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Mechanism of Human Immunodeficiency Virus Type 1 Localization in CD4-Negative Thymocytes: Differentiation from a CD4-Positive Precursor Allows Productive Infection

SCOTT G. KITCHEN,[1] CHRISTEL H. UITTENBOGAART,[1,2,3] AND JEROME A. ZACK[3,4]*

Departments of Microbiology and Immunology,[1] Pediatrics,[2] and Medicine[4] and Jonsson Comprehensive Cancer Center and UCLA AIDS Institute,[3] University of California Los Angeles School of Medicine, Los Angeles, California 90095

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Human immunodeficiency virus (HIV) infection of the thymus could have profound effects on development of the immune response, particularly in children. We and others have established that in addition to infecting and depleting CD4-bearing thymocytes, functional HIV proviruses are found in thymocytes lacking surface CD4 expression. Using in vitro thymocyte cultures, we show that neither HIV-mediated down regulation of CD4 nor CD4-independent infection contributes to the localization of HIV in cells lacking the primary virus receptor. Rather, infection of a CD4-positive precursor cell (CD4 positive/CD8 positive) with subsequent differentiation into a mature CD4-negative phenotype results in productively infected CD4-negative cells. This novel mechanism may contribute to pathogenesis by distributing viral sequences into functional subsets of T cells typically refractory to HIV infection and could account for the presence of viral DNA in CD8-positive lymphocytes recently observed in patients.

The thymus plays an important role in the developing immune system in children as well as in the reconstitution of the cellular immune response in immunocompromised young adults (29). This organ harbors a vast number of both mature and immature CD4-bearing T cells that are susceptible to infection by human immunodeficiency virus type 1 (HIV-1). Thymic abnormalities and infection of thymocytes have been reported in fetuses aborted from HIV-infected women as well as in HIV-1-infected children and adults. HIV-1 infection of fetuses is associated with a greater spontaneous abortion rate and thymic histological changes associated with lymphocyte depletion (24, 33). Further, HIV-1 infection of the thymus may have a dramatic effect on the rapid progression to AIDS observed in perinatally and in utero-infected children as well as in the inability of some infected individuals to reconstitute the peripheral T-cell pool following antiretrovirus therapy (17, 48).

T-lymphocyte development within the thymus is characterized by the appearance and disappearance of various cell surface markers that are functionally indicative of the stages of T-cell maturation (41). Cell surface expression of CD4 during different stages of development renders the thymocyte susceptible to infection by HIV-1 (25). CD4 is expressed transiently early in development, soon after the progenitor cell enters the thymic environment (41). As T-cell development progresses, CD4 is coexpressed with CD8, and with subsequent maturation, selective termination of expression of one of these accessory molecules during lineage commitment results in T lymphocytes that express either CD4 or CD8. In some cases, CD4 expression and CD8 expression are both lost in the mature cell (10). HIV-1 infection of thymocytes at any stage of development can potentially wreak havoc on T-cell differentiation by killing HIV-1-infected cells, directly and/or indirectly disrupting the thymic microenvironment, and possibly allowing export of HIV-1-infected cells into the peripheral lymphocyte pool.

We and others have previously examined the effect of HIV-1 infection in the human thymus and in primary thymocytes both in vivo and in vitro (2, 4, 16, 39, 44). In vivo HIV-1 infection of human fetal thymus/liver (Thy/Liv) implants in the SCID-hu mouse causes a rapid and severe depletion of CD4-bearing thymocytes (2, 4, 39). Virus is found distributed primarily in the CD4 single-positive (SP) and CD4CD8 double-positive (DP) thymocytes, with limited but detectable proviral sequences in CD8 SP thymocytes, at early stages before and during depletion (2, 39, 40). Our observations in vitro (44) as well as those of Stanley et al. (39) in vivo, using Thy/Liv implants in SCID-hu mice, indicate that late in infection of the human thymus, CD8 SP thymocytes harbor and express HIV-1 proviral DNA sequences. In addition to establishing the presence of HIV proviral DNA in CD8 SP thymocytes, we established that viral sequences are also harbored in CD4CD8 double-negative (DN) thymocytes in cells infected and cultured in vitro (44). In the present study, we used the in vitro culture system to elucidate the mechanism behind the localization of HIV-1 in cells that lack the CD4 cell surface receptor. After evaluating several potential mechanisms, we conclude that infection of a CD4-positive precursor cell (CD4CD8 DP) followed by subsequent differentiation into a CD4-negative thymocyte is the major mechanism responsible for this phenomenon. It is thus likely that HIV-1 infection in the human thymus can lead to infection of several T-cell subsets and play a profound role in pathogenesis. Recent studies have demonstrated the presence of HIV-1 in CD8 SP cells in the lungs of infected patients and shown that late in HIV disease progression, the major reservoir for proviral DNA in the peripheral blood is the CD8 SP population (27, 38). A mechanism such as that identified here may be responsible for this phenomenon.

* Corresponding author. Mailing address: Division of Hematology-Oncology, 11-934 Factor Building, Department of Medicine, UCLA School of Medicine and Jonsson Comprehensive Cancer Center, Los Angeles, CA 90095-1678. Phone: (310) 794-7765. Fax: (310) 825-6192.

MATERIALS AND METHODS

Virus stocks. HIV-1_{NL4-3}, HIV-1_{JR-CSF}, HIV-$1_{NL-\Delta nef}$, and HIV-1_{NL-thy} have been previously described (1, 21, 22, 34), and viral stocks were obtained by electroporation (7) of plasmid containing infectious cloned DNA (30 μg) into COS cells. High-titer HIV-1_{NL4-3} and HIV-1_{NL-thy} virus stocks were obtained by electroporation of CEM cells followed by coculture with uninfected CEM cells. Quantitation of $p24^{gag}$ content in viral supernatants was performed by enzyme-linked immunosorbent assay (ELISA; Coulter, Hialeah, Fla.), and titers were obtained by limiting dilution analysis on 3-day phytohemagglutinin-stimulated human peripheral blood mononuclear cells (leukopacks were obtained from the American Red Cross).

SCID-hu mice. CB-17 mice homozygous for the SCID defect were bred at the University of California at Los Angeles and housed as described previously (20). Construction of the SCID-hu (Thy/Liv) mouse, using fetal tissue between 18 and 23 weeks of gestational age, has been described previously (2, 30, 32). Four to six months following implantation of the human fetal liver and thymus, mice were injected with 100 tissue culture infectious units of HIV-1_{NL4-3} in 50 μl of medium (2, 20). Mock-infected mice were injected with 50 μl of mock-electroporated COS or CEM cell supernatant, as appropriate. Mice were sacrificed 6 weeks postinfection, and Thy/Liv implants were removed.

