving potent combination that has profound impact on the CD8 T cell responses in infected children (Spiegel et al., 2000; Scott et al., 2001). We therefore decided to perform a retrospective analysis of HIV-specific CD8 T cells children that never received combination therapy including protease or nonnucleosidic reverse transcriptase inhibitors (HAART naïve). At that time, CD8 T cells responses were measured by the $^{51}$Cr release assays performed after in vitro expansion of PBMC, and recognition of four HIV proteins (Env, Gag, Pol, and Nef) was systematically tested on each blood sample (Buseyne et al., 1993, 1998a, 2002a).

Sixty-three patients that never received nNRTI or PI have been tested for their cytolytic activity. The following analyses were based on the assay performed at study entry. Percent of children with positive responses were 44, 63, 62 and 25 for Env, Gag, Pol and Nef, respectively. Magnitude of positive memory CTL assays, expressed as AUC, was 826±416, 898±601, 983±461 and 870±592 for Env, Gag, Pol, and Nef, respectively (ANOVA $p=0.66$). For the forty-seven children with documented infection with HIV-1 subtype B, percent of children with positive responses were 47, 70, 64 and 19% and mean intensities of positive CTL assays, expressed as AUC, were 853±408, 891±605, 1008±475 and 906±533 for Env, Gag, Pol, and Nef, respectively (ANOVA $p=0.48$). Thus, in absence of potent combination therapy, CTL responses from HIV-infected children targeted more frequently the three structural proteins than the Nef protein.

During chronic viral infection, recognition pattern of antigens by CD8 T cells evolved differently according to their specificity (Goulder et al., 2001). We therefore investigated whether age, which is approximately equal to the duration of

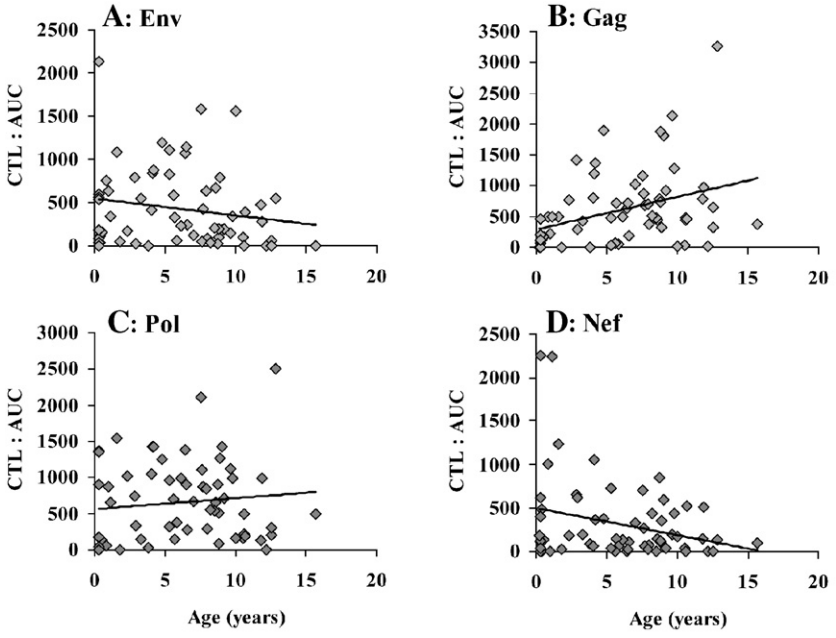


Fig. 3. Correlation between memory CTL and age in HAART naive children. Results from CTL assays from 63-infected children are expressed as area under the curve (AUC) and are presented as a function of age (expressed in years). Linear regression curves are shown. (A) Env-specific memory CTL; (B) Gag-specific CTL; (C) Pol-specific CTL; (D) Nef-specific CTL.

