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CD8 T cell effector maturation in HIV-1-infected children

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Abstract

HIV-1 infection generates maturational responses in overall CD4 and CD8 T cell populations in adults, with elevated expression of lytic effector molecules perforin and granzyme B, and reduced expression of CCR7 and CD45RA. Here, we have found that these marked effects were significantly less pronounced in children, both in terms of the skewed CCR7/CD45RA expression profile as well as the increased perforin expression. Similar to adults, HIV-specific CD8 cells in children were largely CD27+ CD45RA− and lacked perforin. However, one pediatric subject with late-stage infection displayed robust expansion of Gag 77–85-specific CD8 T cells which were perforin+ and lytic, but lacked expression of CD27 and IFNγ. Our data indicate that the T cell effector maturation induced by HIV-1 infection is markedly weaker in children as compared to adults. The data also suggest, however, that the perforin-deficient state of HIV-specific CD8 T cells in children may be reversible.

Keywords: Human; T cells; CD8; CD4; HIV; Viral infections; AIDS; Pediatric; Perforin; Granzyme

Introduction

Adaptive CD8 T cell responses are necessary for clearance or control of many viral infections in humans. Several observations together support a pivotal role for these cells in the partial immune control of Human Immunodeficiency Virus-1 (HIV-1) replication. HIV-specific CD8 T cell responses occur concurrently with control of peak viremia in primary infection (Borrow et al., 1994; Koup et al., 1994; Musey et al., 1997). These responses exert a strong selection pressure on the virus, as evidenced by the rapid appearance of viral escape variants with amino acid substitutions in MHC class I-presented epitopes (Borrow et al., 1997; Goulder et al., 1997; Jones et al., 2004; Price et al., 1997). Slow disease progression can be associated with strong CD8 T cell responses and a healthy capacity to express antiviral cytokines and lytic effector molecules (Klein et al., 1995; Migueles et al., 2002; Pantaleo et al., 1995; Rinaldo et al., 1995). Conversely, progressive HIV-1 disease and CD4 T cell loss may be paired with progressive loss of CD8 T cell functions (Kostense et al., 2002; Sandberg et al., 2003; Shankar et al., 2000).

Based on the finding that HIV-specific CD8 T cells defined by HLA-tetramers are largely low in perforin, it was suggested that these cells may fail to mature into effector cells and they would therefore be functionally incompetent when compared to CD8 T cells specific for other viruses that cause chronic infections such as Cytomegalovirus (CMV) (Appay et al., 2000; Champagne et al., 2001). Although HIV-specific and CMV-specific CD8 T cells differ in surface phenotype, this may reflect normal differences in the nature of immune responses to diverse pathogens rather than a defect in the maturation of HIV-specific T cells (Catalina et al., 2002; Hislop et al., 2002; Ravkov et al., 2003; Tussey et al., 2003; van Leeuwen et al., 2002; Wills et al., 2002; Zhang et al., 2003). However, the recent observations that

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patients with nonprogressive disease have higher perforin expression in HIV-specific CD8 T cells (Migueles et al., 2002) and a bias towards an effector-like surface phenotype in these cells (Hess et al., 2004), suggest that the lack of effector cell properties in HIV-specific CD8 T cells contributes to lack of immune control of HIV-1.

It is also clear that HIV-1 affects the entire CD8 T cell population. Several reports have indicated a close association between the activation status of CD8 T cells and HIV-1 disease progression (Deeks et al., 2004; Giorgi et al., 1999; Hazenberg et al., 2003; Mocroft et al., 1997), and the level of CD8 T cell activation in early infection is an independent predictor of the rate of CD4 T cell decline (Deeks et al., 2004). Immune activation is likely to be directly linked to immune differentiation, and it is clear that HIV-1 infection is accompanied by loss of naive CD4 and CD8 T cells and expansion of T cells with down-modulated CD27 and CD28 expression (Brugnoni et al., 1996; Gruters et al., 1991; Papagno et al., 2004; Roederer et al., 1995). In addition, HIV-1 infection may drive the differentiation of not only HIV-specific CD8 T cells, but also of CD8 cells specific for CMV and EBV (Aandahl et al., 2004).

The functional status of CD4 and CD8 T cells throughout the course of HIV-1 infection, and in particular the ability of these cells to perform their antiviral functions through perforin-mediated lysis of infected cells, continues to be a central issue to the understanding of immune success and failure in HIV-1 infection. In this paper, we have compared HIV-1-infected adult

