

## **Productive Infection of Neonatal CD8<sup>+</sup> T Lymphocytes by HIV-1**

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### **Summary**

CD8<sup>+</sup> T lymphocytes confer significant but ultimately insufficient protection against HIV infection. Here we report that activated neonatal CD8<sup>+</sup> T cells can be productively infected in vitro by macrophage-tropic (M-tropic) HIV-1 isolates, which are responsible for disease transmission, whereas they are resistant to T cell-tropic (T-tropic) HIV strains. Physiological activation of CD8- $\alpha/\beta^+$  CD4<sup>-</sup> T cell receptor- $\alpha/\beta^+$  neonatal T cells, including activation by allogeneic dendritic cells, induces the accumulation of CD4 messenger RNA and the expression of CD4 Ag on the cell surface. The large majority of anti-CD3/B7.1-activated cord blood CD8<sup>+</sup> T cells coexpress CD4, the primary HIV receptor, as well as CCR5 and CXCR4, the coreceptors used by M- and T-tropic HIV-1 strains, respectively, to enter target cells. These findings are relevant to the rapid progression of neonatal HIV infection. Infection of primary HIV-specific CD8<sup>+</sup> T cells may compromise their survival and thus significantly contribute to the failure of the immune system to control the infection. Furthermore, these results indicate a previously unsuspected level of plasticity in the neonatal immune system in the regulation of CD4 expression by costimulation.

CD8<sup>+</sup> T lymphocytes play a protective role during the acute and chronic phases of HIV-1 infection. In addition to eliminating productively infected cells through their cytotoxic activity, CD8<sup>+</sup> T cells also release soluble factors (macrophage inflammatory protein [MIP]-1 $\alpha$ , MIP-1 $\beta$ , regulated on activation, normal T cell expressed and secreted [RANTES], IL-16, and other unidentified factors) that inhibit cell entry and intracellular replication of HIV-1 (for review see references 1 and 2). In most adult patients, HIV triggers a rapid and strong cytotoxic CD8<sup>+</sup> T cell response that appears to limit viral replication during the initial acute phase of the infection (3, 4). The efficiency of the initial CD8<sup>+</sup> T cell response, as reflected by the viral bur-

den at the end of the acute episode, is thought to determine the rate of disease progression to AIDS (5, 6). In the majority of adults, the viral load declines rapidly after primary HIV infection and the disease progresses slowly, with a median clinical latent phase of about 9 yr. In contrast, the evolution of neonatal HIV infection, resulting from maternal-infant transmission, is often much faster, with the highest incidence of pediatric AIDS occurring during the first year of life (7). In perinatally infected infants, plasma HIV levels peak at 1 to 2 mo of age and decline only very slowly during the first 2 yr of life (8). This may be related to an insufficient neonatal HIV-specific CD8<sup>+</sup> T cell response characterized by a smaller amplitude and a more restricted TCR repertoire than in adults (9). Here we provide a mechanism that may contribute to the inefficient response of neonatal CD8<sup>+</sup> T cells by showing that these cells can be productively infected in vitro by HIV.

L.P. Yang and J.L. Riley contributed equally to this study.

## Materials and Methods

**Lymphocyte Preparation and Culture Conditions.** CD8<sup>+</sup> and CD4<sup>+</sup> T cell subsets were isolated from heparinized umbilical cord blood as described (10). In brief, mononuclear cells obtained by centrifugation on Ficoll-Metrizoate gradients were treated with l-leucine methyl ester to remove monocytes and NK cells; enriched T cells were isolated by treating cells forming rosette with SRBCs by means of Lympho-Kwik T (One Lambda, Canoga Park, CA). CD8<sup>+</sup> T cells were positively selected with Dynabeads M-450 CD8 (Dyna, Great Neck, NY) and further depleted of CD4<sup>+</sup> cells with Dynabeads M-450 CD4; CD4<sup>+</sup> T cells were obtained by treating enriched T cells with Lympho-Kwik T helper (One Lambda). Neonatal T cell subsets were >98% CD3<sup>+</sup> and <1% CD14, CD20, CD56, or CD16 positive, respectively; the CD8<sup>+</sup> subset was >95% TCR- $\alpha/\beta$ <sup>+</sup> CD8- $\alpha/\beta$ <sup>+</sup>, >99% CD45RA<sup>+</sup>/RO<sup>-</sup>, and contained no detectable CD4 or CD29 positive cells. T cells were submitted to one or two cycles of activation and IL-2 expansion as described (11). At each cycle, T cells were activated with anti-CD3 mAb (UCHT-1, 200 ng/ml; a gift from P. Beverley, University College and Middlesex School of Medicine, London, UK) and irradiated CD32, B7.1 transfected L cells for 3 d, and cells were then washed twice and expanded in fresh medium supplemented with 50 IU/ml of IL-2 for 4 d. T cells were then washed and either stored in liquid nitrogen, until use for HIV infection, or subjected to another cycle of activation/expansion.

