Generation of $CD3^+CD8^{low}$ Thymocytes in the HIV Type 1-Infected Thymus[1]

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Infection with the HIV type 1 (HIV-1) can result both in depletion of $CD4^+$ T cells and in the generation of dysfunctional $CD8^+$ T cells. In HIV-1-infected children, repopulation of the peripheral T cell pool is mediated by the thymus, which is itself susceptible to HIV-1 infection. Previous work has shown that MHC class I (MHC I) molecules are strongly up-regulated as result of IFN-α secretion in the HIV-1-infected thymus. We demonstrate in this study that increased MHC I up-regulation on thymic epithelial cells and double-positive $CD3^{-/int}CD4^+CD8^+$ thymocytes correlates with the generation of mature single-positive $CD4^-CD8^+$ thymocytes that have low expression of CD8. Treatment of HIV-1-infected thymus with highly active antiretroviral therapy normalizes MHC I expression and surface CD8 expression on such $CD4^-CD8^+$ thymocytes. In pediatric patients with possible HIV-1 infection of the thymus, a low CD3 percentage in the peripheral circulation is also associated with a $CD8^{low}$ phenotype on circulating $CD3^+CD8^+$ T cells. Furthermore, $CD8^{low}$ peripheral T cells from these HIV-1^+ pediatric patients are less responsive to stimulation by Ags from CMV. These data indicate that IFN-α-mediated MHC I up-regulation on thymic epithelial cells may lead to high avidity interactions with developing double-positive thymocytes and drive the selection of dysfunctional $CD3^+CD8^{low}$ T cells. We suggest that this HIV-1-initiated selection process may contribute to the generation of dysfunctional $CD8^+$ T cells in HIV-1-infected patients. ***The Journal of Immunology,*** **2002, 169: 2788–2796.**

Given that HIV type 1 (HIV-1)[4] infection results in disease secondary to a loss of peripheral $CD4^+$ T cells, the ability of the immune system to regenerate functional $CD4^+$ T cells is likely to have significant impact on the course and progression of HIV-induced disease. In principle, such cells could be replenished by expansion of the peripheral naive T cell population and/or by de novo generation of T cells in the thymus. Although there is mounting evidence that the thymus can be active into adulthood (1–5), it is clearly most active in childhood. Consequently, children and young adults should be able to more readily replace peripheral T cells lost to HIV-1 infection. However, this advantage may be offset by the possibility that the $CD3^-CD4^+CD8^-$ intrathymic T cell progenitor pool within the thymus may itself be infected by HIV-1 (6), resulting in further adverse effects on T cell production.

HIV-1 infection of the thymus has been demonstrated in autopsy specimens from HIV-1-infected fetuses and patients (7–12) as well as in experimental infection of both ex vivo thymic tissue and the SCID-hu Thy/Liv mouse (13, 14). In children with perinatal HIV-1 disease, thymic infection is associated with low peripheral T cell counts of both the CD4 and CD8 lineages and with rapid disease progression. Patients with such "thymic dysfunction" (TD) represent a substantial number (~25–30%) of all perinatally infected children and bear many clinical features in common with infants born with congenital thymic defects (15)

In addition to the possibility of direct HIV-1 infection of thymocytes, qualitative defects in thymic maturation may also arise. Noncytopathic HIV-1 infection of thymic epithelial cells (TEC) or of thymic myeloid cells might result in HIV-1 peptides being presented in the context of host MHC class I (MHC I) or class II (MHC II) Ags, driving positive and/or negative selection in a nonphysiologic manner. By example, changes in thymic selection leading to defective immune responses have been noted previously in experimental murine infections with gross murine leukemia virus (16), mouse mammary tumor virus (17), and lymphocytic choriomeningitis virus (18).

We have recently published data confirming and extending earlier reports (19) that MHC I is up-regulated in the HIV-1-infected thymus (20, 21). Because MHC I-TCR interactions are critical in the selection of developing thymocytes, we hypothesized that increased MHC I density on cells in the thymus might lead to high-avidity interactions with TCRs on developing thymocytes and, hence, supranormal levels of negative selection. In this study, we show that HIV-1 infection of the human thymus is associated with up-regulation of MHC I on TEC and with the preferential selection of $CD4^-CD8^+$ (SP8) thymocytes with a low level of expression of CD8 on the cell surface. In the peripheral blood of HIV-1-infected children, particularly those with low numbers of peripheral $CD3^+$

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[4] Abbreviations used in this paper: HIV-1, HIV type 1; TD, thymic dysfunction; TEC, thymic epithelial cell; MHC I, MHC class I; MHC II, MHC class II; SP8, $CD4^-CD8^+$ thymocyte; FTOC, fetal thymic organ culture; PR, protease; $TCID_{50}$, tissue-culture ID_{50}; ddI, didanosine; MFI, mean fluorescence intensity; pp, phosphoprotein; SFC, spot-forming cell; DP, $CD3^{-/int}CD4^+CD8^+$ thymocyte; SP4, $CD4^+CD8^-$ thymocyte; HAART, highly active antiretroviral therapy.

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T cells and possible thymic HIV-1 infection, low expression of CD8 on $CD3^+$ T cells is also associated with poor responses to Ags from CMV. We speculate that this outcome of thymic infection might contribute to the immunodeficient state induced by HIV-1.

Materials and Methods

Patient cohort

$HIV\text{-}1^+$ pediatric patients were treated and followed at the Jacobi Medical Center (Bronx, NY). All subjects were infected through vertical transmission and were diagnosed according to established guidelines, generally by at least two positive viral cultures or by two or more positive HIV-1 DNA PCR results; some (e.g., see those studied in Fig. 5) were also seropositive for CMV. This study was approved by the Institutional Review Board of the Albert Einstein College of Medicine (Bronx, NY) and informed consent was obtained from the parent or legal guardian of each participating child. A patient cohort was selected that met the following criteria: age of 6 years or less at the time of sample collection with the sample processed within 24 h of collection. Patients were assessed as fitting the thymic dysfunction phenotype by comparison of absolute CD4 and CD8 counts at the time of sample to previously published control patients (22). Patients fitting the TD criteria were defined as having absolute peripheral $CD4^+$ and $CD8^+$ T cell counts below the fifth percentile of HIV-1-uninfected controls born to HIV-1-infected mothers (15).