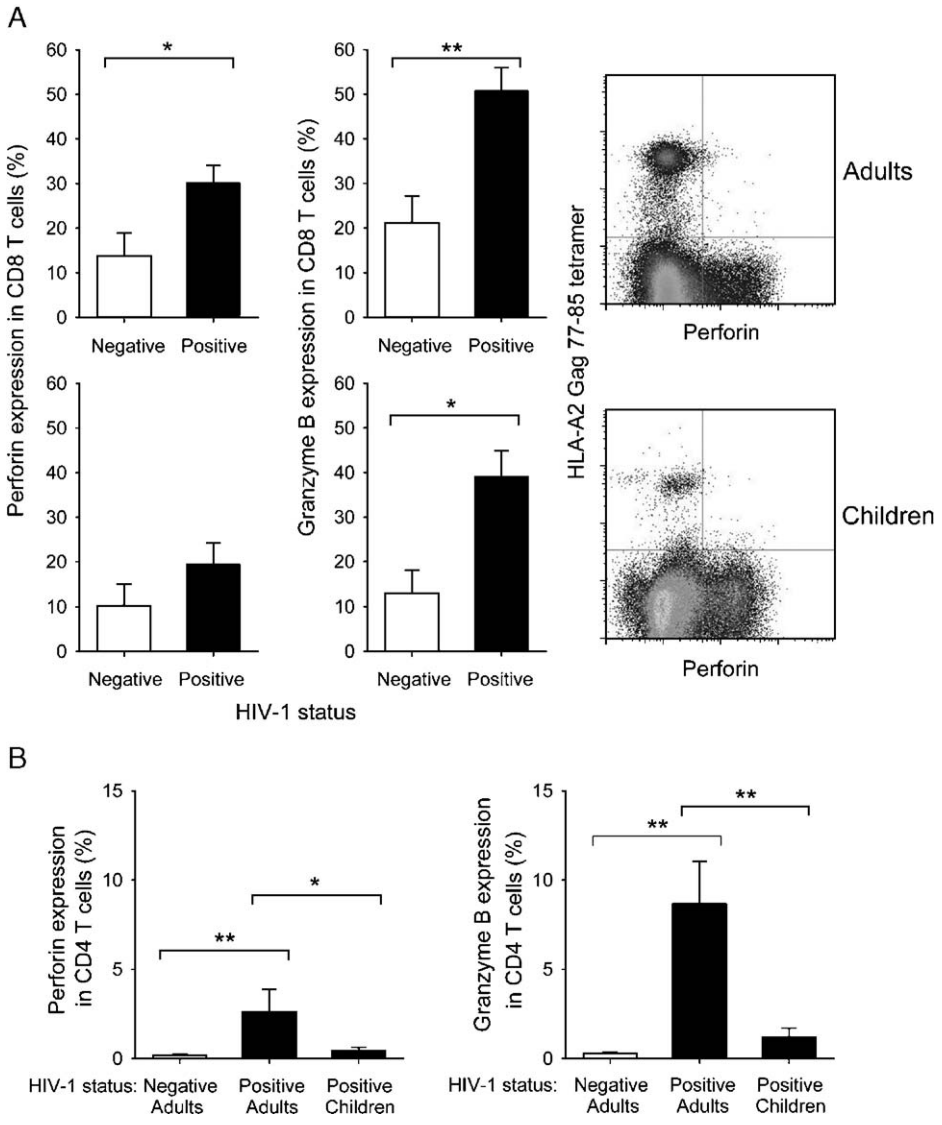

Fig. 1. Differences in expression of perforin and granzyme B in peripheral blood T cells from adult and pediatric subjects with chronic HIV-1 infection. (A) Comparison of 11 HIV-1-infected and 10 uninfected adult subjects (upper panels), and 11 HIV-1-infected and 4 uninfected pediatric subjects (lower panels) with regard to perforin and granzyme B expression in peripheral blood CD3+8+ cells assessed by flow cytometry. Mean and standard error is shown. *Indicates $P < 0.05$, and ** indicates $P < 0.005$ as determined by the t test. The panels to the right show perforin expression in CD3+8+ cells stained with HLA-A2 Gag 77–85 tetramer. One representative out of five adult subjects and one representative out of five pediatric subjects are shown. (B) CD4 T cells express lytic effector molecules in HIV-infected adult subjects. Comparison of 11 HIV-1-infected and 10 uninfected adult subjects, and 11 infected pediatric subjects with regard to perforin and granzyme B expression in CD3+4+ cells. Mean and standard error is shown. *Indicates $P < 0.05$ as determined by the Mann–Whitney rank sum test.

and pediatric hosts with a focus on the T cell maturation status and expression of effector molecules. We find that while HIV-1 infection in adults drives marked maturational responses in CD4 and CD8 T cells as assessed by CD45RA and CCR7 expression, these changes are much less pronounced in children. There is a general enhancement in perforin and granzyme B expression in both the CD8 and CD4 T cell compartments of HIV-infected adult subjects compared to age-matched uninfected controls. In children, this effect is weaker and confined to the CD8 T cell compartment. We can confirm the finding published previously that HIV-specific CD8 T cells are generally low in perforin (Appay et al., 2000; Zhang et al., 2003). However, we also present results indicating that expansion of late-stage differentiated and lytic HIV-specific CD8 T cells is possible even at advanced stages of disease, in support of a model where the lack of perforin in HIV-specific CD8 T cells represents a state of differentiation that may be reversible. The differences we observe between adults and children, and ability of CD8 T cells to focus on certain effector mechanisms and to refocus during an ongoing infection are discussed.

Results

Expression of lytic effector proteins in broad T cell populations and antigen-specific CD8 T cells in chronically HIV-1-infected children and adults

Direct lysis of target cells by CD8 T cells is primarily mediated by the perforin-dependent pathway. Here, we initially assessed the overall expression of the cytolytic effector molecules perforin and granzyme B in peripheral blood of pediatric and adult HIV-positive subjects, and uninfected controls. HIV-1 infection in adults was associated with increased expression of perforin and granzyme B in the overall CD8 T cell compartment relative to healthy controls ($P = 0.018$ and $P = 0.001$, respectively). A similar, but less distinct pattern was observed in children with a trend towards increased perforin expression ($P = 0.29$) and significantly higher granzyme B expression ($P = 0.023$) in HIV-1-infected subjects as compared with uninfected children (Fig. 1A). Granzyme B was more frequently expressed in CD8 T cells than perforin, and granzyme B+/perforin− CD8 T cells were generally CD45RO+, in contrast to the CD45RA+ phenotype observed in perforin+ cells (data not shown). We could confirm the previous finding that significant populations of perforin ($P = 0.002$) and granzyme B ($P < 0.001$) expressing CD4 T cells appear in HIV-1-infected adult subjects (Fig. 1B) (Appay et al., 2002; Norris et al., 2004). Granzyme B was more commonly expressed in these cells than was perforin ($P < 0.001$). Interestingly, CD4 T cell populations expressing perforin and granzyme B were significantly less prevalent in HIV-1-infected pediatric subjects as compared to adult subjects ($P = 0.006$ and $P = 0.001$, respectively) (Fig. 1B).