In some experiments, CD8<sup>+</sup> T cells ( $5 \times 10^5$  cells/ml) were stimulated with irradiated allogeneic dendritic cells ( $5 \times 10^4$  cells/ml) in the presence of IL-2 (50 IU/ml). Dendritic cells were derived from adult blood monocytes cultured for 9 d with IL-4, GM-CSF, and TNF- $\alpha$  exactly as described (12).

**Flow Cytometry.** One- or two-color flow cytometric analysis was performed on a FACSort<sup>®</sup> (Becton Dickinson, Montreal, Canada) according to standard procedures. The following FITC- or PE-labeled mAbs or isotype-matched mouse Ig were purchased from Becton Dickinson: anti-CD8- $\alpha$  (Leu 2a), anti-CD4 (Leu 3a), anti-CD3, anti-CD20, anti-CD14, anti-CD56, and anti-CD16. CD4 was also stained with OKT4 (American Type Culture Collection, Rockville, MD); mAb to CD8- $\alpha/\beta$  heterodimer (2ST8) was given by E. Reinherz (Harvard Medical School, Boston, MA); the anti-CXCR4/fusin mAb (12G5) has been described (13).

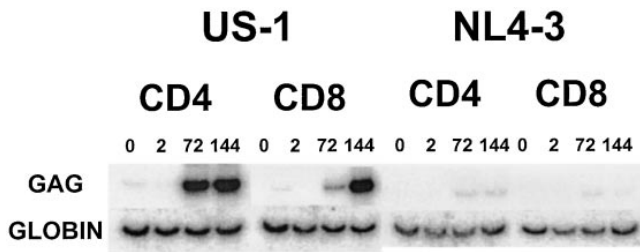
**Reverse Transcriptase PCR.** Total cellular RNA was extracted from resting or activated cord blood CD4<sup>+</sup> or CD8<sup>+</sup> T cells using RNA ease Total RNA Kit (Qiagen, Chasworth, CA) and pretreated with 10 U/ml RNase-free DNase (GIBCO BRL, Gaithersburg, MD) before amplification. Synthesis of cDNA and ensuing PCR amplification were performed using Titan<sup>™</sup> reverse transcriptase (RT)-PCR kit (Boehringer Mannheim, Indianapolis, IN) according to the supplier's instruction manual. PCR products were separated on a 1% agarose gel and stained with ethidium bromide. Chinese hamster ovary cell RNA served as negative control for CD4, CXCR4, and CCR5 genes; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was amplified to confirm equal efficiency of amplification of each RNA. No PCR product was detected in control amplification containing no added RNA, Taq, or RT DNA polymerase. Primers for amplification were as follows: CXCR4: 5'-AGAATTC AACCAGCGGTTACCATGGAGGGG-ATC, 5'-AGAATTCGCTTTTACATCTGTGTTAGCTG-GAG; CCR5: 5'-TCGATCCGGTGAACAAGATGGATTAT-CAAG, 5'-TCGGATCCAAGCCATGTGCACAACCTCTGA-CTG; and CD4: 5'-GCAGTGGCGAGCTGTGGT, 5'-GGG-TCCCCACACCTCACAGG.

**HIV Infection and Quantitative Gag PCR.** Either freshly activated or cryopreserved neonatal CD4<sup>+</sup> and CD8<sup>+</sup> cells were infected with HIV-1<sub>US-1</sub> or HIV-1<sub>NL4-3</sub> as previously described (14, 15). In brief, for each infection  $5 \times 10^6$  cells were centrifuged, washed, and resuspended in 400  $\mu$ l of media containing  $1-3 \times 10^4$  tissue culture and infectious dose (TCID)<sub>50</sub> of HIV-1. The cells were incubated at 37°C for 2 h, washed three times to remove excess virus, and resuspended in 50% conditioned media at  $10^6$ /ml. At  $t = 0, 2, 72,$  and 144 h after infection, 1,000,000 cells were pelleted and frozen at -70°C. The cell pellets were lysed, amplified by PCR using HIV gag-specific primers, and the amplified sequences were detected by hybridization to a radiolabeled oligonucleotide specific for internal gag sequences. The hybridized products were separated by gel electrophoresis and exposed to a PhosphorImager screen for 1 h. To ensure that the reactions were performed within the linear range of the assay, log increments HIV gag plasmid standards were amplified at the same time. To show that equivalent levels of input DNA were present in each PCR reaction, human  $\beta$ -globulin sequences were PCR amplified as described (15).