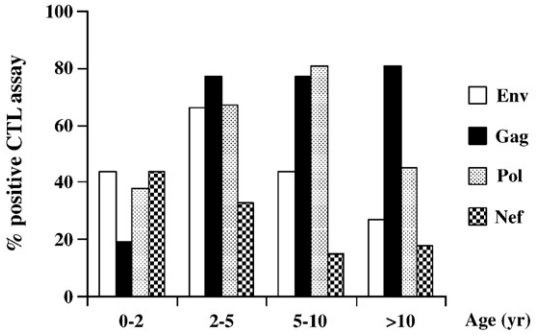


Fig. 4. Frequency of HAART-naive children with positive memory CTL response. The sixty-three children presented in Fig. 2 were stratified into four age groups (0–2 years old, $n=16$; >2 and ≤5 years old, $n=9$; >5 and ≤10 years old, $n=27$; >10 years old, $n=11$). Plasma viral load from the four age groups were (from the youngest to the oldest): 5.7±0.4, 4.5±0.4, 4.2±0.8 and 4.1±1.3 log(HIV-RNA per ml of plasma), and their CD4+% were: 34±13, 33±10; 22±10, and 27±10. Percent of positive responses against each of the four HIV proteins are presented for each age group.

infection for the perinatally infected children, was related to the magnitude of memory CTL. Intensities of Gag-specific memory CTL were positively correlated with age (Pearson's $r=0.346$, $p=0.006$; Fig. 3B). In contrast, Nef-specific memory CTL were negatively correlated with age (Pearson's $r=-0.296$, $p=0.02$, Fig. 3D). The correlation between age and Gag-specific memory CTL was confirmed using the Spearman rank test, but the correlation between age and Nef-specific memory CTL was not. Intensities of Env- and Pol-specific memory CTL were not correlated with age (Pearson's $r=-0.176$, $p=0.16$ and $r=0.113$, $p=0.38$ for Env- and Pol-specific memory CTL, respectively, Figs. 3A and C).

Then, children were stratified according to their age at time of CTL assay and rates of children with positive memory CTL responses were calculated for each target protein (Fig. 4). In children less than 2 years old, rates of patients with positive memory CTL assays against Env, Pol and Nef were similar, around 40%. Rate of responder patients against Gag was twice lower (19%). Rates of positive memory CTL responses against Env- or Pol followed a bell-shaped curve as age increased, with rates of responders being higher in children from the median age groups (2–5 years old for Env, and 5–10 years old for Pol), than in young (less than 2 years) or old children (>10 years). Rate of responders against Nef declined with age. In contrast, rate of responders against Gag was much higher after 2 years of age (over 70% in the 3 age groups) than for children below 2 years of age (19%). Thus, the pattern of HIV antigens recognized by CD8 T cells from HAART naive HIV-infected children varies according to age and to the target protein.

## Discussion

The first part of this study was designed to determine the targets of the HIV-specific CD8 T cell with ex vivo effector function in HIV-1-infected children. The highest rates of children with positive responses were observed for Env, Gag, and Pol proteins. Nef and Vif proteins were recognized by lower rates of children and no response against Tat or Rev was observed. Furthermore, for positive responses, frequencies of IFN-γ producing cells specific for the three structural proteins were higher than those of cells specific for Nef and Vif. In adult patients, the regulatory (Tat and Rev) and accessory proteins (Vif, Vpu, Vpr) contribute modestly to the total magnitude of the CD8 T cell response and/or were less frequently targeted when compared to Env, Gag, Pol, and Nef (Addo et al., 2003; Cao et al., 2003; Draenert et al., 2004; Masemola et al., 2004). In HIV-infected children, weak recognition of Vif, Tat, and Rev was reported as well (Feeney et al., 2003; Sandberg et al., 2003). Thus, the weak recognition of Vif, Tat, and Rev in the present report is in line with previous reports on the HIV-specific CD8 response from both infected children and adults.

One striking feature of our results was the low frequency of children with detectable CD8 T cell responses against Nef, and the low magnitude of these Nef-specific responses using the Elispot assay. We provided evidence that low-level recognition of Nef was not due to low-level expression of the protein by the rVV. Reduced Nef recognition in HIV-infected children could be due to the expression of functional Nef by rVV leading to down-regulation of MHC-I molecule. However, higher level of Gag-specific than Nef-specific cells was observed when peptide pools spanning the whole protein sequence were used. This result excludes that poor Nef recognition is due to MHC-I down regulation induced by the functional Nef protein encoded by rVV. Nevertheless, we cannot formally exclude that the Nef-induced MHC-I down-regulation in rVV-infected cells further dampen detection of Nef-specific CD8 T cells.