It was previously observed that HIV-specific CD8 T cells in adult patients express little perforin protein and may be inefficient in lysis of target cells (Appay et al., 2000; Zhang et al., 2003). We confirmed the observation that HIV-specific CD8 T cells were mostly negative for perforin using HLA-A2 tetramers with the Gag 77–85 epitope in adults, and also found a similar perforin-negative profile in tetramer-defined CD8 cells in children (Fig. 1A). Thus, the generalized increase in lytic effector cell differentiation in the peripheral blood CD8 and CD4 T cells of HIV-1-infected adults is less pronounced in

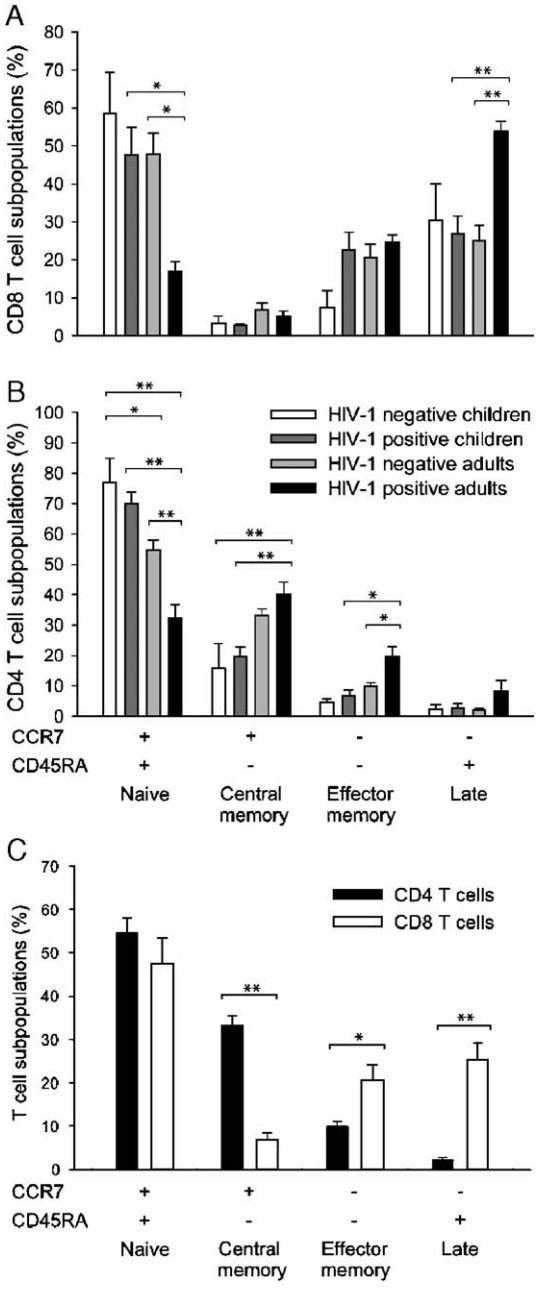

Fig. 2. State of differentiation in the CD8 and CD4 T cell compartments is less affected by HIV-1 infection in children than in adults. PBMC from 6 HIV-1-infected and 10 uninfected adults, and 10 infected and 4 uninfected children was analyzed by flow cytometry with regard to CD45RA and CCR7 expression in (A) CD3+8+ cells and (B) CD3+4+ cells, respectively. (C) Direct comparison of CD45RA and CCR7 expression in CD3+8+ cells and CD3+4+ cells in healthy adult subjects. Mean and standard error is shown. *Indicates $P < 0.05$ and **indicates $P < 0.005$ as determined by the t test or the Mann–Whitney rank sum test as appropriate.

pediatric subjects, and does not extend to HIV-specific CD8 T cells.

Analysis of naïve and memory T cell subsets defined by CD45 isoform and CCR7 in HIV-1-infected children and adults

The impact of HIV-1 infection on T cell maturation and differentiation has been a matter of considerable debate (Lieberman et al., 2001; Nixon et al., 2003; van Baarle et al., 2002). Here, we examined subpopulations of CD4 and CD8 T lymphocytes defined by their expression of CD45RA and CCR7 in peripheral blood from HIV-1-infected adults and children, and compared these to healthy controls (Fig. 2). As expected (Roederer et al., 1995), HIV-1 infection in adults was associated with reduced proportion of naive CD45RA+ CCR7+ cells in both the CD4 and CD8 T cell compartments. However, the two compartments differed in that CD8 T cells were enriched in CD45RA+ CCR7− late-stage differentiated cells (Fig. 2A), whereas the CD4 T cells were predominantly biased towards CD45RA− CCR7+ central memory and CCR7− CD45RA− effector memory phenotypes (statistical significance indicated in figure) (Fig. 2B). Interestingly, this HIV-induced skewing of both CD8 and CD4 T cell subsets was clearly less pronounced in children than in adults, with significant differences between HIV-1-infected adults and children (Figs. 2A and B). The differential bias of CD4 and CD8 T cells seemed to represent an elevation of the bias already present in HIV-1-negative adult subjects (Fig. 2C). These data are compatible with the view that HIV-1 infection drives differentiation of T cells (Aandahl et al., 2004), and also indicate that the responses of CD4 and CD8 T cells to the antigenic burden in HIV-1 infection differ in this regard. The maturational response of T cells to this antigenic burden is less pronounced in children than in adults.