Generation of viral stocks

Stocks of NL4-3, NL4-3 (210WT), NL4-3 (210P), and Ba-L were generated as previously described (23, 24). High-titer NL4-3 stocks (contributed by M. Martin, National Institutes of Health AIDS Research and Reference Reagent Program, Rockville, MD) for inoculation of fetal thymic organ cultures (FTOC) were generated by transfection of 293T cells with pNL4-3 DNA, and virus stocks for inoculation of SCID-hu Thy/Liv implants were produced by electroporation of PBMCs with pNL4-3 and subsequent culture in PBMC blasts over a 4- to 7-day period. NL4-3 (210WT) and NL4–3 (210P) are NL4-3-recombinants containing the protease (PR) domains of patient plasma HIV-1 RNA obtained before (210WT) and after (210P) ritonavir monotherapy; 210P contains the I54V and V82A mutations in PR that confer resistance to PR inhibitors (25) and is highly impaired for replication in human thymus (24). Stocks of 210WT and 210P for SCID-hu inoculation were produced by transfection of HeLa cells. Ba-L stocks (contributed by S. Gartner, M. Popovic, and R. Gallo, AIDS Reagent Program) were generated in monocyte-derived macrophages. Supernatants were collected after 8 days of culture and were frozen as aliquots. All virus stocks were analyzed for p24 content and titrated by limiting dilution assay for 50% tissue-culture ID_{50} ($TCID_{50}$) in PBMC blasts.

HIV-1 infection of FTOC

Cultures of human fetal thymus were prepared and infected as previously described (20, 24). Briefly, thymi were dissected into small pieces and transferred directly into HIV-1 viral stocks ($\sim 10^6$ $TCID_{50}$) or conditioned RPMI medium from mock-infected PBMC cultures. Thymus pieces were inoculated with virus for 4 h at 37°C in a 5% CO_2 incubator. After inoculation, pieces were transferred to sterile filters (Millipore, Bedford, MA) placed on gelfoam (Pharmacia-Upjohn, Kalamazoo, MI) rafts in 700 μl Yssel's medium (Gemini Bio-Products, Calabassas, CA) in 24-well plates. HIV-1-infected thymic cultures were incubated 7–8 days and the medium was changed every 2 days. At the termination of culture, individual thymus pieces were treated with 0.4 μg/ml collagenase B and 100 U/ml DNase (both from Roche, Indianapolis, IN) for 45 min to 1 h at room temperature. Thymic fragments were then triturated and strained through a 70-μm nylon cell strainer. The thymic digest was then stained with Abs against CD3, CD4, CD8, CD45, CDw90 (Thy-1), CD118 (IFN-$\alpha\beta$ receptor), MHC I, and MHC II for FACS analysis.

For IFN-α-treated FTOCs, thymus pieces were placed on filters on gelfoam rafts in 24-well plates (three thymic pieces per well). At the initiation of culture, 1000 U/ml IFN-α (Schering-Plough, Kenilworth, NJ) were added to 700 μl Yssel's medium in each well. FTOCs were harvested 5, 7, and 9 days after the initiation of culture, dispersed by trituration, and stained for CD3, CD4, CD8α, CD8β, and MHC I for FACS analysis.

HIV-1 infection of SCID-hu Thy/Liv mice

All procedures and practices associated with the use of SCID-hu Thy/Liv mice were approved by the University of California (San Francisco, CA) Committee on Animal Research. SCID-hu Thy/Liv mice were generated as previously described (26–28) and maintained under pathogen-free conditions. Mice in a given cohort were constructed using human fetal tissue from a single donor. Implants were directly inoculated with 50 μl virus (2000–3000 $TCID_{50}$) or sterile tissue culture medium. In the experiments shown in Fig. 3, treatment of infected mice with zidovudine (30 mg/kg/day), 3TC (15 mg/kg/day), and indinavir (500 mg/kg/day) was initiated at 21 days after inoculation with NL4-3. Mice were treated twice daily by oral gavage (200 μl/dose). In the noted experiments, didanosine (ddI) treatment (100 mg/kg/day by once-daily i.p. injection) was started the day before inoculation with Ba-L and continued until implant collection. Implants were harvested at the indicated time points, placed into sterile PBS-FCS, and dispersed into single cell suspensions. Thymocytes were then counted and aliquoted for p24 ELISA, bDNA, and FACS analysis, as previously described (28).

FACS staining and analysis

Dispersed thymocytes from FTOC cultures and SCID-hu Thy/Liv implants were stained as previously described (20). PBMCs were isolated from whole blood by centrifugation over a Ficoll gradient. For FACS staining, cells were washed, pelleted, and resuspended in 50 μl of mAbs diluted in 1 mg/ml of human gamma globulin and incubated on ice for 30 min. After incubation, cells were rinsed, pelleted, and resuspended in 200 μl of PBS-FCS for immediate FACS analysis.

Four-color FACS staining was done with the indicated combinations of the following fluoresceinated Abs against: CD3-FITC, CD4-FITC, CD3-PerCP, CD4-PerCP, CD8-PerCP, HLA-DR-PerCP (all from BD Biosciences, San Jose, CA), HLA-A, B, C (pan-MHC I; W6/32)-PE (DAKO, Carpinteria, CA), CD8β-PE (Corixa, Hialeah, FL), CD45-FITC, CD45RA-FITC, CD118-PE, CD8-tricolor, CD3-allophycocyanin, $CD4^-$ allophycocyanin (all from Caltag Laboratories, Burlingame, CA), and CDw90 (Thy-1)-PE (SyStemix, Palo Alto, CA). To best visualize gradations in the mean fluorescence intensity (MFI) of CD8 staining, it was important to use bright fluorochromes (e.g., tricolor and PE) on the anti-CD8 Abs.

Samples were analyzed immediately after staining on a FACSCalibur (BD Biosciences). Virus-infected and uninfected SCID-hu Thy/Liv implants shown in Table I were collected and stained on the same day and analyzed together to minimize between-run variation in CD8 MFI. For the pediatric patient samples, Quantibrite Rainbow Beads (Spherotech, Libertyville, IL) were used to normalize FL2 (PE) levels to a constant setting matched to bead fluorescence.

IFN-γ ELISPOT assay

HIV-specific and CMV-specific $CD8^+$ T cell responses were measured using the recombinant vaccinia IFN-γ ELISPOT assay (29). Each well of a sterile multiscreen 96-well filtration plate (Millipore) was coated with 50 μl anti-IFN-γ mAb (Mabtech, Stockholm, Sweden) at a concentration of 10 μg/ml in 1 M sodium bicarbonate buffer (pH 9.5). After an overnight incubation at 4°C, each well was washed 4 times with PBS (Cellgro, Herndon, VA) and blocked with 50 μl 5% pooled human serum in RPMI (Cellgro) for 1 h at 37°C. PBMCs (1.5×10^5) were added to each well and recombinant vaccinia viruses expressing HIV-1 IIIB Pol, Nef, Gag, Env, or CMV phosphoprotein (pp)65 (Therion Biologics, Cambridge, MA) were added at a multiplicity of infection of 2:1 directly to the cell solution. Vaccinia strain TK^- was used as negative control, and staphylococcal enterotoxin B (Sigma-Aldrich, St. Louis, MO) was used as a positive control. After an overnight incubation at 37°C, plates were washed four times using PBS with 0.05% Tween 20 (Fisher Biotech, Fair Lawn, NJ). Biotinylated anti-IFN-γ mAb 7-B6-1 (Mabtech, Cincinnati, OH) was added at 1 μg/ml in 100 μl PBS and the plate was incubated for 2 h at 37°C. Plates were washed four times using 0.1% Tween 20 in PBS and then treated with avidin-conjugated HRP H (Vector Laboratories, Burlingame, CA). After 1 h, plates were washed four times with 0.1% Tween 20 in PBS. Stable diaminobenzidene tetrahydrochloride substrate (50 μl; Research Genetics, Huntsville, AL) were added to each well for 5 min and then washed away with water. IFN-γ spot-forming cells (SFC) were visualized and counted using an AID ELISPOT reader system (Autoimmun Diagnostika, Strassberg, Germany). Raw counts were standardized to express the frequency of SFC per microliter of blood. Background frequencies obtained with vaccinia strain TK^- were subtracted from Ag-specific frequencies to obtain the final count.