There are potential limitations for interpreting our data. One of these is sequence mismatching between HIV strains infecting patients and the HIV strain used for CD8 detection. This caveat is inherent to any CD8 T cell study and leads to underestimation of either frequency of positive response or intensity of response. We previously evaluated cross-recognition of clade B and nonclade B HIV isolates by CTL lines from the same study group, and found that cross-recognition was the predominant pattern (Buseyne et al., 1998b). Level of cross-recognition were higher for Nef (83%) than for Gag (67%) (Buseyne et al., 1998b), in accordance with another report based on 250 individuals from 4 continents (Coplan et al., 2005). In the present study, around 20% of patients harbor nonclade B virus, reflecting their diverse geographic origin. When we limited the analysis to patients with documented clade B infection, percentage of patients with positive responses and magnitude of responses varied modestly, and hierarchy of HIV proteins recognition was unchanged. Therefore, it is unlikely that low level of Nef-specific CD8 responses compared to Gag-specific CD8 responses could be explained by the diversity of HIV-1 strains infecting the patients.

The poor Nef-specific response can be explained by its shorter size compared to those of the three structural proteins and its higher sequence variability when compared to Gag and Pol. In our study, dividing the percentage of responding patients to each protein by the protein length expressed in amino acids,

as performed by others (Addo et al., 2003), gave scores of 0.21, 0.16, 0.07 and 0.07 for Nef, Gag, Pol, and Env responses with the Elispot assay and 0.12, 0.12. 0.06, and 0.05 with the $^{51}$Cr release assay. So, when the size of the Nef protein is taken into account, its recognition by CD8 T cells is similar or greater than that of structural proteins. Nevertheless, the level of Nef recognition by our studied patients was low relative to other published results in adults, as discussed below.

In adult patients, magnitude of responses and frequencies of responders against HIV proteins varied according to reports, but the relative dominance of Env, Gag, Pol, and Nef was constantly observed, with rate of patients responding to Nef ranging from 57 to 95% (Betts et al., 2001; Addo et al., 2003; Bansal et al., 2003; Cao et al., 2003; Currier et al., 2003; Draenert et al., 2004; Masemola et al., 2004). The reduced recognition of Nef by perinatally infected patients was not observed by Feeney et al. (2003), but a study from Sandberg and colleagues reported Nef-positive responses in only 25% of children, whereas 50 to 70% of them recognized Env, Gag, or Pol (Sandberg et al., 2003). Another report showed that CD8 T cell from pediatric patients respond less frequently to the Nef protein and their responses were of lower magnitude when compared to adult patients (Chandwani et al., 2004). This study used both peptides and Vaccinia viruses, excluding biases linked to the source of antigen in the in vitro assay. Parallel study of patients matched for therapy history and duration of infection are required to definitively prove a reduced CD8 T cell response to Nef during perinatally acquired HIV infection, when compared to infection during adulthood. But our observation and those of other investigators (Sandberg et al., 2003; Chandwani et al., 2004), both suggest that HIV infection as newborn or as adult are associated with different repertoires of the CD8 T lymphocytes during chronic infection.