In vitro thymocyte cell culture. Fetal thymus tissue ranging in gestational age from 18 to 24 weeks was obtained from Advanced Bioscience Resources (Alameda, Calif.) or Anatomical Gift Foundation (Woodbine, Ga.). Tissue was minced, and single-cell suspensions were nylon wool purified as previously described (44, 45). Up to 2×10^8 thymocytes were then either exposed to mock-electroporated cell supernatant or infected with HIV-1 as described previously (45). Briefly, virus was added in the presence of Polybrene (10 μg/ml) for 2 h with periodic shaking. Cells were then washed and cultured at a concentration of 2×10^7/ml in Iscove's modified Dulbecco medium (Irvine Scientific, Santa Ana, Calif.) containing bovine serum albumin (1.1 mg/ml; Sigma Chemical Co., St. Louis, Mo.), transferrin (85 μg/ml), 2 mM glutamine, penicillin-streptomycin (25 mg/ml; Irvine Scientific), recombinant human interleukin-2 (IL-2; 20 U/ml) and recombinant human IL-4 (20 ng/ml; R & D Systems, Inc.) as described previously (44). The protease inhibitor Indinavir (Merck, West Point, Pa.) was used in some experiments at a concentration of 100 nM. For differentiation studies, 10^7 thymocytes were cultured in the presence of IL-2 and IL-4 as described above. Some cultures contained hydroxyurea (Sigma) at a concentration of 10 mM for the entire culture period.

Flow cytometry and fluorescence-activated cell sorting (FACS). HIV-1-infected and mock-infected Thy/Liv implants from SCID-hu mice were subjected to biopsy, teased into single-cell suspensions, and stained with monoclonal antibodies specific for CD4 (Leu3a or v4) and CD8 (Leu2a) conjugated with phycoerythrin (PE) and fluorescein isothiocyanate (FITC) (Becton Dickinson, San Jose, Calif.), respectively (20). Staining with Leu3a or v4 yielded similar results with respect to both staining and quantitative PCR for viral distribution, indicating that epitope masking of the Leu3a antibody was not occurring (data not shown). Cells were then placed in serum-free medium, analyzed, and sorted into respective populations, using a FACStar^{Plus} flow cytometer. Immunophenotypic analysis was performed with the Cellquest program (Becton Dickinson). Live cells from the Thy/Liv implants were gated by using forward versus side scatter analysis.

In vitro-cultured thymocytes were removed from culture, washed with serum-free medium, stained similarly with 3 μl each of antibodies specific for CD4 (PE) and CD8 (FITC) per 10^6 live thymocytes (as determined by trypan blue exclusion), and washed twice more with serum-free medium. 7-Amino-actinomycin D was added at a concentration of 1 μg/ml to the in vitro-cultured cells before analysis and sorting for dead cell discrimination (36). Experiments investigating HIV-1 expression used anti-Thy1.2 monoclonal antibody conjugated with allophycocyanin (APC) at a concentration of 1 μg/million live thymocytes (Caltag, South San Francisco, Calif.). Studies involving analysis of differentiation in vitro used monoclonal antibodies specific for CD1a (PE), CD5 (FITC), CD3 (PE), and CD45RA (FITC).

Quantitative PCR. Unsorted or sorted cells were pelleted, lysed in urea lysis buffer (4.7 M urea, 1.3% sodium dodecyl sulfate, 0.23 M NaCl, 0.67 M EDTA, 6.7 mM Tris-HCl [pH 8.0]) and then subjected to phenol-chloroform extractions followed by ethanol precipitation. Purified DNA was subjected to 25 cycles of quantitative PCR using the AA55-M667 primer pair for HIV-1 detection and quantitation in conjunction with a primer pair specific for human β-globin. One primer from each pair was end labeled with ^{32}P as described previously (51). Following resolution on a 6% polyacrylamide gel, quantitation was performed by value comparison to standard curves of known amounts of both HIV-1 plasmid DNA and human genomic DNA, using an Ambis radioanalytic imager (San Diego, Calif.). The percentage of viral DNA in described subsets was calculated as [(amount of proviral DNA in subset)(% of subset in total culture)]/total amount of proviral DNA in culture.

RNA purification and Reverse transcriptase-PCR (RT-PCR). Sorted cells were examined for the presence of CD4 mRNA following extraction of the total RNA from cell pellets. Cell pellets were resuspended in RLT lysis buffer (Qiagen, Chatsworth, Calif.) and frozen at −70°C until the extraction procedure was performed. Extraction of RNA was performed with the use of the RNeasy column extraction procedure (Qiagen). Total RNA was eluted from the column in 30 μl of double-distilled, RNase-free water. A fraction of the eluted sample, which contained amounts of contaminating DNA, was removed for direct quantitative DNA PCR for HIV and human β-globin sequences as described above. The remainder of the sample was DNase treated (12.5 mM Tris [pH 7.5], 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 RNasin [1 U/μl; Promega, Madison, Wis.], template DNA RQ1 RNase-free DNase [1 U/μg; Promega]) for 15 min at 37°C to remove contaminating DNA sequences. RNA was then repurified by RNeasy column purification (Qiagen) and eluted from the column in 30 μl of RNase-free water.

RT-PCR was performed for CD4 and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA, using a GeneAmp Thermostable rTth Reverse Transcriptase RNA PCR kit (Perkin-Elmer, Foster City, Calif.), with slight modifications. RT-PCR for CD4 mRNA was performed with primers previously described (28). An RNA splice site exists between the two amplification primers, and chromosomal DNA or other sequences are not amplified (26, 28). Briefly, the reverse transcription step involved incubating antisense primer (CD4.2, 16 μM) with sample RNA and reaction components in a total volume of 10 μl for 5 min at 55°C to allow more efficient primer annealing and initiation of reverse transcription. Samples were then incubated at 70°C for 15 min to allow optimal reverse transcription elongation and completion. Chelating/DNA amplification buffer containing the sense primer (CD4.1, 4.725 μM) and other reaction components was added to a total reaction volume of 50 μl. DNA amplification was then performed, using 35 cycles of 1 min at 94°C for denaturation and 2.5 min at 60°C for annealing and elongation. For detection of the final product, a third primer (CD4.3) (28) was ^{32}P labeled as described previously (51) and added as a hybridization probe to the amplified reaction product at a concentration of 0.02 pM. A portion of the reaction product (12 μl) was analyzed by polyacrylamide gel electrophoresis as described previously (51) to detect a 299-bp fragment.

To determine cellular RNA input, RT-PCR was performed with primers to a cellular housekeeping gene, *GAPDH*. Sense (GAPDH1; 5′-GACCCCTTCATT GACCTCAAC-3′) and antisense (GAPDH2; 5′-CTTCTCCATGGTGGTGAA GAC-3′) primers were used in the reverse transcription and amplification reactions, employing the RT-PCR system described above with other modifications. During the reverse transcription step, sample RNA, the antisense primer (GAPDH2; final concentration, 15 μM), and the other reaction components were incubated at 70°C for 20 min. Following reverse transcription, the chelating/DNA amplification solution, containing ^{32}P-labeled GAPDH1 (4.24 μM), was added, and amplification took place, using 25 cycles of 1 min at 94°C and 2 min at 60°C preceded by 3 min at 94°C, yielding a 222-bp product. The sample was subsequently analyzed by acrylamide gel electrophoresis as described above. Standards for both CD4 and *GAPDH* mRNA were made by using RNA derived from the CD4-positive CEM cell line.

In vitro infection of CD4-negative thymocytes. To examine direct infection, CD8 SP and CD4 SP cells from thymocytes cultured for 1 week were sorted as described above. Following sorting, CD8 SP or CD4 SP thymocytes were exposed to HIV-1_{NL-thy} at a multiplicity of infection of 1 infectious unit per cell for 2 h in the presence of Polybrene (10 μg/ml). Cells were then washed twice, cultured for 1 more week, and subsequently immunophenotypically analyzed. To examine cell-cell infection, CEM cells infected with HIV-1_{NL-thy} producing >1,000 ng of $p24^{gag}$/ml/day were added to an equal number of sorted CD8 SP thymocytes. Cells were cultured for 1 week and subsequently immunophenotyped and analyzed by excluding CD4-positive cells (CEM cells). $p24^{gag}$ in the culture was measured by ELISA. Mock-infected thymocytes were exposed to cell-free CEM supernatant in parallel.