Robust expansion of Gag 77–85-specific CD8 T cells can occur late in chronic pediatric HIV-1 infection

We were next interested in assessing the function and maturation status of T cells responding to HIV-1 antigens in infected children. We investigated HIV-specific CD8 T cell responses in the three pediatric subjects P59, P30 and P33 in

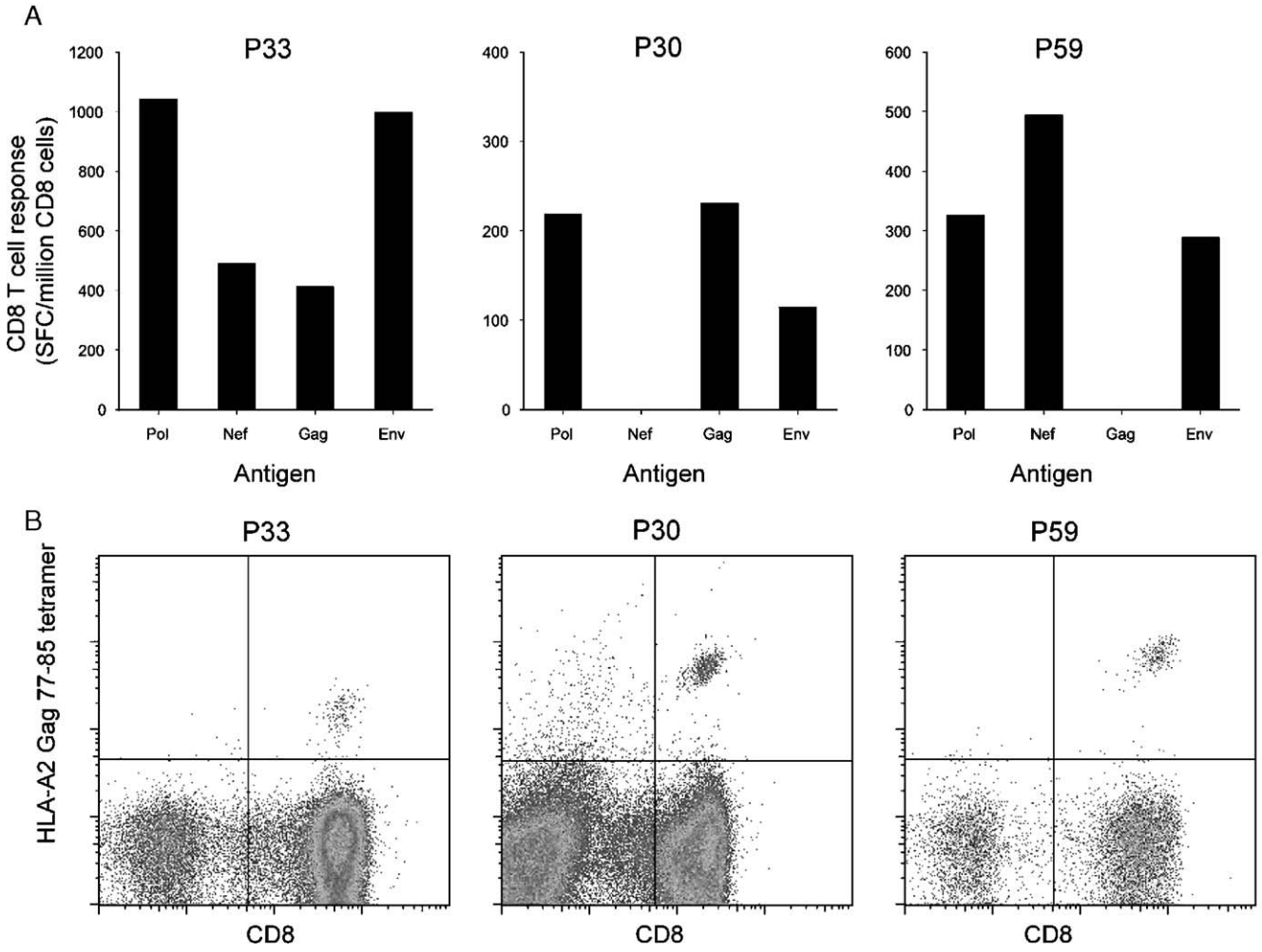

Fig. 3. CD8 T cell responses to HIV-1 antigens in three pediatric patients. (A) CD8 T cell responses in subjects P33, P30, and P59 against vaccinia constructs expressing HIV-1 IIIB Env, Gag, Pol, and Nef as measured in an IFNγ Elispot assay. (B) Identification of CD8 T cells specific for the HLA-A2 presented Gag 77–85 epitope by HLA tetramer staining and flow cytometry.

more detail. All three subjects were infected at or near birth and were HLA-A2-positive. Subject P59 was infected by blood transfusion shortly after birth, but not diagnosed as HIV-1-positive until age 13 when she presented with severe immune depletion. Her initial virologic response to antiretroviral treatment (ART) was transient, and CD4 T cell counts remained below 200 cells/μl. The relatively long survival of subject P59 in spite of severe CD4 T cell loss and treatment failure prompted us to investigate the status of CD8 T cell responses to HIV-1 in this patient. Vigorous CD8 T cell responses to HIV-1 Pol, Env, and Nef were detected using a recombinant vaccinia virus IFNγ Elispot assay (Fig. 3A). However, no responses to HIV-1 Gag-expressing vaccinia constructs were observed in P59 by this assay. Subject P33 displayed responses to all four HIV-1 gene products tested, and P30 to all but Nef (Fig. 3A). All three subjects had detectable populations of CD8 T cells specific for the HLA-A2-restricted Gag 77–85 epitope (Fig. 3B). For subject P59, this was unexpected given the lack of IFNγ production to the Gag vaccinia construct. Over a period of several years, patient P59 experienced an expansion of CD8 T cells specific for Gag 77–85, up to 18.3% of total CD8 T cells (Fig. 4A). This was accompanied by a two log10 drop in viral load to below 10^4 copies/ml (Fig. 4B), and decay of CD4 T cell counts from around 200 cells/μl down to approximately 15 cells/μl, corresponding to around 3% of PBMC (Fig. 4C). Subject P59 was before, during, and after this expansion of Gag 77–85-specific cells on ART including a protease inhibitor. The frequency of Gag-specific cells later declined, although remained at elevated frequency, and viral load increased to around 10^5 copies/ml while CD4 T cell counts reached a low of 6 cells/μl. Absolute CD8 T cell numbers also declined and reached 72 cells/μl at the last time-point analyzed. In contrast, subjects P30 and P33 displayed stable levels of Gag 77–85-specific cells, CD4 counts and viral loads (data not shown). These data indicate that Gag-specific CD8 T cells maintain the capacity for robust expansion in late-stage chronic pediatric HIV-1 infection.