The study of ex vivo IFN-γ producing cells by the Elispot assays began in mid-1999. At that time more than half of tested children received potent ARV therapy including at least a protease inhibitor or a nonnucleosidic reverse transcriptase inhibitor. Suppression of viral replication leads to reduced antigenic stimulation of CD8 responses that usually decrease in treated children (Luzuriaga et al., 2000; Spiegel et al., 2000; Buseyne et al., 2002b, 2005b). Therefore, we performed a retrospective analysis of memory CTL from HAART naive children, whose CD8 responses and disease progression were not affected by potent therapy. One major difference with the Elispot assay was that the $^{51}$Cr release assay was performed after in vitro culture on selected lymphocytes with proliferative capacity. The proliferative activity of CD8 T cells is associated with high perforin content and control of disease progression (Migueles et al., 2002). As the specificity of the CTL response is known to change during the course of HIV infection (Goulder et al., 2001; Alter et al., 2004), we presented results from CTL assays as a function of age, which is almost equal to the duration of infection in children. This second set of data confirmed the low recognition of Nef by HIV-infected children. In addition, it showed that in absence of potent ARV therapy intensities of memory CTL responses and rates of positive responses were correlated with age. For the three structural proteins, children younger than two responded less frequently than older children. The difference between young and old children was especially marked for Gag-specific CTL. In children older than 10 years, which are mostly slow progressors, the Gag-specific response was dominant. This result is in line with the stronger inverse correlation between Gag-specific CTL and plasma viral load, when compared to CTL of other specificities (Buseyne et al., 2002a). In sharp contrast to CTL targeting structural proteins, Nef-specific memory CTL were more frequently detected in children before the age of two, than after. Age in vertically infected children reflects the duration of infection, and our results are consistent with earlier detection of Nef-specific CTL when compared to Env, Gag, or Pol-specific CD8 T cells in adults (Alter et al., 2002; Addo et al., 2003; Lichterfeld et al., 2004). However, CTL decrease with disease progression has been described in untreated adults (Chouquet et al., 2002), but preferential loss of Nef-specific CTL has never been reported. Low rates of Nef-specific responses were observed in oldest children compared to CTL of other specificities. This suggests that Nef-specific CTL disappear earlier than CTL specific for structural proteins. A longitudinal study will be required to formally demonstrate different kinetics of HIV-specific CTL according to their specificity. As for each child, a single CTL assay against Env-, Gag, Pol and Nef was performed on the same blood sample, our data definitely show that the recognition pattern of HIV proteins by CD8 T cells differs according to age/duration of infection over two decades and in absence of treatment.

Our results raise the question of mechanisms that could lead to difference in the HIV-specific CTL repertoire across the age groups. Appearance of epitope escape mutation is usually followed by the decay of specific CTL (Evans et al., 1999; Jamieson et al., 2003). In vitro, emergence CTL escape mutations is more rapid and consistent in Nef than in Gag or RT sequences (Yang et al., 2003a). So, CTL specific for structural proteins may be detected over longer period than Nef-specific CTL, due to a lower rate of escape mutation in the former. Interestingly, it was shown that the frequency of mutations within CTL epitopes correlates positively with the viral set-point (Barouch et al., 2002), which is higher for pediatric patients than for adults and may accelerate the loss of CTL directed to nonstructural proteins in infected children (Mofenson et al., 1997; Rouzioux et al., 1997; Shearer et al., 1997; Palumbo et al., 1998). A more speculative explanation for the difference in rate of CD8 responses to Nef and Gag according to age, could be their different sensitivity to the deleterious effect of antigen persistence and antigen level, as shown for other proteins in murine viral infection (Probst et al., 2003). Evolution of the CTL response could be linked to changes in viral sequence affecting expression level and function. In pediatric infection, age is approximately equal to duration of infection and older children are more likely to be non/slow progressors than younger ones. Accumulation of mutations affecting Nef expression have been reported in nonprogressing perinatally infected children (Geffin et al., 2000; Casartelli et al., 2003a, 2003b) and could lead to loss of specific CD8 T cells. Alternatively, reduced Nef-mediated

MHC-I down-regulation may enhance recognition of late proteins, such as Gag (Casartelli et al., 2003b).

In conclusion, we characterized the pattern of recognition of HIV proteins by CD8 T cells from infected children using two functional assays and during two different study periods. We show that the repertoires of HIV-specific CD8 T cells differ across age groups. The results suggest that attrition of Nef-specific CD8 T cells occurs faster in infected children when compared to adults. Furthermore, generation of Gag-specific CTL appeared to be delayed in the youngest children compared to CTL with other specificities, although Gag-specific responses are dominant after 2 years of age and associated with lower viral load (Buseyne et al., 2002a, 2005c).