RESULTS

HIV-1 proviral distribution in thymocytes. To determine the cellular distribution of HIV-1 proviral sequences in thymocytes, Thy/Liv implants were infected with the syncytium-inducing, T-cell-tropic molecular clone HIV-1_{NL4-3} (1) by intrathymic injection. Thymocytes were harvested up to 7 weeks postinoculation, immunophenotypically analyzed for cell surface expression of CD4 and CD8, and subsequently sorted into CD4 SP, CD8 SP, CD4CD8 DP, and CD4CD8 DN populations by FACS. Sorted cells were subjected to quantitative PCR to assess levels of proviral DNA in the various subsets (51). As we have previously shown, HIV-1_{NL4-3} infection caused a depletion of the CD4-bearing cell populations, particularly the CD4CD8 DP population (Fig. 1A). HIV-1 proviral DNA was found distributed in every thymocyte population, including the CD8 SP population and the CD4CD8 DN population (Fig. 1B).

To investigate the mechanisms behind HIV-1 localization in phenotypically CD4-negative thymocyte populations, we used an in vitro thymocyte culture system that allows a greater amount of manipulation than the SCID-hu system. Purified

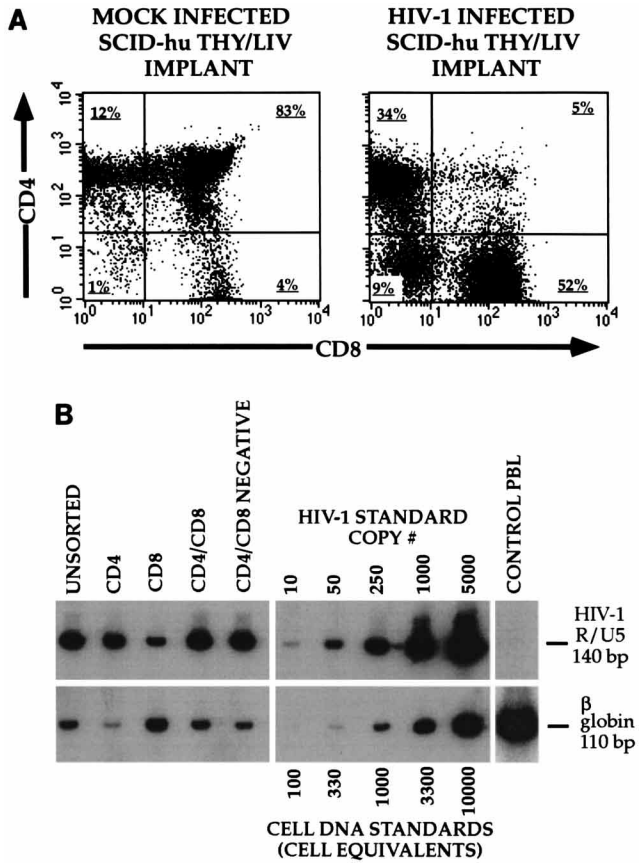

FIG. 1. HIV-1-induced depletion and proviral distribution of thymocytes in vivo. (A) HIV-1-induced depletion in the Thy/Liv implant. Six weeks after infection with HIV-1_{NL4-3}, the entire Thy/Liv implant was removed, and thymocytes were immunophenotyped by using monoclonal antibodies specific for CD4 (PE) and CD8 (FITC) and analyzed by flow cytometry. (B) Proviral distribution in an HIV-1-infected Thy/Liv implant in the SCID-hu mouse. Thymocytes were surface stained for CD4 and CD8 and sorted into CD4 SP, CD8 SP, CD4CD8 DP, and CD4CD8 DN populations by FACS. Live unsorted thymocytes and uninfected human peripheral blood lymphocytes (PBL), gated only on forward versus side scatter, were run through the flow cytometer and collected in the absence of phenotypic sorting. DNA from these populations was isolated and subjected to quantitative PCR using primers to detect the presence of the HIV-1 R/U5 region of the long terminal repeat (140 bp) and β-globin sequences (110 bp). The human PBL lane was purposely overloaded and run on a distant region of the gel to verify the absence of contaminating HIV-1 sequences.

human fetal thymocytes were infected with either HIV-1_{NL4-3} or the non-syncytium-inducing HIV-1_{JR-CSF} molecular clone (22) and cultured in the presence of IL-2 and IL-4, two cytokines present in the normal thymus that function in the proliferation and differentiation of thymocytes (42, 43, 46). We have previously shown that these two cytokines have a synergistic effect in promoting HIV-1 replication in this in vitro system (16). Infected and mock-infected thymocytes were removed at regular time points and analyzed by flow cytometry for CD4 and CD8 cell surface expression.

Six days after infection with either virus, thymocytes demonstrated a decrease in the percentage of CD4-bearing cell populations (Fig. 2A). Within 12 days postinfection, thymocytes infected with HIV-1_{NL4-3} were depleted of most of the CD4 SP and CD4CD8 DP cells. Thymocytes infected with HIV-1_{JR-CSF} demonstrated a less severe depletion of CD4-bearing cells; depletion of cells with low levels of surface expression of CD4 and CD8 (CD4CD8 DP dim) was most apparent. Analysis of replication kinetics by ELISA for viral p24 antigen production in the supernatant revealed that the replication rate of HIV-1_{NL4-3} was slightly greater than that of HIV-1_{JR-CSF} (data not shown), as has been observed in infected Thy/Liv implants in SCID-hu mice (20).

Quantitative PCR for HIV-1 proviral DNA in sorted cells was performed to determine if infection of certain thymocyte subsets was related to their depletion. Within 1 week postinfection with either viral strain, proviral DNA sequences were found primarily in the CD4 SP and the CD4CD8 DP subsets, with 96% of the total HIV-1_{NL4-3} and 92% of the total HIV-1_{JR-CSF} found in these subsets at this time (Fig. 2B) (percentages calculated as described in Materials and Methods). Within 2 weeks postinfection, the distribution of proviral DNA shifted into the CD4-negative populations (CD8 SP and CD4CD8 DN). At this time, there was an approximately ninefold increase in the percentage of total HIV-1_{NL4-3} (from 4% within week 1 to 36% within week 2) and a fivefold increase in the percentage of total HIV-1_{JR-CSF} (from 9% within week 1 to 45% within week 2) in the CD4-negative subsets, with the largest increase found in the CD4CD8 DN population. This phenomenon was observed following sorting with monoclonal antibodies specific for CD4 that bind either within (Leu3a) or outside (v4) the HIV-1 CD4 binding domain, indicating that epitope masking was not occurring (data not shown). Thus, early during HIV-1 infection of fetal thymocytes in vitro, HIV-1 proviral DNA is found primarily in the CD4-bearing subsets, consistent with the early depletion of these subsets. Following depletion, proviral DNA distribution shifts into cells that lack CD4 cell surface expression, similar to what was observed in vivo.