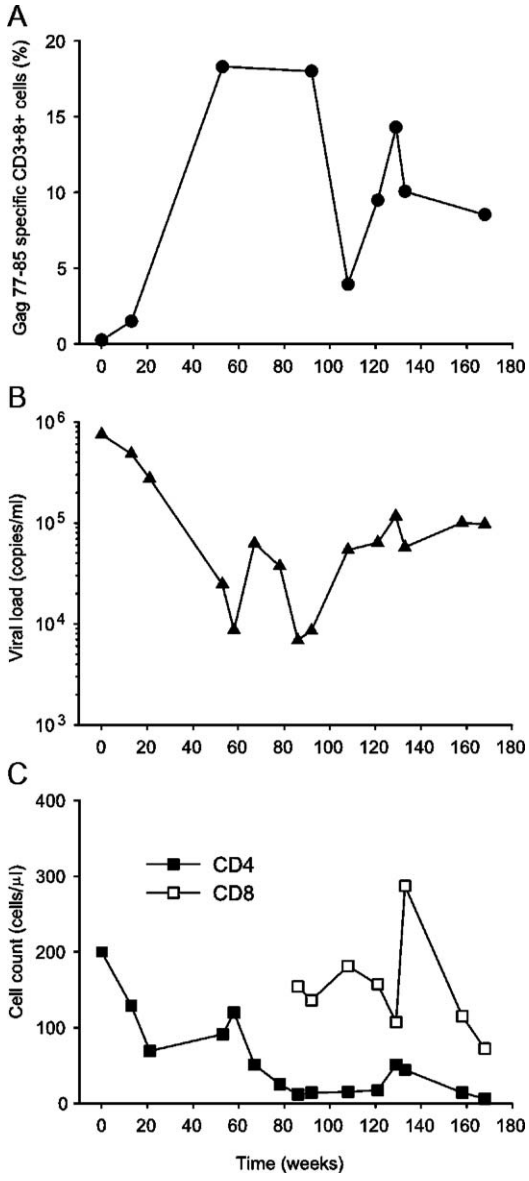

Fig. 4. Robust expansion of Gag-specific CD8 T cells in a pediatric subject with chronic HIV-1 infection and severe CD4 T cell loss. (A) Changes over time in the frequency of CD8 T cells in PBMC from subject P59 specific for the HLA-A2 presented Gag 77–85 epitope determined by HLA tetramer staining and flow cytometry. (B) HIV-1 load changes with time in subject P59. (C) Absolute CD4 and CD8 cell counts over time in subject P59.

Expanded Gag 77–85-specific CD8 T cells in pediatric subject P59 lack IFNγ, express perforin, and are cytolytic directly ex vivo

The perforin-mediated pathway for lysis of target cells is important for CD8 T cell-dependent immune defense against many viruses (Harty et al., 2000). The finding that HIV-specific CD8 T cells are generally low or negative for perforin in chronic infection has been interpreted to indicate that this pathway may be defective in the response to HIV-1. In contrast to this, the expanded Gag 77–85-specific CD8 T cells in subject P59 were predominantly perforin+ (Fig. 5). In a ^{51}Cr-release assay against Gag 77–85 peptide-pulsed HLA-A2+ BCL, fresh PBMC from P59 displayed significant peptide-specific cytolytic activity ex vivo (Fig. 5). Strong ex vivo HIV-specific cytolytic activity is not commonly observed in infected subjects, as exemplified by our two control subjects P30 and P33 (Fig. 5).

CD8 T cells utilize several effector mechanisms other than perforin-dependent lysis to combat viral infections, including cytokines with antiviral effects. To further investigate the lack of detectable IFNγ production in response to the Gag protein in subject P59 (Fig. 3A), we used Gag 77–85 peptide as antigen in an Elispot assay. The frequency of IFNγ expression in response to peptide in subject P59 corresponded to only 1% of circulating Gag 77–85-specific CD8 T cells, whereas considerably higher numbers were obtained with subjects P30 and P33 (Fig. 6). Inclusion of 1 ng/ml of exogenous IL-7 and IL-15 in the assay had only a minor effect in increasing the response by P59 to the Gag 77–85 peptide. Together, these data show that the Gag 77–85-specific CD8 T cells that were expanded in subject P59