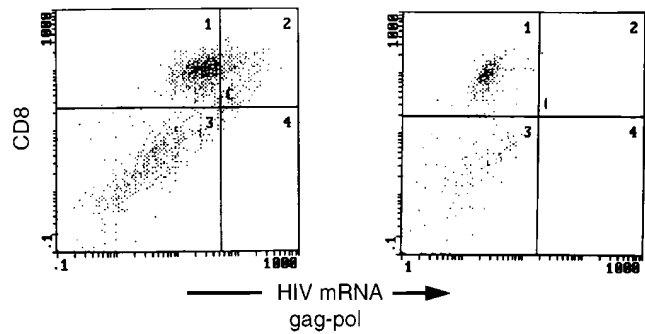
**Intracellular HIV Gag-pol Staining.** After washes  $1-2 \times 10^6$  cells were stained with optimized concentrations of cell surface marker antibodies for 1 h at 4°C. The cells were washed twice with sterile PBS, pH 7.4, and fixed in 100  $\mu$ l of PermeaFix (Ortho Diagnostics, Raritan, NJ) for at least 1 h at room temperature. Cells were washed in sterile PBS, pH 7.4, pelleted by centrifugation, and then washed again in  $2 \times$  standard saline citrate (SSC). After centrifugation, the cell pellet was resuspended in hybridization solution ( $2 \times$  SSC, 30% formamide, sonicated salmon sperm, yeast transfer RNA) containing 500 ng of 5-carboxy-fluorescein double-end-labeled, gag-pol-specific oligonucleotide probes or gag-pol sense oligonucleotides as a negative control probe cocktail (16). The intracellular hybridization was performed at 42°C for 1 h followed by successive washes in  $2 \times$  SSC, 0.5% Triton X-100, and  $1 \times$  SSC, 0.5% Triton X-100 at 42°C. The cells were resuspended for analysis in PBS, pH 8.3, and analyzed on a flow cytometer (Epic XL; Coulter, Miami, FL).

## Results and Discussion

Highly purified umbilical cord blood CD8<sup>+</sup> T cells (>98% CD3<sup>+</sup>, >95% TCR- $\alpha/\beta$ <sup>+</sup>, CD8- $\alpha/\beta$ <sup>+</sup>, no detectable CD4<sup>+</sup> cells) and CD4<sup>+</sup> T cells (>98% CD3<sup>+</sup> CD4<sup>bright</sup>, no detectable CD8<sup>+</sup> cells) were activated with anti-CD3 mAb cross-linked on CD32 and B7.1 transfected L cells, expanded in IL-2-supplemented medium, and then infected with the macrophage-tropic HIV<sub>US-1</sub> or with the T cell-tropic (T-tropic) HIV<sub>NL4-3</sub> virus; HIV replication was evaluated at days 3 and 6 after infection by quantitative PCR analysis of gag DNA accumulation. Both CD8<sup>+</sup> and CD4<sup>+</sup> T cell subsets supported significant replication of the macrophage tropic (M-tropic), but not the T-tropic, strain of HIV (Fig. 1), although the initial infection was slower in CD8<sup>+</sup> than in CD4<sup>+</sup> T cells, as judged from the day 3 values. That HIV infected CD8<sup>+</sup> T cells, and not contaminating CD4<sup>+</sup> cells was supported by 3 lines of evidence. First, neonatal CD8<sup>+</sup> T cells were directly shown to contain intracellular HIV. After infection with HIV<sub>US-1</sub> for 6 d, CD8<sup>+</sup> T cells were surface stained for CD8 expression and stained intracellularly for HIV gag and pol RNA sequences.



**Figure 1.** Infection of neonatal CD4<sup>+</sup> and CD8<sup>+</sup> cells. Umbilical cord blood CD4<sup>+</sup> and CD8<sup>+</sup> T cells were activated with anti-CD3 and B7-1-transfected CD32 L cells for 3 d, washed, expanded in IL-2, and infected with either a CCR5-dependent virus, HIV<sub>US1</sub>, or a CXCR4-dependent virus, HIV-1<sub>NL4-3</sub>. The PhosphorImage shows the accumulation of gag DNA in CD4 and CD8 cultures at 0, 2, 72, and 144 h after infection. To ensure equivalent amounts of DNA were in the PCR reaction, human  $\beta$ -globulin sequences were amplified.