There are three possible mechanisms to explain the localization of HIV-1 in CD4-negative populations. First, HIV may induce down regulation of CD4 cell surface expression on infected thymocytes, resulting in phenotypically CD4-negative cells. HIV-1-mediated down regulation of CD4 by *nef* or *vpu* and envelope has been well documented (8, 9, 13, 14, 18, 35, 37, 49). Second, since HIV-1 has been shown to infect non-CD4-expressing cell types either through direct or cell-cell infection, a non-CD4-mediated entry mechanism could be operating. Third, since thymocyte differentiation has been shown to occur in both the SCID-hu mouse and our in vitro system (30, 46), an immature thymocyte could be infected at a CD4-positive stage of T-lymphoid development and further differentiate into a cell lacking CD4 surface expression which harbors HIV-1 proviral DNA.

Role of HIV-1 *nef* in proviral distribution. The HIV-1 *nef* gene product has been shown to down regulate the display of CD4 at the cell membrane through a posttranslational endocytic mechanism (13, 14, 35, 37). To determine if *nef* is involved in HIV-1 proviral DNA localization in CD4-negative thymocytes, thymocytes were infected with either wild-type HIV-1_{NL4-3} or an isogenic strain which contains a deletion in the *nef* gene (HIV-$1_{NL4-3\Delta nef}$) (19). Removal of *nef* did not alter the overall distribution of proviral DNA; however, the kinetics of depletion were slightly delayed (not shown), consistent with the attenuated phenotype of HIV-$1_{NL4-3\Delta nef}$ in Thy/Liv implants in SCID-hu mice (3, 19). Thus, the appearance of HIV proviral DNA in phenotypically CD4-negative cells was not caused by *nef*-mediated CD4 down regulation.

Since a deletion of the *nef* gene did not alter the viral distribution from that of the wild type, HIV-1_{NL4-3} containing the murine *thy1.2* gene within the *nef* open reading frame (HIV-1_{NL-thy}) (34) was used to introduce a virally encoded marker gene to examine virus expression in cultured thymocyte subsets. Mock-infected and thymocytes infected with either

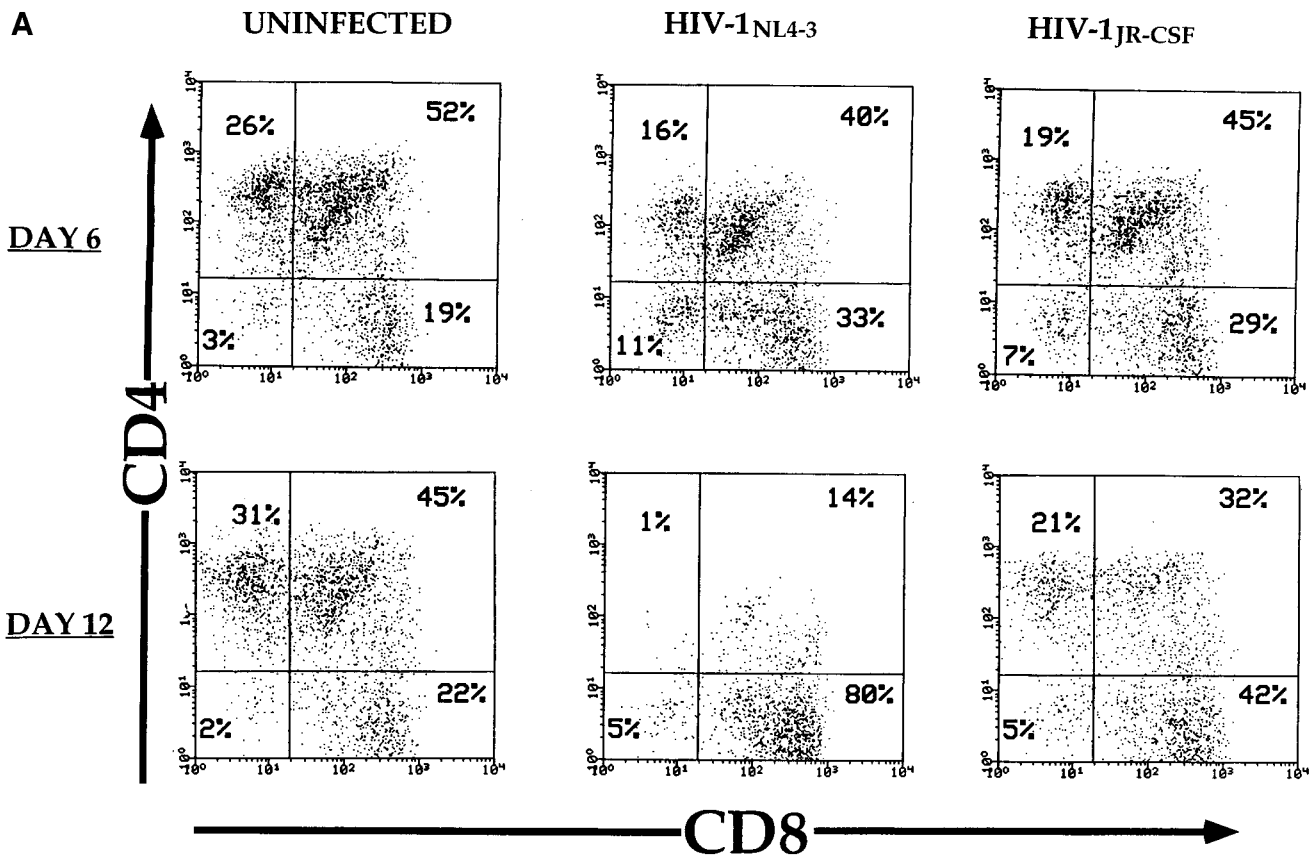

FIG. 2. HIV-1-induced depletion and proviral distribution of thymocytes in vitro. (A) HIV-1-induced depletion of thymocytes in vitro. Fresh nylon wool-purified fetal thymocytes were infected with either HIV-1_{JR-CSF} or HIV-1_{NL4-3} and cultured in serum-free medium containing IL-2 and IL-4 for 12 days. On days 6 and 12, thymocytes were immunophenotyped for CD4 (PE) and CD8 (FITC) and subsequently analyzed by flow cytometry. Percentages of cells found in each quadrant are indicated. (B) HIV-1 proviral distribution in fetal thymocytes in vitro. Thymocytes from infected cultures were surface stained and sorted by a FACStar^{Plus} on the days indicated into the indicated populations. Due to depletion of the CD4CD8 DP population at day 11 in thymocytes infected with HIV-1_{NL4-3}, DP bright and DP dim populations were not separable; therefore, the entire DP population was analyzed. As a control, murine lymphoma cells were passed through the FACStar^{Plus} between individual sorts to detect the presence of contaminating viral and cellular DNA sequences and determine the potential of sort to sort contamination. Murine control cells contained no significant contaminating DNA sequences. DNA from the sorted populations was isolated and subjected to quantitative PCR as described above. Quantitative standards amplified in parallel are indicated.