Results

MHC I is up-regulated on TEC after HIV-1 infection

MHC I expression is dramatically up-regulated on thymocytes from HIV-1-infected thymi (19, 20). We have recently shown that

MHC I up-regulation on thymocytes is the result of IFN-α secretion by type 2 predendritic cells resident in the medulla of the infected thymus (20). IFN-α induces MHC I up-regulation on thymocytes expressing high levels of the IFN-$\alpha\beta$ receptor expression, including intrathymic T cell progenitor and CD3$^{-/int}$CD4$^+$CD8$^+$ (DP) thymocytes (Fig. 1*A*).

MHC I on thymic epithelial cells is essential for the selection of developing CD8$^+$ thymocytes and has been shown to be critical to the positive selection of developing thymocytes in murine models (21, 30, 31). To determine whether MHC I expression is up-regulated on TEC in the HIV-1-infected thymus, forward scatter-high side scatter-high CD3$^-$CD45$^-$ thymic epithelial cells were isolated from mock- and HIV-1-infected FTOCs. All of these cells were positive for CDw90 (Thy-1), a marker of TEC (32) and, depending on the donor, 30–70% were positive for MHC II (data not shown). As shown in Fig. 1*B*, all cells in this subpopulation also showed up-regulation of MHC I expression after HIV-1 infection.

MHC I up-regulation on TEC may be mediated by IFN-α, as is the case for DP thymocytes (20). Consistent with this possibility, TEC were found to express high levels of IFN-$\alpha\beta$ receptor (Fig. 1*C*) and IFN-α treatment of FTOCs led to MHC I up-regulation on thymocytes and on TEC (data not shown). Additionally, there was a strong correlation between MHC I up-regulation on DP thymocytes and on TEC isolated from mock- ($n = 3$) and HIV-1-infected ($n = 4$) FTOCs from the same donor ($r^2 = 0.774$, $p = 0.009$). These data indicate that DP MHC I up-regulation can be used as a surrogate for MHC I up-regulation on TEC from HIV-1-infected thymi.

CD8 expression is down-regulated on SP8 after HIV-1 infection

Given the importance of high-avidity interactions between MHC I and TCR during the course of thymocyte development (21), we reasoned that up-regulation of MHC I could have profound effects on the positive or negative selection of developing thymocytes. MHC I expression levels on TEC have been shown to be critical in the selection of CD8$^+$ thymocytes in mice, with small (1.5-fold) increases in MHC I expression leading to negative rather than positive selection in TCR transgenic models of thymic selection (33). To determine whether such an alteration in thymic selection might occur in the HIV-1-infected human thymus, the expression of CD3, CD4, and CD8 was monitored on mature CD4$^+$CD8$^-$ (SP4) and SP8 thymocytes from mock- and HIV-1-infected thymi. CD8 expression on SP8 thymocytes, but not CD4 expression on SP4 thymocytes, was found to decrease after inoculation of SCID-hu Thy/Liv mice with the HIV-1 isolates NL4-3 (X4) (Fig. 2*A*) or Ba-L (R5) (Fig. 2*B*). To better define the kinetics of such down-regulation, cohorts of SCID-hu Thy/Liv mice implanted with tissue from a single donor were harvested at serial time points after inoculation with NL4-3 or Ba-L. As previously reported (19), increased MHC I expression was observed on DP thymocytes 14–21 days after inoculation with NL4-3 and Ba-L (Fig. 2, *C* and *D*, *left panels*). During the same time frame, there was a corresponding decrease in the MFI of CD8 expression on SP8 thymocytes, but no significant change in the MFI of CD4 expression on SP4 thymocytes from thymus implants (Fig. 2, *C* and *D*, *center* and *right panels*, respectively). Staining with Abs against CD3 (Fig. 2*E*) indicated that CD4$^-$CD8^{low} thymocytes were CD3^{high} single-positive thymocytes, ruling out the possibility that they represented DP thymocytes that had down-regulated CD4 due to HIV-1 infection.

To determine whether these modest changes in CD8 expression on SP8 thymocytes were statistically significant, two additional experiments were analyzed (Table I). In one, SCID-hu Thy/Liv mice were inoculated with NL4-3 (210WT) or with NL4-3 (210P), an isogenic recombinant of NL4-3 carrying a PR gene resistant to ritonavir; the latter virus isolate has been previously shown to replicate well in activated PBMC, but poorly, if at all, in the human thymus (24). Collected 14, 21, and 28 days after inoculation, Thy/Liv implants inoculated with NL4-3 (210P) showed undetectable levels of viral replication, no increase in the expression of MHC I, and no change in the expression of CD8 on SP8 thymocytes. By contrast, CD8 was down-regulated on SP8 thymocytes in NL4-3 (210WT)-infected Thy/Liv implants ($p = 0.0039$ at day 21 and $p = 0.0062$ at day 28), a change that was associated with up-regulation of MHC I and signs of viral replication. Similarly, Ba-L-infected Thy/Liv, but not mock-infected, implants or mice treated with ddI before inoculation with Ba-L showed down-regulation of CD8 on SP8 thymocytes at day 21 ($p = 0.011$).

CD8 down-regulation is induced by IFN-α and is reversible

We used IFN-α treatment of FTOCs in vitro to mimic HIV-1-induced IFN-α-mediated MHC I up-regulation on TECs. Notably, all of the CD3$^+$CD8^{low} thymocytes from IFN-α-treated FTOCs expressed the CD8$\alpha\beta$ heterodimer and not the CD8$\alpha\alpha$ homodimer which can be selected under high-avidity conditions (34) (data not shown). Taken together, these data demonstrate a time-dependent decrease in CD8$\alpha\beta$ expression on SP8 thymocytes that is related to HIV-1-induced IFN-α mediated up-regulation of MHC I expression in the infected human thymus.