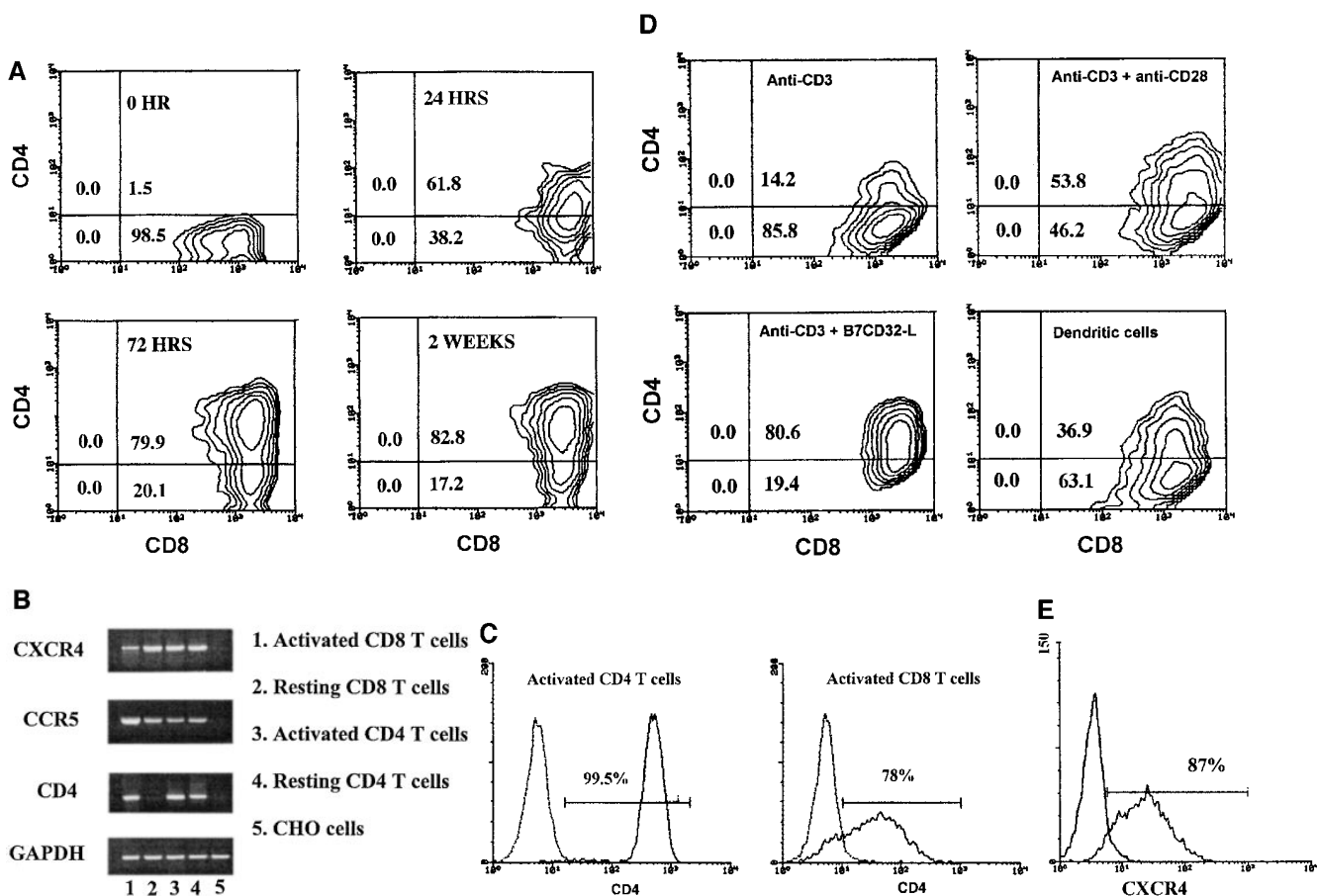


**Figure 2.** Intracellular HIV-1 staining of neonatal CD8<sup>+</sup> cells. Neonatal CD8<sup>+</sup> T cells were harvested 6 d after infection and stained with both anti-CD8 and labeled HIV DNA probes. *Left*, FACS<sup>®</sup> analysis of cells infected with HIV<sub>US1</sub>; *right*, a mock-infected control.