HIV-$1_{NL4-3\Delta nef}$ or HIV-1_{NL-thy} were analyzed at various times postinfection by flow cytometry for CD4, CD8, and Thy1.2 expression. In HIV-1_{NL-thy}-infected cultures (Fig. 3), Thy1.2 expression was detectable 6 days postinfection, with 2% of the total thymocyte population expressing Thy1.2, primarily in the CD4CD8 DP population. Expression of Thy1.2 in the bulk population peaked at day 14 postinfection, where 14% of total thymocytes expressed the marker gene. As depletion occurred, relative expression in the CD8 SP and the CD4CD8 DN populations increased; at day 18, virus expression occurred predominantly in these populations, paralleling the shift observed in proviral DNA. In contrast, cells staining for Thy1.2 in either mock-infected or HIV-$1_{NL4-3\Delta nef}$-infected cultures never exceeded 0.58% of the total cells at all time points tested (not shown). These results indicate that a subset of the viral DNA found in the CD4-negative populations is transcriptionally active and is not a defective or transcriptionally silenced form of the virus.

Role of CD4 down regulation in proviral localization. In addition to Nef, gp160 and Vpu have been shown to down regulate CD4 cell surface expression by posttranslational mechanisms (8, 9, 18, 49). If posttranslational CD4 down regulation was occurring, CD4 mRNA would be present at greater levels in cell surface CD4-negative, HIV-1-infected cells than in cell surface CD4-negative, uninfected cells. To determine if CD4 down regulation was occurring via a posttranslational mechanism in phenotypically CD4-negative cells, HIV-1-infected and mock-infected CD4-negative thymocytes were sorted and examined for levels of CD4 mRNA, using RT-PCR (Fig. 4A). CD4 mRNA levels in unsorted, uninfected cells were much higher than in unsorted, HIV-1_{NL4-3}-infected cells, indicating that HIV-1 was depleting cells that express CD4. CD4 mRNA levels in infected CD4-negative cells were not higher than in uninfected CD4-negative cells. Quantitative PCR for HIV-1 proviral DNA determined that, assuming one copy of HIV-1 DNA per cell, approximately 31% of the unsorted thymocytes, 25% of the CD8 SP cells, and 46% of the CD4CD8 DN cells harbored proviral DNA in this experiment (Fig. 4B). Thus, if posttranslational CD4 down regulation was operating, CD4 RNA would have been easily detectable in our assay. The evidence indicates that HIV-1 is cytopathic to cells that express CD4 RNA and that posttranscriptional CD4 down regulation does not appear to be the major mechanism involved in the appearance of proviral DNA in cells that lack cell surface CD4 expression.

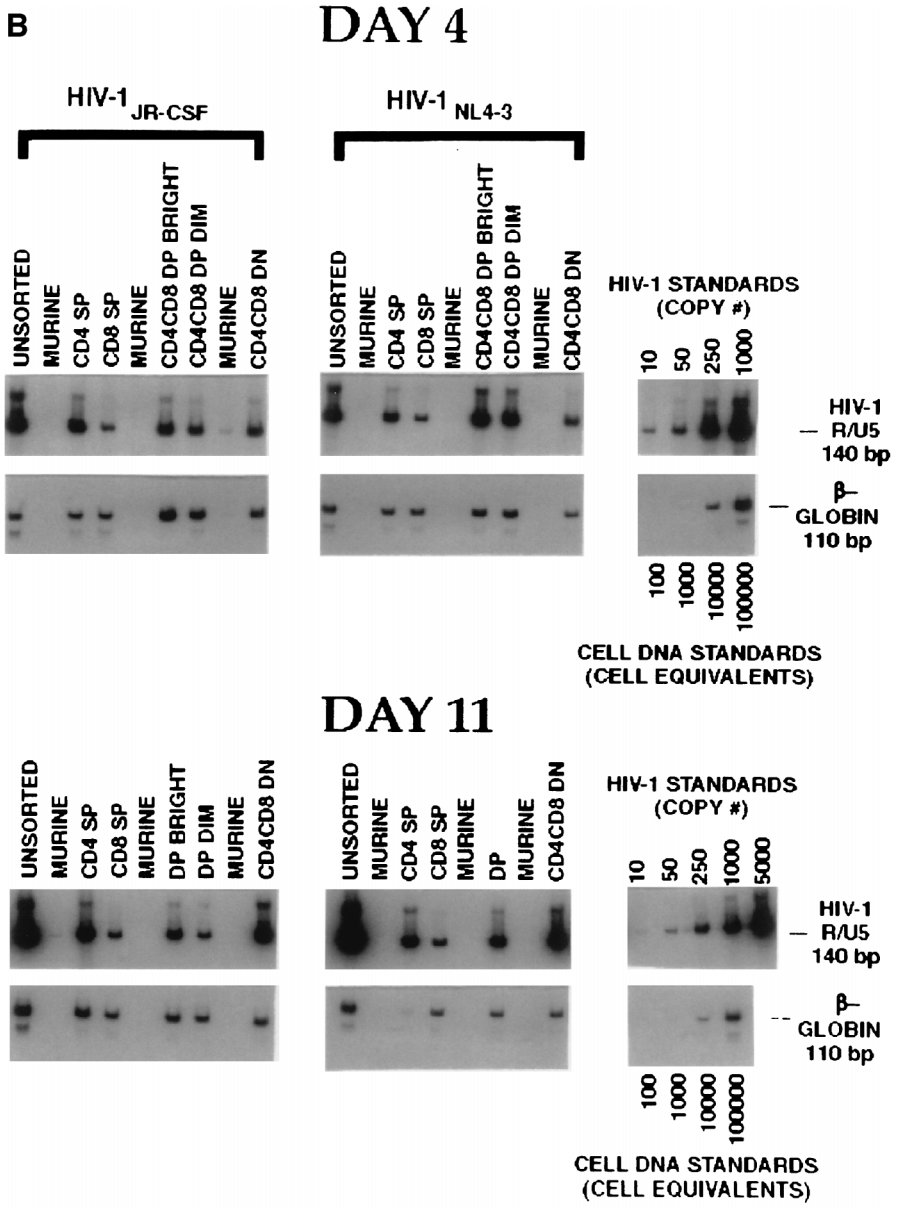

FIG. 2—*Continued.*

Role of CD4-independent infection. To determine if either direct infection or cell-to-cell transfer of HIV-1 into CD4-negative cells was occurring in fetal thymocytes, CD8 SP fetal thymocytes were sorted and either mock infected, directly exposed to cell-free HIV-$1_{NL\text{-}thy}$, or cocultured with HIV-$1_{NL\text{-}thy}$-infected CEM cells. Thymocytes were then cultured for 1 week and subsequently analyzed by flow cytometry for Thy1.2 expression. While Thy1.2 expression was easily detected in CD4 SP cells sorted and infected in parallel (not shown), Thy1.2 expression was not detected in CD8 SP cells exposed to virus in either fashion (Fig. 5). Thus, CD8 SP thymocytes are not directly susceptible to infection by HIV-1 in vitro, and this is not the mechanism responsible for the appearance of HIV-1 in CD4-negative thymocytes.