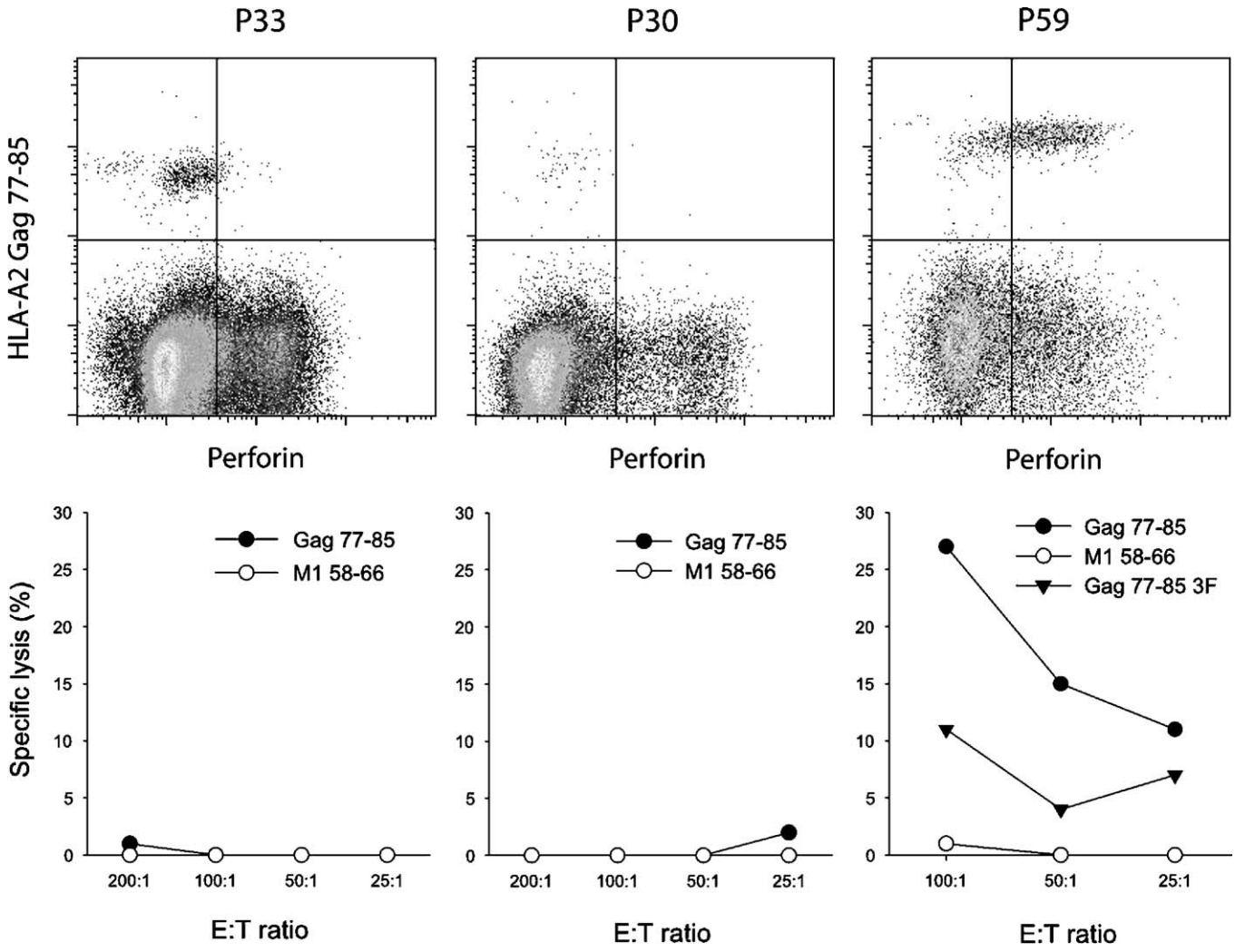

Fig. 5. CD8 T cells specific for Gag 77–85 in subject P59 express perforin and are cytolytic. Assessment of perforin expression (upper panels) and ex vivo cytolytic capacity (lower panels) in CD8 T cells specific for the Gag 77–85 epitope in subjects P30, P33, and P59. Perforin expression was assessed by intracellular flow cytometry combined with specific HLA-A2 tetramer staining. Cytolysis was determined in a ^{51}Cr assay using BCL pulsed with Gag 77–85 peptide or an influenza virus control peptide. For subject P59, target cells pulsed with Gag 77–85 peptide with the Y at position 3 substituted with an F, were also included. Data shown is from week 92 of study with subject P59, which is representative out of three experiments from different time points.

expressed perforin and were lytic directly ex vivo, but expressed almost no IFNγ.

Expanded Gag 77–85-specific CD8 T cells in pediatric subject P59 switch to a CD27− CD45RO+ phenotype

The generally low expression of perforin in HIV-specific CD8 T cells in adults has been suggested to associate with a block in full effector maturation as indicated by expression of the co-stimulatory receptor CD27 (Appay et al., 2000). Gag 77–85-specific CD8 T cells in subject P59, as well as the corresponding cells in subjects P30 and P33, displayed this phenotype at the early time points measured before the expansion of these cells took place. However, at a later time point (week 92), these cells were CD27 negative in subject P59 (Table 1). Thus, the expansion of Gag-specific CD8 T cells was associated with a maturational shift from CD27 positive to CD27 negative, while they remained CD45RO+.

Discussion

The long-term failure of adaptive immunity in HIV-1 infection has prompted investigators to analyze the functionality of CD8 T cell responses in infected subjects. Suggested CD8 T cell dysfunctions include poor cytolytic activity, low expression of perforin, impaired cytokine production, and a maturational block in HIV-specific cells (Appay et al., 2000; Champagne et al., 2001; Kostense et al., 2002; Sandberg et al., 2003; Shankar et al., 2000). We have revisited some of these aspects of T cell biology in pediatric and adult subjects to help improve our understanding of how these factors may influence the ability of the immune system to control HIV-1. It is clear that HIV-1 infection drives a broad activation and differentiation of T cells in adults, pushing an increased number of cells into late-stage differentiation. The elevated number of T cells expressing the cytolytic effector molecules perforin and granzyme B, and the relative expansion of cells expressing more differentiated

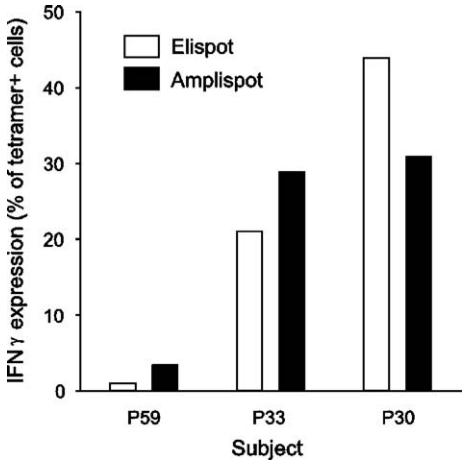

Fig. 6. Poor expression of IFNγ in expanded Gag 77–85-specific CD8 T cells in subject P59. CD8 T cell responses in subjects P33, P30, and P59 against Gag 77–85 peptide as measured in an IFNγ Elispot assay expressed as % of the frequency observed with HLA-A2 Gag 77–85 tetramer staining. Responses were measured using a standard IFNγ Elispot assay (white bars), and the Amplispot assay with the addition of 1 ng/ml of exogenous IL-7 and IL-15 in order to amplify weak responses (black bars). One representative out of six experiments with P59 is shown.

immunophenotypes are in line with this. Given the broad changes in CCR7 and CD45RA expression in both CD8 and CD4 T cell compartments in adult patients, it seems likely that the sequence of activation and differentiation is driven by antigens derived not only from HIV-1, but also from other pathogens. The markedly less pronounced effector and memory T cell maturation in HIV-1-infected children as compared to adults could be due to the combined effect of weaker immune responses to HIV-1 in children (Luzuriaga et al., 1995; Sandberg et al., 2003; Scott et al., 2001), and a greater regenerative capacity in the young immune system (Douek et al., 1998).