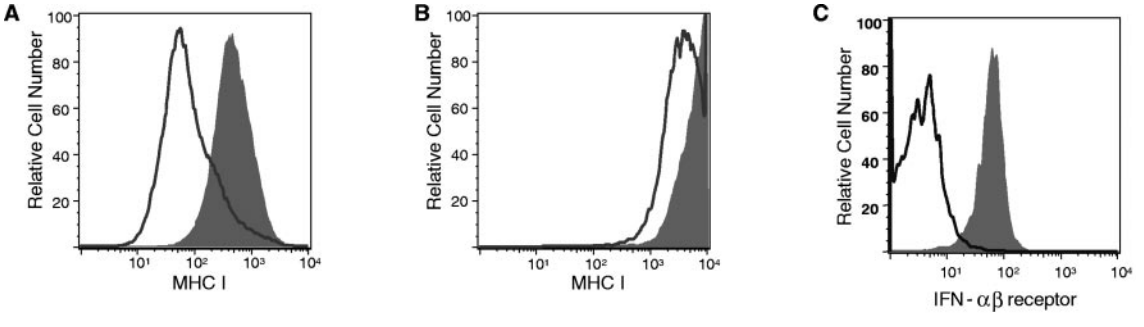

FIGURE 1. TEC express the IFN-$\alpha\beta$ receptor and up-regulate MHC I after HIV-1 infection. TEC from collagenase-digested fetal thymi were identified as CD3$^-$CD45$^-$ cells with high forward scatter and side scatter profiles. Thymocytes and TEC were isolated from mock-infected (line histogram) or HIV-1-infected (shaded histogram) FTOCs 7 days after inoculation. An increase in MHC I surface expression was observed on both (*A*) DP thymocytes and (*B*) CD3$^-$CD45$^-$ TEC. These histograms are representative of results from two independent experiments using two HIV-1 isolates (NL4–3 and Ba-L). *C*, TEC were positive for expression of IFN-$\alpha\beta$ receptor (CD118). Staining for CD118 (shaded histogram) is representative of results from two fetal thymi and is compared with stains using an isotype control Ab (line histogram).

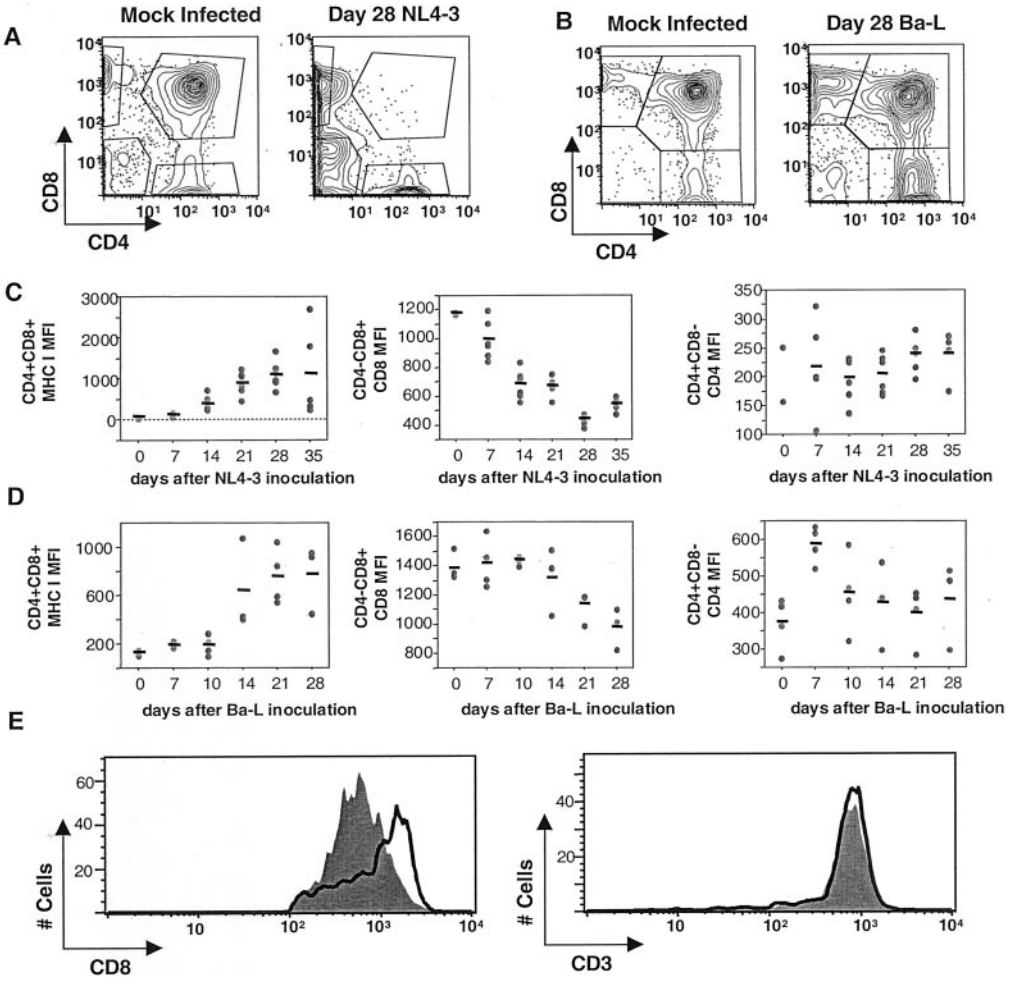

FIGURE 2. After HIV-1 infection, CD8 expression on SP thymocytes declines as MHC I expression increases in the SCID-hu Thy/Liv mouse. *A*, CD8 expression on SP thymocytes declines over the course of X4 HIV-1 infection. Implants were collected from mock- and NL4-3-infected SCID-hu Thy/Liv mice 28 days after inoculation and thymocytes were stained for CD4 and CD8. The CD8 MFI on SP8 thymocytes in the mock- and NL4-3-infected implants was 1181 and 475, respectively. *B*, CD8 expression on SP8 thymocytes declines over the course of R5 HIV-1 infection. Mock- and Ba-L-infected SCID-hu Thy/Liv mice were analyzed as in *A*. The CD8 MFI on SP8 thymocytes in the mock- and Ba-L-infected implants was 1328 and 815, respectively. Histograms in *A* and *B* are representative of two to five mice per group collected 28 days after inoculation. *C*, MHC I expression on DP thymocytes increases and CD8 MFI on SP8 thymocytes declines over the duration of NL4-3 infection. SCID-hu Thy/Liv implants were mock-infected or injected with 3000 $TCID_{50}$ of NL4-3. Infected implants were harvested at the indicated times and analyzed for MHC I expression on DP cells, CD8 expression on SP8 cells, and CD4 expression on SP4 cells. CD8 MFI on SP8 thymocytes declined during the same time frame that MHC I expression on DP thymocytes increased, while CD4 MFI on SP4 thymocytes remained unchanged. Each data point (●) represents an individual implant from mice in the same cohort. Means are shown as bars. *D*, MHC I expression on DP thymocytes increases and CD8 MFI on SP8 thymocytes declines over the duration of Ba-L infection. SCID-hu Thy/Liv implants were inoculated with 2000 $TCID_{50}$ of Ba-L. Implants were harvested at the indicated times and analyzed as described in *C*. *E*, $CD4^{-}CD8^{low}$ thymocytes are $CD3^{high}$. Mock- and Ba-L-infected SCID-hu Thy/Liv implants were harvested at 21 days after inoculation and stained for CD4, CD8, and CD3. SP8 thymocytes demonstrated a similar $CD3^{high}$ profile in mock-infected (open histogram) and Ba-L-infected (filled histogram) implants. In all cases analyzed in Fig. 2 and in Table I, SP8 thymocytes were defined as being $CD4^{low}$ whereas SP4 thymocytes were defined as being $CD8^{low}$ (see gating strategy shown in *A*).