Role of differentiation of infected CD4CD8 DP thymocytes. To investigate if the mechanism allowing HIV-1 expression in CD4-negative cells was the result of infection of immature CD4-positive cells which differentiated into cells lacking CD4 expression, we initially performed studies to determine if differentiation of fetal thymocytes occurred in our in vitro culture system. Freshly isolated human fetal thymocytes were analyzed for expression of thymocyte cell surface markers which are indicative of different stages of T-cell maturation. CD1a is expressed on less mature thymocytes, whereas high levels of CD5 and CD45RA are expressed on more mature thymocytes. In addition, cell surface expression of CD3 increases as a thymocyte becomes more mature (41). Fresh thymocytes were cultured for 1 week in the presence of IL-2, IL-4, and hydroxyurea (to prevent proliferation [46]) and analyzed for differentiation markers. Total survival of the cultured thymocytes was approximately 30% after 1 week in culture, and analysis by flow cytometry at this time revealed the increased presence of a CD1a-negative, CD5-positive population (Fig. 6A). Selective survival of this CD1a-negative, CD5-positive population could

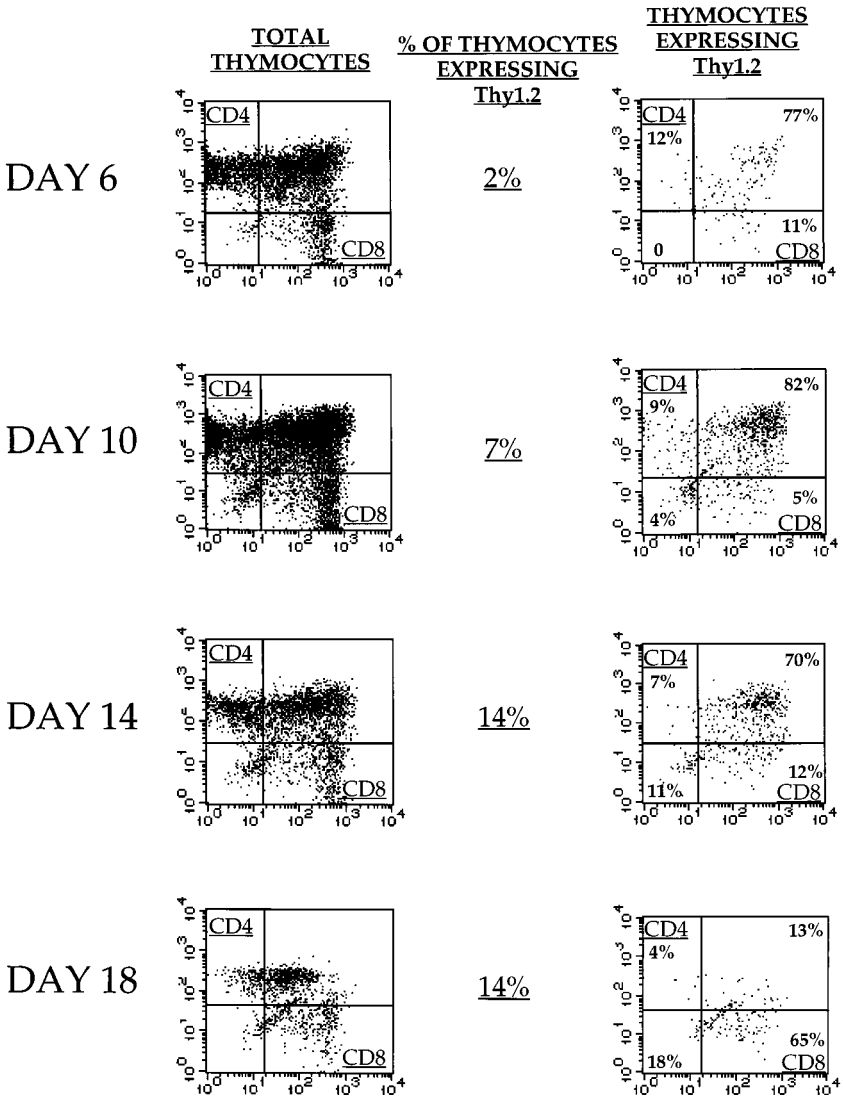

FIG. 3. Kinetics of viral expression of HIV-$1_{NL\text{-}thy}$. At the indicated times after infection with the virus containing murine *thy1.2* in the *nef* region, cells were immunophenotyped for CD4 (PE), CD8 (FITC), and Thy1.2 (APC) surface expression and analyzed by flow cytometry. The percentage of cells positive for Thy1.2 expression is represented in the middle column. The phenotype of thymocytes expressing Thy1.2 was determined by gating on the cells that were positive for Thy1.2 at a given time point and analyzing this population for CD4 and CD8 cell surface expression (right column).

account for the changes observed in the total population. However, when these thymocytes were analyzed for CD45RA and CD3 expression, a fairly mature population (CD3 dim, CD45RA positive) that was essentially undetectable (0.7% of total thymocytes) increased in relative frequency more than 40-fold (to 30% of total thymocytes) in the absence of proliferation. This was accompanied by a decrease in CD3 dim, CD45RA-negative cells as well as a fivefold increase in the CD3 bright, CD45RA-positive population. This major 40-fold increase in a more mature phenotype cannot be explained by selective survival if total thymocyte number decreased only 3-fold. Thus, the culture of fetal thymocytes in the presence of IL-2 and IL-4 resulted in the increased expression of multiple markers of T-cell maturation, which cannot be explained by selective survival of mature thymocytes. This finding firmly establishes that a process of induced differentiation is occurring in our in vitro system.

To examine if HIV-1 was infecting a CD4-positive cell type which further differentiated into a CD4-negative cell type harboring and expressing the virus, CD4CD8 DP fetal thymocytes were sorted 1 week following infection with HIV-$1_{NL\text{-}thy}$ or HIV-$1_{NL\text{-}\Delta nef}$. Postsort analysis revealed that the purity of the sorted populations was greater than 98% CD4CD8 DP bright. These cells were then cultured for 1 additional week to allow differentiation and subsequently analyzed by flow cytometry for Thy1.2 expression (Fig. 6B). Analysis 7 days after the sort revealed the presence of an increased percentage of CD4 SP and CD8 SP populations, indicating that differentiation had occurred in vitro. Expression of Thy1.2 was found in the newly differentiated CD8 population in HIV-$1_{NL\text{-}thy}$-infected cultures. In these experiments HIV-$1_{NL\text{-}\Delta nef}$-infected cultures showed undetectable levels of Thy1.2 expression (not shown). The distribution of cells in each population expressing HIV-1 in the sorted, newly differentiated culture recapitulated that observed in an unsorted culture. We also found in two of three experiments that CD4CD8 DP cells could differentiate into