Several studies have indicated that CD8 T cells specific for HIV-1 antigens tend not to differentiate to late-stage perforin+ effector cells (Appay et al., 2000; Champagne et al., 2001; Zhang et al., 2003). The perforin+ cells that accumulate in HIV-1-infected adult subjects, and to a lesser extent in pediatric subjects, may thus be specific for other antigens to which these subjects are exposed. However, here, we have observed in one pediatric subject that the classical perforin− CD27+ phenotype of Gag-specific CD8 T cells can be overturned in late-stage HIV-1 infection resulting in the expansion of perforin+ CD27− epitope-specific CD8 T cells. The mechanism preventing this type of effector differentiation in many infected subjects remains unknown. However, our data suggest that it may be possible to find a way to overcome this block and obtain full effector differentiation of HIV-specific CD8 T cells.

The terms “function” and “dysfunction” are widely used in the analysis of CD8 T cell responses in infectious diseases, including HIV-1 infection. However, what is a true “dysfunction” can be very hard to establish. The phenotype of Gag-specific CD8 T cells in subject P59 illustrates the problem of defining a “functional” versus a “dysfunctional” response; at early time points, the Gag-specific cells expressed little perforin, but later on they expanded vigorously, expressed perforin, and became lytic. These cells were very poor at producing IFNγ, but this may represent a focusing on cytolytic function rather than a dysfunction in IFNγ production (Sandberg et al., 2001). The lack of IFNγ expression in these cells is reminiscent of the “stunned” phenotype observed in Hepatitis C virus (HCV)-specific CD8 T cells (Klenerman et al., 2002). Another aspect that complicates the issue is that lytic effector mechanisms may be restricted in some tissues (Shacklett et al., 2004). An understanding of what governs up- or down-modulation of specific functions and refocusing of T cell function could be very valuable for the development of effective immunotherapy. Our observations also adds to the debate of whether full cytolytic effector differentiation of CD8 T cells and the strongest possible T cell response is always beneficial for the HIV-1-infected host (Deeks and Walker, 2004). In subject P59, the expansion of lytic Gag-specific CD8 T cells coincided with a drop of CD4 counts down to very low levels. It is tempting to speculate that these cytolytic CD8 T cells, in an attempt to control HIV-1 infection, actually contributed to the elimination of CD4 T cells in circulation. Interestingly, in vitro experiments recently suggested that the Gag 77–85 epitope might be helper-independent (Kan-Mitchell et al., 2004). Such properties may allow CD8 T cells with this specificity to persist and expand despite severe CD4 T cell loss.

In this study, we have used surface expression of CD45RA isoform and CCR7 to characterize the differentiation status of the CD4 and CD8 T cell compartments in HIV-1-infected adults and children and their uninfected counterparts. The CD8 T cell compartment of HIV-1-infected adults displayed a marked skewing towards a late-stage CD45RA+ CCR7− phenotype, while the CD4 T cells were biased towards a CD45RA− and CCR7+ or CCR7-memory-like phenotype. These relative changes in peripheral blood T cells probably represent an expansion of memory and effector populations specific for many different antigens, as well as loss of naive cells. In contrast, HIV-1-infected children displayed no significant changes in the distribution of effector and memory populations as compared to uninfected children, perhaps indicative of a higher regenerative capacity paired with relatively weaker immune responses (Douek et al., 1998; Luzuriaga et al., 1995; Sandberg et al., 2003; Scott et al., 2001). Interestingly, in adults, it was recently observed that the majority of CD4 T cells producing cytokines in response to HIV-1 antigens displayed the CD45RA− CCR7− phenotype (Harari et al., 2004).

The differential bias of the CD8 and CD4 T cell compartments seems to reflect an enhancement of a pattern present in

Table 1
Maturation phenotype of HIV-1 Gag-specific CD8 T cells

Subject	CD45RA	CD45RO	CD27	CD62L	CCR7
P59 week 13	12%[a]	88%	90%	2%	3%
P59 week 92	16%	84%	8%	n.d.	n.d.
P33	10%	90%	92%	1%	5%
P30	10%	90%	95%	1%	5%

[a] Expression of surface receptors as indicated in Gag 77–85-specific CD3+CD8+ cells identified using the specific HLA-A2 tetramer.

the healthy host (Sallusto et al., 1999). Down-regulation of CCR7 and preferential homing to peripheral sites in CD8 T cells could relate to the main task of these cells, namely to go to the periphery, scan MHC class I-presented peptides, and eliminate infections. CD4 T cells on the other hand may do most of their job in secondary lymphoid tissues where MHC class II-mediated antigen presentation occurs, and CCR7 is used to enter the T cell zones of these tissues. The finding that CD4 T cells may start to express perforin in response to HIV-1 infection (Appay et al., 2002; Norris et al., 2004 and this study) is intriguing, and these cells may have a cytolytic function in the antiviral response. Interestingly, MHC class II-restricted killing in vivo was recently observed in mice infected with lymphocytic choriomeningitis virus (Jellison et al., 2005).