## Patients and methods

### *Patients*

The patients were followed at Necker Hospital in Paris. Legal guardians gave informed consent before inclusion of the children in the longitudinal follow-up of their CD8 T cells activities and the study was approved by local ethic committee. The presented results are based on two sets of data. For the first study, results from Elispot assays in response to HIV-1 antigens were obtained from June 1999 to April 2001. Children included in the longitudinal follow-up were tested providing there were sufficient PBMC to perform the assay. Among the 51 children tested, 28 were receiving potent ARV therapy at time of assay. Characteristics of patients (mean±SD) were as follows: 9.1±5.0 years for age, 3.6±1.4 log (HIV RNA/ml) for plasma viral load, 30±11 for CD4 percentages. This study group comprises 33 boys and 18 girls. Geographic origins were European (32), sub-Saharan African (9), and others (10). HIV-1 subtypes were determined using heteroduplex mobility assay HMA, as previously described (Buseyne et al., 1998b), or using phylogenetic analyses performed by estimating the relationships among *pol* sequences and reference sequences of HIV-1 genetic subtypes and circulating recombinant forms obtained from the Los Alamos Database (http://hiv-web.lanl.gov) (Chaix et al., 2003). The patients harbored subtype A ($n=2$), subtype B ($n=36$), subtype D ($n=1$), subtype F ($n=2$), subtype G ($n=1$), or subtype CRF02 ($n=2$) HIV-1 strains. HIV-1 subtype was not documented for 7 children.

The second study analyzed CTL assays from 63 HAART-naive children, most of them obtained before 1996. We selected CTL assays performed at study entry and before any treatment including nonnucleosidic reverse transcriptase or protease inhibitors. Children with progression to CDC stage C or death before 12 months of age were excluded, as they have poor CTL responses (Buseyne et al., 1998a). Characteristics of patients (mean±SD) were as follows: 6.0±4.1 years for age, 4.7±1.0 log(HIV RNA/ml) for plasma viral load, 27±12 for CD4+ percentages. This study group comprises 29 boys and 34 girls. Geographic origins were European (38), sub-Saharan African (13), and others (12). The patients harbored subtype A ($n=3$), subtype B ($n=47$), subtype D ($n=5$), subtype F ($n=3$), or subtype G ($n=1$) HIV-1 strains. HIV-1 subtype was not documented for 4 children.

### *Vaccinia viruses*

Recombinant Vaccinia viruses (rVV) encoding individual HIV genes from the HIV-$1_{Lai}$ strain were obtained from Transgène, Strasbourg, France. vvTG1139, vvTG1144, vvTG3167, vvTG1147, vvTG1160, vvTG3196 and vvTG4113 encode the Env, Gag, Pol, Nef, Vif, Tat, and Rev proteins, respectively (Buseyne et al., 1998b; Chouquet et al., 2002). The parental Copenhagen Vaccinia strain was used as a negative control. Expression of Vaccinia and HIV-1 antigens was evaluated on Epstein-Barr transformed B lymphocytes infected for 16 h at a m.o.i. of 10. Cells were fixed with paraformaldehyde and labeled with an anti-Vaccinia-specific polyclonal ascite, a monoclonal Gag-specific antibody (MAb1542, Biorad, provided by Dr. B. Parekh), or a Nef-specific antibody (MATG0020 (Sol-Foulon et al., 2002)), and stained with a FITC-labeled anti-mouse IgG (Biosys). Cells were analyzed on a FACScalibur (Becton-Dickinson).

### *Elispot assay*

Elispot assays were performed as previously described with freshly isolated PBMC (Buseyne et al., 2002b, 2005a). Results presented were obtained at a cell input of $5\times10^5$ PBMC/well. rVV encoding HIV proteins were used at a final concentration of $2\times10^8$ pfu/ml. Pools of overlapping peptides (15 mers overlapping by 11 amino acids), corresponding to clade B consensus sequence were obtained from the NIH (#8117 for Gag; #5189 for Nef) and used at a final concentration of 1–2 μg/ml for each peptide. The number of spots forming cells (SFC) was determined with computer-assisted image analysis software (KS-Elispot, Zeiss, Munich, Germany). An assay was considered positive if (1) a minimum of 10 spots/well was reach in the presence of antigen, and (2) the spot number obtained in the presence of antigen exceeded the mean plus 3 SD of the spot number obtained with control antigen, and (3) the spot number obtained in the presence of antigen was at least twice the spot number obtained with control antigen. Net SFC was calculated as: (SFC obtained with antigen)−(SFC obtained with control antigen). Results were expressed as SFC/$10^6$ PBMC$=10^6\times$[(SFC number per well)/(number of cell per well)].