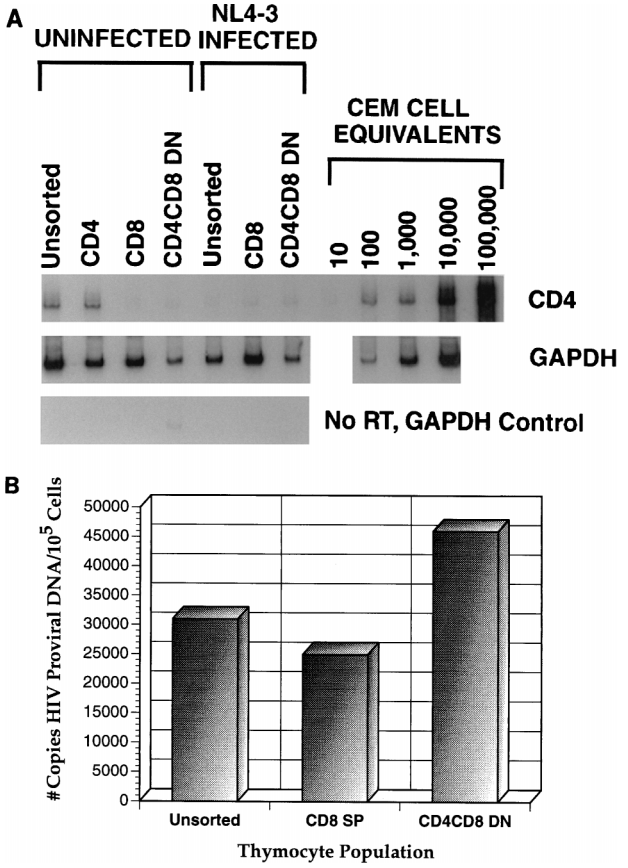

FIG. 4. CD4 expression in sorted thymocytes. (A) HIV-1-infected and uninfected cultured fetal thymocytes were sorted 12 days postinfection into CD8 SP and CD4CD8 DN populations as described in Materials and Methods. Unsorted thymocytes were passed through the sorter without phenotypic selection. RNA was isolated from the sorted populations and subjected to RT-PCR. Primers specific for CD4 were used to detect CD4 mRNA levels (28), and primers specific for the housekeeping gene *GAPDH* were used to determine cellular RNA input. A PCR amplification lacking an RT reaction was performed to detect the presence of contaminating cellular DNA in each reaction. Standards consisting of RNA derived from cultured CEM cells were amplified in parallel and are indicated. (B) Quantitative PCR for HIV-1 proviral DNA in thymocyte populations sorted prior to RT-PCR. Data obtained from radioanalytic image analysis of the samples shown in panel A are presented graphically, showing the proviral burden of each subset.

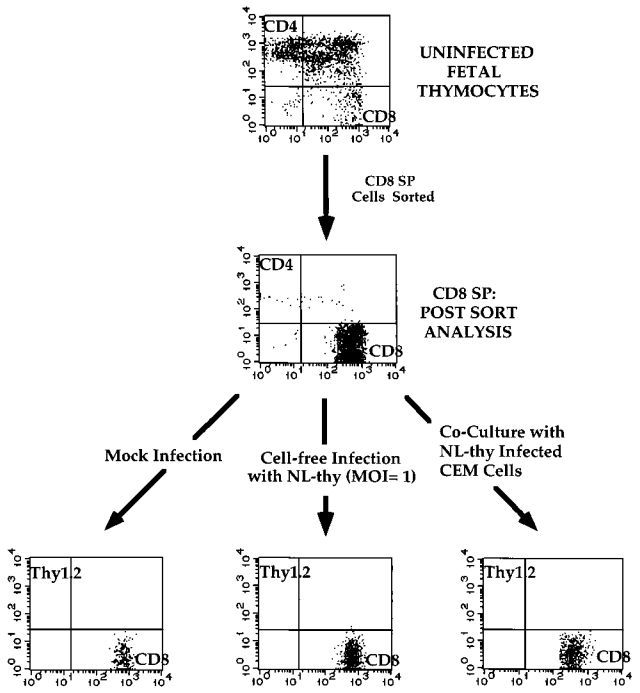

FIG. 5. Cell-cell and Cell-free HIV-1 infection of CD8 SP cells. CD8 SP cells were sorted from cultured thymocytes and split into three aliquots. The cells were either mock infected with CEM cell culture supernatant, exposed to cell-free HIV-$1_{NL\text{-}thy}$ at a multiplicity of infection (MOI) of 1, or exposed to HIV-$1_{NL\text{-}thy}$-infected CEM cells. All cells were cultured for 7 days and immunophenotyped for Thy1.2 and CD8 cell surface expression. CD8-negative cells, the virus-producing cell line, were eliminated by gating. Cocultured CD4-positive cells are not shown, as they cannot easily be differentiated from producer cells.

CD4CD8 DN cells (data not shown). In one of these experiments in which protease inhibitor was added to prevent potential viral spread, quantitative PCR of the sorted CD8 and CD4CD8 DN populations showed the presence of 2 copies per 100 cells in the CD8 SP population and 23 copies per 100 cells in the CD4CD8 DN population. This result indicates that differentiation of an infected CD4CD8 DP precursor was responsible for localization of proviral sequences in both CD4-negative subsets. A smaller percentage of cells expressed Thy1.2 14 days postinfection than harbored proviral DNA, suggesting that a portion of the viral DNA in these subsets may be defective. Nevertheless, some of these cells lacking CD4 are productively infected.

In the experiment illustrated in Fig. 6B, total survival of sorted CD4CD8 DP cells from infected cultures was 35% 7 days after the sort. Therefore, selective survival of the small number of contaminating cells in SP quadrants (1% in each quadrant) could not give rise to the increased numbers of CD4 SP and CD8 SP cells seen following culture. In addition, at the time of sort, the level of Thy1.2 expression in the SP populations was below 1%. Thus, in the absence of a CD4-independent infection mechanism, selective expansion of Thy1.2-expressing cells in the CD8 SP population cannot account for the 27% expression observed following differentiation. Taken together, these results indicate that following productive infection, CD4CD8 DP cells can differentiate into cells lacking CD4 expression and result in the expression of the virus in these CD4-negative cells.

DISCUSSION

HIV-1 infection of non-CD4-bearing cells has been shown to occur in implants in the SCID-hu mouse and in vitro in cultured thymocytes (2, 39, 44). Others have reported HIV-1 infection of CD4-negative thymocytes, but these studies did not rule out involvement of a CD4-mediated pathway in infection (15, 47). HIV-1 has also been found harbored in CD8 SP lymphocytes in vitro in various culture systems and in the peripheral blood and lungs of patients with AIDS (11, 27, 31, 38). We have examined the mechanism behind distribution of HIV-1 into CD4-negative thymocytes. Our data exclude epitope masking, HIV-1-mediated CD4 down regulation, and cell-cell and cell-free infection of CD8 SP cells as the mechanisms involved in this phenomenon. Our data demonstrate that an immature CD4CD8 DP thymocyte becomes infected with HIV-1 and further differentiates into CD8 SP cells and likely into CD4CD8 DN cells which contain proviral DNA and can express viral genes following differentiation. It is of interest that we were unable to detect viral expression in CD4 SP cells