Fig. 2 shows that only CD8<sup>+</sup> T cells contained HIV-1 sequences. In addition, to confirm that a spreading infection was occurring in the neonatal CD8<sup>+</sup> T cells, samples were collected on both days 3 and 6 after infection and analyzed. At day 3, 3.4% of the cells were double positive for CD8 and HIV RNA, and by day 6 the number grew to 12.3%, which is consistent with a productive infection (data not shown). Second, CD8<sup>+</sup> T cell cultures did not contain detectable CD4 single positive cells at day 6 after infection (data not shown). Third, CD4 Ag and CD4 messenger RNA (mRNA) were undetectable in freshly prepared CD8<sup>+</sup> T cells, as assessed by flow cytometry and RT-PCR analysis respectively (Fig. 3, A and B). Thus, activated neonatal CD8<sup>+</sup> T cells can be productively infected by primary M-tropic HIV isolates, responsible for disease transmission, but not by T-tropic HIV. The apparent resistance of neonatal CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets to T-tropic HIV could be related to the recent finding that anti-CD3/B7-activated naive CD4<sup>+</sup> T cells isolated from adult blood are also resistant to T-tropic HIV, as a result of postentry block in viral replication (17–20).

To be susceptible to HIV infection, a cell must express CD4, the primary HIV receptor, together with either CCR5 (the coreceptor for M-tropic strains), or CXCR-4/fusin (the coreceptor for T-tropic strains) (for review see reference 21). According to this paradigm, activated neonatal CD8<sup>+</sup> T cells should express CD4 together with at least CCR5. Indeed, we found that neonatal single positive CD8<sup>+</sup> T cells started to express low to moderate levels of CD4 within 24 h after anti-CD3/B7.1 activation (Fig. 3 A); CD4 expression reached a plateau at 72 h (60 to >90% double positive cells,  $n = 24$ ) and remained stable for at least 2 wk (the duration of the present experiments). Flow cytometric analysis of anti-CD3/B7.1-stimulated CD8<sup>+</sup> T cells stained with anti-CD4 revealed a unimodal histogram, suggesting that the ability to coexpress CD4 is a common property of most, if not the entire, neonatal CD8<sup>+</sup> T cell population (Fig. 3 C). Quantitatively, CD4 was expressed at significantly lower levels on activated CD8<sup>+</sup> T cells than

on CD4 single positive T cells (Fig. 3 C). RT-PCR analysis revealed that CD4 mRNA was undetectable in freshly isolated CD8<sup>+</sup> T cells, but clearly induced after anti-CD3/B7.1 stimulation, suggesting transcriptional activation of the CD4 gene (Fig. 3 B). In experiments designed to determine the minimal requirements for CD4 expression on neonatal CD8<sup>+</sup> T cells, we found that: (a) TCR/CD3-mediated signals were sufficient to induce low levels of CD4, but that CD4 induction was markedly enhanced by CD28 costimulation (Fig. 3 D), and (b) cellular proliferation was neither sufficient or necessary. Indeed, CD4 was not expressed on proliferating CD8<sup>+</sup> T cells stimulated with anti-CD2 together with anti-CD28 mAbs and IL-2, whereas it was induced on irradiated (nonproliferating) anti-CD3/B7.1-stimulated cells (data not shown). Finally and importantly, CD4 was coexpressed on neonatal T cells activated under more physiological conditions, i.e., by antigenic stimulation with allogeneic dendritic cells (Fig. 3 D). Thus, physiological activation of umbilical cord blood CD8<sup>+</sup> T cells is associated with the coexpression of low to moderate levels of CD4, most likely as a result of CD4 gene transcriptional activation. Moreover, effective costimulation is required for optimal induction of CD4 coexpression, presumably explaining why this phenomenon went unnoticed in earlier studies. Consistent with their susceptibility to infection with M-tropic HIV, activated neonatal T cells expressed CCR5 mRNA (Fig. 3 B). Interestingly, they also expressed CXCR4/fusin protein and mRNA (Fig. 3 E). As already mentioned, it is suggested that their resistance to T-tropic HIV infection results from a postentry block in viral replication (17–20). The ability to coexpress CD4 is not a unique feature of neonatal CD8<sup>+</sup> T cells, and indeed a variable proportion (10–40%,  $n = 5$ ) of CD8<sup>+</sup> T cells isolated from adult blood expressed low levels of CD4 after anti-CD3/B7.1 activation (data not shown). Since neonatal CD8<sup>+</sup> T cells are immunologically naive whereas their adult counterparts contain both naive and memory cells, it will be of interest to examine CD4 expression and susceptibility to HIV infection in naive and memory CD8<sup>+</sup> T cell