Treatment of SCID-hu Thy/Liv mice with highly active retroviral therapy (HAART) has been shown to significantly suppress active HIV-1 replication and to reverse thymocyte depletion in the SCID-hu Thy/Liv mouse model (35). Reasoning that suppression of viral replication by HAART should also result in the down-regulation of MHC I and the normalization of CD8 expression on SP8 thymocytes, HIV-1-infected SCID-hu Thy/Liv mice were provided combination antiretroviral therapy 21 days after HIV-1 inoculation. As shown in Fig. 3, *A* and *B*, SCID-hu Thy/Liv mice displayed increased MHC I expression and decreased CD8 expression on the surface of SP8 thymocytes 21 days after inoculation of NL4-3. Twenty-one days after the initiation of HAART treatment (6 wk after virus inoculation), MHC I expression in these animals decreased to levels indistinguishable from those found in mock-infected controls (Fig. 3, *A*, *C*, and *D*). Concomitantly, levels of CD8 expression on SP8 thymocytes increased to levels equal to or higher than those found in mock-infected controls. Before and after treatment, the MFI of CD4 expression on SP4 thymocytes remained unchanged (Fig. 3*D*, *right*). These data indicate that the appearance of $CD3^{+}CD8^{low}$ thymocyte populations is reversible and dependent upon ongoing viral replication.

$CD8^{low}$ T cells are also observed in HIV-1-infected children

Although the selection of $CD3^{+}CD8^{low}$ thymocytes is strongly associated with HIV-1 infection of the thymus in the SCID-hu Thy/Liv mouse model, it is not clear whether mature $CD8^{low}$ thymocytes move into peripheral T cell pools. However, it is notable that CD8 expression has previously been found to be decreased on

Table I. *Reduction in CD8 expression on CD4$^-$CD8$^+$ thymocytes in HIV-1-infected Thy/Liv implants*

Virus	Days After Inoculation	Mice Per Group	p24 Per Million Thymocytes (pg)	Log_{10} Copies HIV-1 RNA Per Million Thymocytes	CD4$^+$CD8$^+$ MHC I MFI	CD4$^-$CD8$^+$ CD8 MFI	*p* Value
NL4-3 (210WT)	14	6	72 ± 29	4.6 ± 0.4	230 ± 38	990 ± 69	0.15
PI-res. NL4-3 (210P)	14	6	Undetectable	2.0 ± 0.2	84 ± 5.5	1100 ± 37	
NL4-3 (210WT)	21	6	420 ± 100	5.4 ± 0.2	380 ± 90	570 ± 57[a]	0.0039
PI-res. NL4-3 (210P)	21	6	Undetectable	2.6 ± 0.4	82 ± 6.7	990 ± 48	
NL4-3 (210WT)	28	6	1000 ± 210	5.9 ± 0.1	810 ± 140	540 ± 49[a]	0.0062
PI-res. NL4-3 (210P)	28	5	Undetectable	2.6 ± 0.4	100 ± 11	1000 ± 20	
Ba-L	21	6	160 ± 33	5.4 ± 0.1	330 ± 66	990 ± 64[a]	0.011
Mock-infected	21	4	Undetectable	Undetectable	92 ± 3.1	1300 ± 20	
Ba-L/ddI[b]	21	5	Undetectable	Undetectable	85 ± 3.1	1300 ± 35	0.33

[a] $p < 0.05$ by Mann-Whitney *U* test for reduction in CD8 MFI in HIV-1-infected vs uninfected implants collected at the same time point.
[b] SCID-hu Thy/Liv mice were treated with 100 mg/kg/day ddI by once-daily i.p. administration beginning 1 day before inoculation of Thy/Liv implants with HIV-1 Ba-L.

mature CD3$^+$CD8$^+$ T cells in the periphery of HIV-1-infected patients (36–38). To address the possibility that HIV-1 infection of the thymus may result in the generation of such cells, we evaluated peripheral blood from a cohort of perinatally infected HIV-1$^+$ children. In particular, we wished to determine whether the CD8^{low} phenotype might be associated with signs of thymic HIV-1 infection. Thus, we identified HIV-1-infected children with evidence of TD (defined as absolute peripheral CD4$^+$ and CD8$^+$ T cell counts below the fifth percentile of HIV-1-uninfected-controls born to HIV-1-infected mothers) (15, 22). TD patients have been found to display a constellation of clinical characteristics (e.g., increased vulnerability to infections caused by viruses, fungi, and *Pneumocystis carinii*) similar to those found in children born with congenital thymic abnormalities (e.g., DiGeorge syndrome). HIV-1$^+$ children with TD have also been noted to progress more rapidly to AIDS. In a cross-sectional analysis, children with HIV-1 disease were separated into TD and non-TD groups based on absolute CD4$^+$ and CD8$^+$ T cell counts taken at the time of sampling and compared with previously published controls (22). As shown in Table II (in boldface type), 5 of 21 patients (24%) met the criteria for TD, a proportion similar to that observed in earlier studies (15). All of these patients were on antiretroviral therapy with varying degrees of virological suppression. If these children had sustained HIV-1 infection of the thymus, we postulated that such infection might be manifest by the subsequent appearance and persistence of CD8^{low} T cells in the peripheral blood.