### *Intracellular cell cytometry*

Freshly isolated PBMC were seeded in RPMI-10% SVF and incubated overnight in the presence of medium, Gag or Nef peptide pools (NIH #8117 for Gag; #5189 for Nef). Brefeldin A was added during the whole incubation period (2 μg/ml, Sigma-Aldrich, Saint-Quentin Fallavier, France). Cells were stained with CD3-FITC (BD bioscience, Le-Pont-de-Claix, France), CD8β-PC5 (Beckman-Coulter, Villepinte, France) and CD4-ECD (Beckman-Coulter) for 30 min at +4 °C. After washing cells were fixed and permeabilized using the Intraprep kit

(Beckman-Coulter), and stained with an anti-IFN-γ-PC7 monoclonal antibody (BD Biosciences) for 30 min at +4 °C, washed and fixed with paraformaldehyde 1%. Labeled cells were analyzed on FC500 flow cytometer (Coulter Electronics Inc., Hialeah, FL). Events accumulation was followed up until to reach 250,000 living cells on the lymphocyte gate which was set up using both forward and right angle scatters. To allow comparison with data from the Elispot assay, results were expressed as percent among lymphocytes. The %peptide-specific IFN-γ+ lymphocytes were calculated as follows: [% IFN-γ+ cells in the presence of peptides] − [% IFN-γ+ cells in the presence of medium]. The cut-off value used to define positive response against peptide pools was the mean + 2 SD of %IFN-γ+ in presence of medium for the study group, that is 0.08%.

### *CTL assays*

Freshly isolated PBMC were stimulated with PHA and expanded in the presence of IL-2 for 3 weeks, and their HIV-specific cytolytic activities were tested with the conventional 4-h $^{51}$Cr release assays as previously described (Buseyne et al., 1998a, 1998b). Target cells were autologous Epstein-Barr transformed B lymphocytes infected with rVV encoding HIV antigens. The CTL responses against HIV antigens were considered positive if specific lysis exceeded the mean of control target lysis by three SD and by 10% of specific lysis, at least at the highest effector to target (E/T) ratio. Positive responses without dose-effect were discarded. Net specific lysis was defined as the specific lysis of target cells expressing HIV-1 antigen minus specific lysis of control target cells. Areas under the curve of net specific lysis (AUC) were calculated as previously described (Lubaki et al., 1999; Buseyne et al., 2002a): $(l_{60}+l_{20})\times(60-20)/2+(l_{20}+l_7)\times(20-7)/2$, where $l_{60}$, $l_{20}$ and $l_7$ are the net specific lysis at E/T ratios of 60:1, 20:1 and 7:1, respectively.

### *Statistics*

Differences in intensities of positive CD8 responses between antigens were evaluated using ANOVA. For both the Elispot and CTL assay, analyzed values followed a normal distribution. The *F* test of variance equality showed that variance of responses to antigens was equivalent for results of positive CTL assays expressed as AUC, allowing use of AUC for ANOVA. When results from positive Elispot assays towards the HIV antigens were expressed as SFC/$10^6$PBMC, variances were significantly different. Thus, results from Elispot assays were expressed as log(SFC/$10^6$PBMC) to obtain equal variance between groups and to perform statistical comparisons using ANOVA. Multiple comparisons were performed with the Bonferonni–Dunn method. Associations between quantitative variables were defined by Pearson's correlations, and confirmed using the nonparametric Spearman rank test. All *p* values were two-tailed and the significance level was set at 0.05. The analysis was performed with Statview software (SAS Institute, Cary, NC).

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