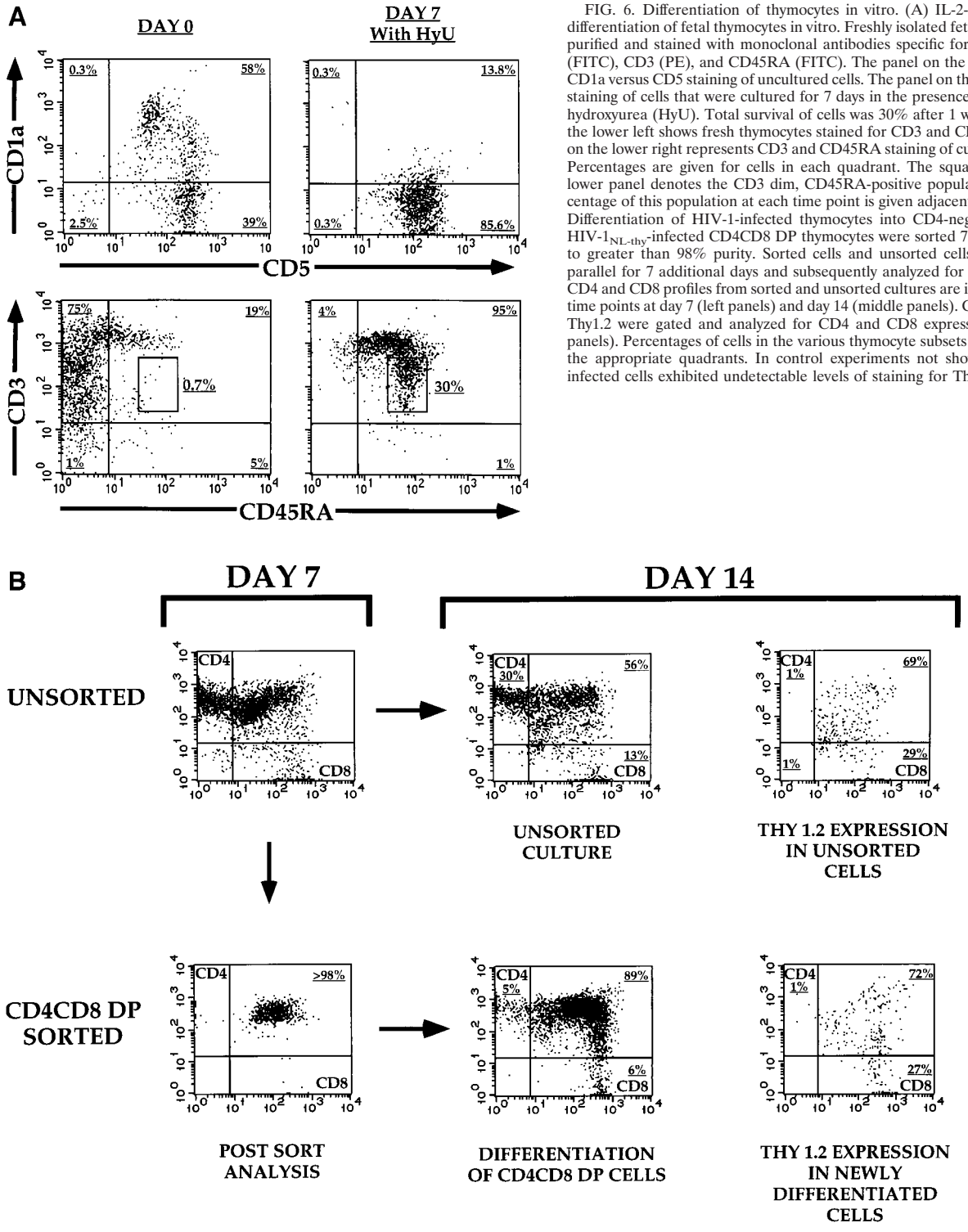

FIG. 6. Differentiation of thymocytes in vitro. (A) IL-2- and IL-4-induced differentiation of fetal thymocytes in vitro. Freshly isolated fetal thymocytes were purified and stained with monoclonal antibodies specific for CD1a (PE), CD5 (FITC), CD3 (PE), and CD45RA (FITC). The panel on the top left represents CD1a versus CD5 staining of uncultured cells. The panel on the top right displays staining of cells that were cultured for 7 days in the presence of IL-2, IL-4, and hydroxyurea (HyU). Total survival of cells was 30% after 1 week. The panel on the lower left shows fresh thymocytes stained for CD3 and CD45RA. The panel on the lower right represents CD3 and CD45RA staining of cultured thymocytes. Percentages are given for cells in each quadrant. The square region on each lower panel denotes the CD3 dim, CD45RA-positive population, and the percentage of this population at each time point is given adjacent to the region. (B) Differentiation of HIV-1-infected thymocytes into CD4-negative populations. HIV-$1_{NL\text{-}thy}$-infected CD4CD8 DP thymocytes were sorted 7 days postinfection to greater than 98% purity. Sorted cells and unsorted cells were cultured in parallel for 7 additional days and subsequently analyzed for Thy1.2 expression. CD4 and CD8 profiles from sorted and unsorted cultures are indicated at various time points at day 7 (left panels) and day 14 (middle panels). Cells that expressed Thy1.2 were gated and analyzed for CD4 and CD8 expression (day 14, right panels). Percentages of cells in the various thymocyte subsets are represented in the appropriate quadrants. In control experiments not shown, HIV-$1_{NL\text{-}\Delta nef}$-infected cells exhibited undetectable levels of staining for Thy1.2.

following differentiation from sorted CD4CD8 DP thymocytes, although we have detected the presence of HIV-1 proviral DNA in these populations. This observation may reflect a CD4-mediated killing mechanism in cells that express both HIV-1 and CD4 and suggest that the infected CD4-positive cells that preferentially survive harbor defective or latent forms of the virus. This possibility is consistent with low levels of expression in surviving CD4-positive cells in unsorted cultures at later time points (Fig. 3).

Infection of an immature progenitor cell followed by differentiation into a mature productively infected CD8 SP cell is a novel mechanism of HIV-1 entry into a non-CD4-expressing cell. It has been reported that CD8 SP cells in the lungs of patients with AIDS-related lower respiratory tract chronic in-

flammation contain HIV-1 proviral sequences (38). Recently, it has been determined that CD8 SP cells in the peripheral blood of AIDS patients with a CD4 cell count of less than 200/mm^3 serve as the primary reservoir for provirus in the majority of the patients examined (27). We believe that a mechanism similar to what we have described could occur in vivo in either thymic or extrathymic T-cell differentiation where production of CD8 SP lymphocytes is preceded by a CD4CD8 DP precursor cell stage. This phenomenon could play an important role in the pathogenesis of AIDS and may have the greatest impact on perinatally and in utero-infected children, where the majority of mature lymphocytes are derived directly from the thymus. Further, the relatively high levels of proviral DNA in CD8 SP cells in the peripheral blood of AIDS patients who have an AIDS-defining CD4 cell count may indicate that the thymus has a significant role in reconstituting the HIV-1-ravaged immune system (27). Further, HIV-1 infection and expression in CD8 cells could alter function and maturation of the cell through direct lysis, alteration of cellular gene regulation and expression, and induction of apoptosis (25).

Differentiation of T cells into a CD4CD8 DN phenotype is less understood (5, 6). We have determined that in our culture system, the majority of these cells bear CD3 and the αβ T-cell receptor (44). Evidence suggests that this subset is a functionally distinct subset of thymocytes which has cytotoxic activity and secretes a variety of cytokines (5, 12). Studies of the origin of CD4CD8 DN thymocytes in mice suggest that this population arises from cells that previously expressed CD4 (12, 23, 50). Differentiation of CD4CD8 DP cells and CD8 SP cells into CD4CD8 DN cells has been demonstrated previously, and CD8 SP cells may be an intermediate cell type in this process (10, 12, 23). Our data indicate that CD4CD8 DN cells can differentiate from CD4CD8 DP cells in vitro, although they are a small percentage of the total thymocyte population. These cells can also harbor and express HIV-1 after differentiation. It is unclear how productive infection of this cell type might influence pathogenesis; nonetheless, infection would likely alter the function of the cell. Thus, infection of an immature cell followed by subsequent differentiation and virus expression might affect many stages of lymphoid development and could contribute to functional immune abnormalities not directly linked to CD4-positive cells.

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