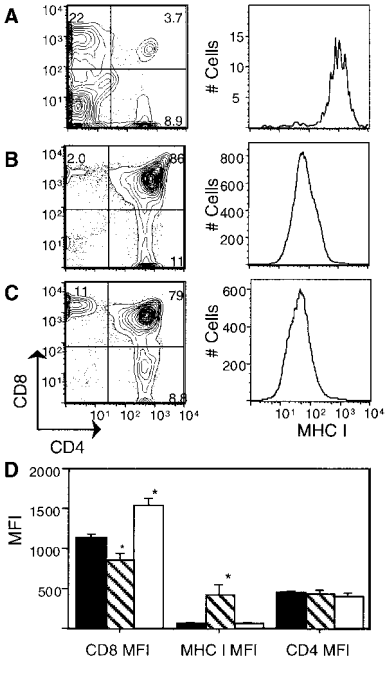

FIGURE 3. CD8 MFI on thymocytes from SCID-hu Thy/Liv implants normalizes after treatment with highly active antiretroviral therapy. SCID-hu Thy/Liv mice were mock-infected or inoculated with 3000 $TCID_{50}$ of NL4-3. Twenty-one days after inoculation, depletion of DP thymocytes, reduction of SP4-SP8 ratios, and increased expression of MHC I were observed in NL4-3-infected thymi (*A*) in comparison to (*B*) mock-infected controls. At 21 days after inoculation, HAART was initiated. *C*, Twenty-one days after the initiation of HAART, DP thymocytes were more abundant and MHC I expression was no longer increased. Aggregate results from six HIV-1-infected and three mock-infected mice are shown in *D*: CD8 MFI on SP8 thymocytes decreased after 21 days of untreated HIV-1 infection (▨) and recovered to normal levels after 21 days of HAART (□). Mock-infected controls are shown as solid bars. Reciprocally, MHC I expression on DP thymocytes increased significantly in the NL4-3-infected thymus and decreased to normal levels after HAART treatment. CD4 MFI on SP4 thymocytes remained unchanged in the presence or absence of HIV-1 infection or treatment. Similar results were obtained in two independent experiments. *, $p < 0.05$ by the unpaired *t* test for all analyses.

Separation of HIV-1$^+$ pediatric patients into TD and non-TD groups allowed an analysis of markers of HIV pathogenesis (Table III). Compared with non-TD patients, those with TD were found to have significantly lower absolute CD8$^+$ T cell counts ($p = 0.0006$), total lymphocyte counts ($p = 0.0027$), and CD3$^+$ T cell percentages ($p = 0.03$), as expected for selection based on TD criteria. Other parameters that were not directly part of TD criteria approached significance, including the CD8 MFI on memory/effector ($p = 0.06$) and naive ($p = 0.10$) CD8$^+$ T cells and the percentage of CD8$^+$ memory/effector T cells ($p = 0.09$). The absolute CD4$^+$ T cell counts, which were used as part of the TD criteria, also approached significance ($p = 0.12$). Interestingly, there was not a striking increase in AIDS-defining illnesses in TD patients, possibly due to the fact that many of the children were followed closely in the clinic and provided chemoprophylaxis for opportunistic infections when necessary.

In these HIV-1$^+$ pediatric patients, there was a significant correlation ($r^2 = 0.27$; $p = 0.01$) between the percentage of CD3$^+$ lymphocytes in the peripheral blood and CD8β MFI on naive (CD3$^+$CD45RA$^+$) CD8$^+$ T cells (Fig. 4*A*). No such association was observed between CD4 MFI on naive (CD3$^+$CD45RA$^+$) CD4$^+$ T cells and CD3$^+$ percentage (Fig. 4*B*), indicating that changes in CD8 MFI are uniquely associated with lowered CD3$^+$ T cell numbers. CD8 MFI on naive CD8$^+$ T cells was also positively correlated with increased percentage of naive CD8$^+$ T cells ($r^2 = 0.30$; $p < 0.01$) and negatively correlated with memory/

Table II. *Clinical and experimental characteristics of pediatric HIV-1$^+$ cohort*

Patient No.	Age at Time of Sample[a] (years, mo)	Sex	HIV-1 Viral Load (copies/ml plasma)	CD4%/CD4 Abs (cells/μl)	CD8%/CD8 Abs (cells/μl)	%CD3$^+$/Total Lymphocyte Counts (cells/μl)	CD8 MFI[b]	Antiretroviral Therapy[c]	Opportunistic Infections/ Clinical Manifestations[d]
1	**2, 1**	**M**	**<50**	**44/1,102**	**12/298**	**54/2,480**	**329**	**d4T, 3TC, NFV**	**None**
2	2, 1	F	402,276	25/1,270	37/1,875	42/5,060	422	ZDV, 3TC, ABC	None
3	**2, 6**	**F**	**235,798**	**27/772**	**17/491**	**24/2,870**	**208**	**Unmedicated**	**None**
4	3, 4	M	1,851	33/1,526	24/1,110	65/2,867	723	d4T, 3TC, NFV	PCP
5	3, 6	M	4,796	30/973	30/973	63/3,250	587	d4T, 3TC	None
6	3, 7	F	54,247	51/2,174	20/849	70/4,300	597	d4T, 3TC, NVP, Kal	PCP
7	**3, 10**	**M**	**2,465**	**33/410**	**45/565**	**72/1,250**	**503**	**ZDV, 3TC, NFV**	**None**
8	4, 7	F	12,480	41/1,301	30/959	79/3,170	466	ZDV, 3TC	None
9	4, 7	F	47,365	27/910	41/1,392	68/3,420	377	d4T, 3TC, NVP	None
10	4, 9	F	<50	31/992	35/1,112	55/3,170	823	ZDV, 3TC, NFV	None
11	4, 11	F	<50	44/1,078	25/627	69/2,470	331	ZDV, 3TC, NFV	None
12	**5, 0**	**F**	**96,250**	**32/791**	**29/703**	**31/2,460**	**359**	**EFV, Kal**	**CMV**
13	5, 8	F	72	45/2,096	37/1,723	79/4,680	1,016	ZDV, 3TC, RTV, SQV	Encephalopathy, cardiomyopathy, HIV-related growth failure
14	**5, 7**	**F**	**1,208**	**36/722**	**34/681**	**54/2,030**	**295**	**d4T, 3TC, EFV, Kal**	**Pneumococcal meningitis**
15	5, 8	M	<400	64/2,395	32/1,196	62/3,770	419	ZDV, FTC, NFV	Encephalopathy
16	5, 9	F	<400	27/883	41/1,353	56/3,320	308	d4T, ABC, Kal	Thrombocytopenia
17	6, 1	M	355,588	1/14	42/934	36/2,220	317	Unmedicated	Bacterial sepsis, pneumococcal pneumonia, lymphocytic interstitial pneumonitis, parotitis
18	6, 1	F	96	23/767	28/949	63/3,330	493	d4T, 3TC, ABC, NFV	None
19	6, 4	M	5,128	37/1,331	31/1,139	72/3,620	534	ZDV, 3TC	None
20	6, 7	M	8,338	27/899	31/1,064	55/3,390	365	d4T, 3TC, ddl	Encephalopathy, parvovirus B19 anemia, abdominal MAC
21	6, 8	F	2,151	13/868		65/	337	d4T, FTC, Kal	Bacteremia with focal infections including splenic abscess, osteomyelitis, pneumonia

[a] Pediatric patients fitting the TD criteria are shown in boldface type.

[b] CD8 MFI was measured on CD3$^+$CD4$^-$CD8β^+CD45RA$^+$ cells falling in the lymphocyte gate.

[c] Antiretroviral therapy at the time blood was obtained for phenotypic analysis. d4T, stavudine; 3TC, lamivudine; NFV, nelfinavir; NVP, nevirapine; Kal, Kaletra (lopinavir/ritonavir); ZDV, zidovudine; EFV, efavirenz; RTV, ritonavir; SQV, saquinavir; FTC, emtricitabine; ABC, abacavir.