In this paper, we observe that the strong effector maturation and differentiation response of CD8 T cells and CD4 T cells driven by HIV-1 infection in adults are significantly weaker in children. CD4 and CD8 T cells respond differently to HIV-1 infection in terms of changes in surface maturation phenotype and this may reflect the different functions of these two major classes of T cells. Furthermore, we identified one pediatric subject who experienced a robust expansion of perforin+ lytic CD8 T cells specific for HIV-1 Gag, indicating that such effector differentiation can occur in late-stage pediatric infection. Interestingly, these cells where focused on cytolysis as they produced hardly any IFNγ in response to antigen. Further investigation into what regulates late-stage differentiation of T cells and their focusing on certain functions remains a high priority.

Materials and methods

Patient samples and viral load measurements

We identified eligible adult and pediatric subjects from two existing cohorts. HIV-1-infected pediatric subjects were treated and followed at the Jacobi Medical Center (Bronx, NY). Median age in this group was 7 years, range 2–14 years, and median CD4 T cell count was 834 cells/μl, range 200–1480 cells/μl. All 13 pediatric patients were on ART but experienced varying degrees of viral suppression due to adherence problems and drug resistance. The median viral load of these subjects was 1400 copies/ml, range 50–750,000 copies/ml. HIV-1-infected adult subjects were from the San Francisco General Hospital (San Francisco, CA). Nine out of 13 patients were on ART, but only two had viral load suppressed below limit of detection. The median CD4 T cell count of these subjects was 411 cells/μl, range 18–911 cells/μl, and the median viral load was 2914 copies/ml, range 50–500,000 copies/ml. Uninfected adult control subjects were healthy volunteers, and uninfected pediatric control subjects were siblings of the infected children. Median age in the uninfected pediatric control subjects was 6.5 years. Heparinized whole blood samples were obtained after informed consent, based on protocols approved by local institutional review boards. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll–Paque PLUS density gradient centrifugation (Amersham Pharmacia Biotech, Uppsala, Sweden). Plasma HIV-1 RNA was measured with the Amplicor HIV-1 Monitor with a lower limit of quantification at 400 copies of RNA/ml (Roche Diagnostic Systems, Branchburg, New Jersey).

Flow cytometry and mAbs

We assessed maturation phenotype and expression of perforin and granzyme B in peripheral blood T cells by six-color flow cytometry. Anti-CD62L FITC, anti-perforin FITC, purified anti-CCR7 IgM, anti-IgM biotin, and streptavidin allophycocyanin (APC) conjugate were purchased from BD Pharmingen, San Jose, CA. ECD-conjugated mAbs against CD45RA and CD45RO were from Coulter Immunotech, and anti-CD8 APC-Cy7, anti-CD4 PE-Cy7, and anti-CD3 PE-Cy7 were from Caltag Laboratories, Burlingame, CA. 1×10^6 PBMC was first stained with the anti-CCR7 IgM, washed, stained with anti-IgM biotin, washed, and stained with streptavidin–APC and other mAbs to surface antigens. After one wash, cells were plated in a 96-well V-bottom plate, permeabilized, washed, and stained for intracellular perforin or granzyme B. Samples were washed three times before acquisition on a FACS Vantage SE (Becton Dickinson, San Jose, CA) equipped with an argon laser delivering 200 mW of power at 488 nm and a helium–neon laser running at 5 mW and 633 nm. The six fluorescent parameters were measured using the following band-pass filters: FITC-525/50, PE-575/26, ECD (PE-Texas Red)-612/25, PE-Cy7-740 LP, APC-685/80, and APC-Cy7-750 LP. Flow cytometry data were analyzed using CellQuest software (Becton Dickinson) or FlowJo software (Tree Star, Ashland, OR).

We identified CD8 T cells specific for HIV-1 Gag using APC or PE-conjugated HLA-A2 tetrameric complexes refolded with the Gag 77–85 peptide epitope (Coulter Immunotech, Marseilles, France), and further phenotyped the cells using mAbs against CD3, CD8, CD27, CD45RO, CD62L, and CCR7 (BD Pharmingen, San Jose, CA). Ex vivo intracellular staining was performed in the absence of any additional stimulation and in combination with HLA-A2 tetramer staining to assess the percentage of perforin+ tetramer+ cells. Subjects were evaluated for HLA-A2 expression by staining with anti-HLA-A2 mAb (One Lambda, Canoga Park, CA). Samples were analyzed using a FACSCalibur Instrument (BD Pharmingen) and CellQuest (BD Pharmingen) or FlowJo software (Tree Star, Ashland, OR).

IFNγ Elispot assay

HIV-specific CD8 T cell responses were measured using the standard IFNγ Elispot assay as described (Larsson et al., 1999; Sandberg et al., 2003), and the Amplispot assay which uses the addition of 1 ng/ml exogenous IL-7 and IL-15 in order to amplify weak responses (Chandwani et al., 2004; Jennes et al., 2002). CD8 T cell responses were assessed using recombinant vaccinia virus expressing HIV-1 IIIB Gag (Therion, Inc., Cambridge, MA) or the HLA-A*0201-restricted Gag 77–85 SLYNTVATL peptide.

Statistical analysis

The flow cytometry data obtained were analyzed by descriptive statistics, *t* test, the Mann–Whitney rank sum test, the paired *t* test, and the signed rank test, as appropriate using Sigma Stat software (SPSS, Chicago, IL).

CTL assay

CTL activity was measured in a standard ^{51}Cr-release assay. Briefly, Gag 77–85 SLYNTVATL peptide-coated, Gag 77–85 SLFNTVATL mutant peptide-coated, or Influenza virus M1 58–66 GILGFVFTL control peptide-coated B cell line (BCL) target cells were prepared by incubating cells with 10 μM peptide or in the absence of peptide for 1 h at 37 °C. These cells were next labeled with 10 μl 10 mCi/ml ^{51}Cr for 1 h at 37 °C. Titrated numbers of effector cells were incubated with 3×10^3 ^{51}Cr-labeled target cells for 4 h at 37 °C, 5% CO_2. After incubation, released radioactivity was measured and specific lysis was calculated according to the formula: % specific release = ((experimental release-spontaneous release) / (maximum release-spontaneous release)) × 100.

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