**Figure 3.** Expression of CD4, CXCR4, and CCR5 on neonatal CD8<sup>+</sup> T cells. Umbilical cord blood CD8<sup>+</sup> T cells were activated with anti-CD3 mAb immobilized on B7.1 and CD32-transfected L cells and expanded in IL-2-supplemented medium. (A) Two-dimensional contour plot showing expression of CD4 and CD8 before T cell activation, after 24 and 72 h of primary activation, and at the end of a second cycle of activation/IL-2 expansion (day 14). Similar results were obtained by staining with OKT4 mAb, recognizing another CD4 epitope than Leu3a mAb. More than 95% of resting or activated CD8<sup>+</sup> T cells were stained brightly with 2ST8 mAb (received from E. Reinherz, Harvard Medical School, Boston, MA) specific for the CD8- $\alpha/\beta$  heterodimer. (B) Expression of CD4, CXCR4, and CCR5 mRNA in resting and activated (day 7) neonatal CD4<sup>+</sup> and CD8<sup>+</sup> T cells. (C) Comparison of CD4 expression on activated CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Cells were stained with anti-CD4 mAb at the end of the first cycle of activation/IL-2 expansion (day 7). (D) CD4 expression on CD8<sup>+</sup> T cells activated with either plate-coated anti-CD3 (10  $\mu$ g/ml), immobilized anti-CD3 together with soluble anti-CD28 (clone 9.3, 500 ng/ml), anti-CD3 (200 ng/ml) immobilized on B7.1 CD32 L cells, or allogeneic dendritic cells together with IL-2 (50 IU/ml). Cells were stained at day 7 with anti-CD4 and anti-CD8 mAbs. (E) CXCR4 expression on activated CD8<sup>+</sup> T cells. Anti-CD3/B7.1-activated CD8<sup>+</sup> T cells were stained with 12G5 mAb at day 7.

subsets. Preliminary results suggest that indeed, naive but not memory adult CD8<sup>+</sup> T cells may be induced to coexpress CD4 and become infectable by HIV.

These observations have potentially important biological and clinical implications. They challenge the paradigm that the CD4 gene is irreversibly silenced in extrathymic mature single positive CD8- $\alpha/\beta$  TCR- $\alpha/\beta$  T lymphocytes and raise the question of the role of CD4 in the biology of CD8<sup>+</sup> T cells. A small fraction (2–3%) of peripheral TCR- $\alpha/\beta$  T cells are CD8<sup>+</sup> CD4<sup>+</sup> double positive (22). In the large majority of the cases, these cells are CD4<sup>bright</sup> CD8<sup>dull</sup> and express CD8 as a CD8- $\alpha/\alpha$  homodimer (23). In three cases however, the double positive T cells were shown to display the same phenotype as activated neonatal CD8<sup>+</sup> T cells, i.e., CD8- $\alpha/\beta$ <sup>bright</sup> CD4<sup>dull</sup>, indicating that cells expressing this phenotype exist in vivo (24). Finally, infection

of CD8<sup>+</sup> T cells by human herpesvirus 6 was previously shown to induce CD4 expression and confer susceptibility to HIV infection (25). From a clinical point of view, our finding may explain the recent reports that CD8<sup>+</sup> T cells of infected patients can harbor HIV-1 (26, 27). It is tempting to relate the HIV susceptibility of neonatal CD8<sup>+</sup> T cells to the rapid progression of the disease in a large proportion of infected neonates. Since viral replication is mainly controlled by CD8<sup>+</sup> T cells, it seems reasonable to assume that the infection of these cells may compromise the immune response to HIV. Productive infection of primary HIV-specific CD8<sup>+</sup> T cells may lead to an early disruption of the Ag repertoire of HIV-specific CD8<sup>+</sup> T cells. Infected CD8<sup>+</sup> T cells may be deleted directly, after supporting high rates of viral replication, or indirectly by cytotoxic HIV-specific CD8<sup>+</sup> T cells. Early restriction of the antigen

repertoire would facilitate the emergence of HIV variants expressing functionally important Ags that were recognized by the deleted T cells (28–30). Future studies aiming to verify in vivo some of these hypotheses and to confirm the

differential susceptibility of naive versus memory CD8<sup>+</sup> T cells to HIV infection should further our understanding of the disease and have important implications for the development of HIV vaccines.

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