[d] Opportunistic infections and clinical manifestations refer to diagnoses made after documented HIV-1 infection. PCP, *Pneumocystic carinii* pneumonia; MAC, *Mycobacterium avium* complex.

effector CD8$^+$ T cells ($r^2 = 0.26$; $p = 0.015$). Additionally, CD8 MFI on naive CD8$^+$ T cells was positively associated with CD4$^+$ T cell absolute counts ($r^2 = 0.25$; $p = 0.017$), in agreement with previously published data from adult HIV-1+ patients (38).

Because CD3$^+$CD8^{low} T cells have been found to be anergic in murine models (39), it was of interest to determine the functionality of CD3$^+$CD8^{low} T cells isolated from the peripheral blood of HIV-1-infected patients. We used the recombinant vaccinia virus ELISPOT assay to determine the magnitude of CD8$^+$ T cell responses against CMV pp65 or against a combination of vaccinia constructs expressing HIV-1 Gag, Env, Pol, and Nef. In the 21 HIV-1$^+$ pediatric patients, total CD8$^+$ T cell responses to HIV-1 Ags did not correlate significantly with the level of CD8 expression of T cells in PBMC ($r^2 = 0.004$, $p = 0.80$; Fig. 5*A*). However, in 16 of these patients who were also CMV-seropositive, there was a correlation between CD8 MFI on the surface of CD3$^+$CD8$^+$ T cells and CD8$^+$ T cell responses to CMV pp65 ($r^2 = 0.43$, $p = 0.008$; Fig. 5*B*): those with a low CD8 MFI were generally less responsive by this assay. Thus, pediatric patients with an accumulation of CD8^{low} T cells in peripheral blood mount weaker CD8$^+$ T cell responses against CMV pp65; this relationship is not observed in the case of responses against epitopes from HIV-1.

Discussion

We demonstrate in this study that HIV-1 infection of the human thymus results in MHC I up-regulation on TEC, that such up-regulation is associated with and possibly mediated by increased levels of IFN-α, and that it is associated with the selection of CD8^{low} T cells. Down-regulation of CD8 on SP8 thymocytes is time-dependent, reproducible, and statistically significant, and it occurs after infection with both X4 and R5 isolates of HIV-1. Treatment of HIV-1-infected thymus with HAART normalizes both MHC I and CD8 expression, further indicating that there is a reciprocal relationship between the expression levels of the two cell surface markers. In HIV-1-infected children, there is a significant correlation between low CD8 expression on naive CD8$^+$ T cells and low peripheral CD3$^+$ T cell percentages, consistent with the possibility that the thymus has been infected in these individuals and that selection of CD3$^+$CD8^{low} T cells has occurred. Finally, we demonstrate that children with low expression of CD8 on their peripheral CD3$^+$CD8$^+$ T cells are more likely to have weak responses against Ags from CMV.

Our observations are consistent with, but do not prove, the hypothesis that CD8^{low} T cells are selected because MHC I is up-regulated in the HIV-1-infected thymus. Thymic selection is critically dependent on the avidity of the interactions between MHC I

Table III. *Comparison between parameters of HIV-1 disease in TD and non-TD pediatric HIV-1$^+$ patients*

Subset	TD	Non-TD	*p* Value
CD8 absolute count (cells/μl)	548 ± 164	1,165 ± 320	0.0006
Total lymphocyte counts (cells/μl)	2,218 ± 617	3,466 ± 730	0.0027
% CD3$^+$	47 ± 19	62 ± 11	0.03
CD8 MFI on memory CD8$^+$	320 ± 98	454 ± 142	0.06
% CD3$^+$CD8$^+$CD45RA$^-$	21 ± 10	13 ± 10	0.09
CD8 MFI on naive CD8$^+$	339 ± 108	505 ± 205	0.1
CD4 absolute count (cells/μl)	759 ± 246	1,199 ± 586	0.12
% CD3$^+$CD8$^+$CD45RA$^+$	58 ± 6	69 ± 15	0.14
% Lymphocytes	39 ± 14	51 ± 16	0.17
% CD3$^+$CD4$^+$	58 ± 13	47 ± 16	0.19
% CD3$^+$CD8$^+$	32 ± 10	40 ± 13	0.2
CD4 MFI on memory CD4$^+$	191 ± 39	198 ± 22	0.61
% CD3$^+$CD4$^+$CD45RA$^+$	69 ± 12	65 ± 18	0.7
% CD3$^+$CD4$^+$CD45RA$^-$	21 ± 10	24 ± 16	0.71
CD4 MFI on naive CD4$^+$	176 ± 35	181 ± 23	0.72
Viral load (copies/ml)	67,154 ± 102,864	58,595 ± 126,760	0.89

and the TCR-CD8 coreceptor signaling complex (21). Given this dependence, significant increases in the expression of MHC I on the surface of both thymocytes and TEC may result in an abnormally high avidity of interactions with the CD8 coreceptor. Such high-avidity interactions, in turn, may favor the selection of thymocytes with a reciprocally low expression of CD8. This hypothesis is supported by studies in high-avidity murine models of T cell selection, in which mature thymocytes are found to display decreased CD8 cell surface density (34). Such decreased cell surface expression of CD8 presumably lowers the overall affinity of the TCR-CD8 signaling complex for its high-avidity MHC I ligand, allowing thymocytes to escape negative selection. If such escape from negative selection were to occur in the HIV-1-infected thymus, mature CD8^{low} T cells would likely be generated and released into the peripheral circulation.

This phenomenon is of interest because CD3$^+$CD8^{low} T cells have been found to be anergic in the mouse. In the context of murine TCR transgenic models, CD3$^+$CD8^{low} T cells have a shortened half-life in vivo and in vitro, as well as a decreased proliferative capacity (39). Cloned CD3$^+$CD8^{low} T cells selected on high-avidity backgrounds have markedly reduced cytotoxicity in in vitro assays compared with CD3$^+$CD8^{high} T cells specific for the same Ag (40). Finally, CD8 blockade results in a similar nonresponsiveness in phenotypically normal CD8$^+$ T cell clones, mirroring the observation that reduced CD8 expression results in reduced responsiveness to Ag (40). In each of these instances, the

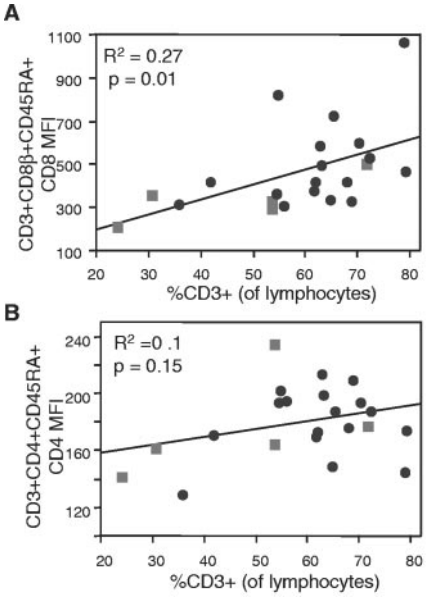

FIGURE 4. A decrease in CD8 MFI on CD3$^+$CD8$^+$CD45RA$^+$ peripheral T cells in HIV-1-infected children is related to declining CD3$^+$ T cell percentages. Lymphocytes isolated from the peripheral blood of HIV-1$^+$ pediatric patients were stained for CD3, CD4, CD8β, and CD45RA. *A*, CD3$^+$CD4$^-$CD8β^+CD45RA$^+$ cells falling in the live lymphocyte gate were analyzed for CD8β MFI. A significant positive relationship (p = 0.01) is observed between CD8 MFI and the percentage of CD3$^+$ lymphocytes in the peripheral blood of TD patients (■) and non-TD patients (●). *B*, There is no significant association between CD4 MFI on CD3$^+$CD4$^+$CD8β^-CD45RA$^+$ peripheral T cells and the total percentage of CD3$^+$ lymphocytes.

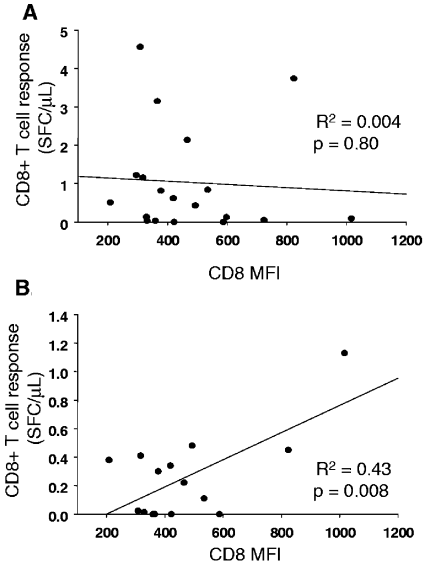

FIGURE 5. Patients with CD8^{low} T cells are weakly responsive to CMV Ags. HIV-specific and CMV-specific CD8$^+$ T cell responses were measured using the recombinant vaccinia IFN-γ ELISPOT assay. Peripheral blood lymphocytes from HIV-1-infected children were stimulated with recombinant vaccinia virus expressing (*A*) HIV-1 or (*B*) CMV Ags. The response was measured by counting the number of IFN-γ SFC per milliliter of peripheral blood. Responses in *A* represent the sum of responses against HIV-1 IIIB Gag, Pol, Env, and Nef. Responses in *B* represent those against CMV pp65 Ag. Children with low expression of CD8 on their peripheral CD3$^+$CD8$^+$ T cells were more likely to have a weak response to CMV Ags, a relationship not observed with HIV-1 Ags.

degree of CD8 down-regulation was relatively modest (e.g., 2- to 4-fold) and similar to the degree of CD8 down-regulation observed in this study in the context of the HIV-1-infected thymus.

It is possible that nonfunctional $CD8^{low}$ peripheral T cells are also generated during the course of HIV-1 disease, perhaps through aberrant thymic selection mediated by enhanced MHC I expression on TEC or in response to increased secretion of IL-4 in the thymus and/or the periphery (41). During primary infection with HIV-1, the $CD8^+$ T cell response successfully controls viral replication and leads to the establishment of a chronic infection. As disease progresses, the ability of the immune system to contain HIV-1 infection gradually erodes. A variety of factors likely contribute to this global defect, including the lack of T cell help provided by $CD4^+$ T cells and defects in Ag presentation. Progressive dysfunction of $CD8^+$ T cells is another key factor in the inability of the immune system to control HIV-1 (42).

Consistent with this idea, peripheral $CD8^+$ T cells in HIV-1-infected patients display a phenotype of chronic activation and reduced function (43–47). As the thymus is the source of naive T cell emigrants that replenishes depleted peripheral pools, its output could be pivotal in maintaining suppression of HIV-1 viral infection. The decline of immunocompetence in HIV-1-infected patients may reflect not just loss of $CD4^+$ T cells, but also the loss of peripheral $CD8^+$ T cells through chronic activation and the inability of the thymus to replenish the supply with competent precursor $CD8^+$ T cells. We observe that a significant population of mature $CD3^+CD8^{low}$ thymocytes arises in the HIV-1-infected thymus of the SCID-hu Thy/Liv mouse model. Possibly, in the HIV-1-infected human, such thymic infection could lead to the generation of a circulating peripheral pool of $CD3^+CD8^{low}$ T cells, as is found in this study of children with the TD phenotype.

Although cells with the $CD8^{low}$ phenotype are nonfunctional in murine models (39), it has also been shown that viral infection of the murine thymus can result in the generation of specific tolerance to Ags from the infecting virus (18). In a small group of patients, we observed a correlation between $CD3^+CD8^{low}$ T cells and lowered $CD8^+$ T cell responsiveness to CMV-associated Ags, consistent with the possibility that the peripheral $CD8^+$ T cell compartment is dysfunctional. In contrast, we observed no correlation between CD8 expression on peripheral T cells and responses to HIV. These discordant observations may be due to a number of mutually nonexclusive reasons, ranging from differential effects of HIV-1 and CMV on the generation of a functional TCR repertoire (42) to differential circulating or maturation patterns of CMV and HIV-1-responsive T cells (48). Our observations are also limited by the small size and heterogeneous composition of the pediatric patient cohort that was studied. More extensive studies of a prospective nature will be required before firm conclusions on this point can be drawn.

Indeed, although we have demonstrated an intriguing relationship between CD8 MFI on naive $CD8^+$ T cells and peripheral markers of HIV-1 disease, further studies on $CD8^+$ T cell function in pediatric HIV^+ cohorts must be conducted to validate the hypothesis that HIV-1 infection of the thymus results in aberrant thymic selection of $CD8^+$ T cells. In particular, it will be important to demonstrate HIV-1 infection of the human thymus and to determine whether $CD8^{low}$ T cells might also be generated within infected peripheral lymphoid organs. Such studies, though technically demanding, appear to be warranted by the data shown in this work.

In conclusion, we demonstrate that $CD3^+CD8^{low}$ thymocytes are generated in HIV-1-infected thymus in association with increased expression of MHC I. HAART treatment normalizes both MHC I expression and CD8 expression on SP8 thymocytes. Preliminary studies in a cohort of $HIV\text{-}1^+$ children support this observation, as the $CD8^{low}$ phenotype on naive $CD8^+$ T cells is significantly correlated with decreasing percentages of $CD3^+$ T cells. $CD3^+CD8^{low}$ T cells appear to show reduced function in response to CMV Ags in in vitro assays. Taken together, these findings suggest that the generation of $CD8^{low}$ T cells from the HIV-1-infected thymus may contribute to the generalized immunosuppression that is a hallmark of HIV-1 disease.

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