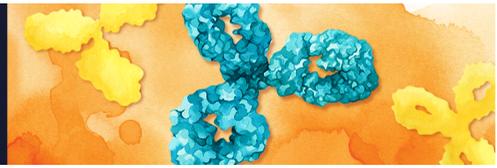


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CXCR4 and CCR5 Expression Delineates Targets for HIV-1 Disruption of T Cell Differentiation¹

Robert D. Berkowitz,* Karen P. Beckerman,*[‡] Thomas J. Schall,[†] and Joseph M. McCune^{2*}

HIV-1 disease is often associated with CD4⁺ T lymphopenia as well as quantitative reductions in naive CD8⁺ T cells and cytopenias involving nonlymphoid hemopoietic lineages. Studies in HIV-1-infected humans as well as in animal models of lentivirus disease indicate that these effects may be secondary to infection and destruction of multilineage and lineage-restricted hemopoietic progenitor cells. To define the stages of T cell differentiation that might be susceptible to HIV-1, we performed flow cytometric analysis of the surface expression of CXCR4 and CCR5 on T cells and their progenitors from fetal tissue, cord blood, SCID-hu Thy/Liv mice, and adult peripheral blood. We found that CXCR4 is expressed at low levels on hemopoietic progenitors in the bone marrow, is highly expressed on immature (CD3⁻CD4⁺CD8⁻) T cell progenitors in the thymus, and then is down-regulated during thymocyte differentiation. As thymocytes leave the thymus and enter the peripheral circulation, the expression of CXCR4 is again up-regulated. In contrast, CCR5 is undetectable on most hemopoietic progenitors in the bone marrow and on intrathymic T progenitor cells. It is up-regulated when thymocytes coexpress CD4 and CD8, then down-regulated either in the thymus (CD4⁺ cells) or during exit from the thymus (CD8⁺ cells). These results indicate that discrete, lineage-related populations of T cell progenitors may vary widely in their potential to respond to chemokines and to be infected by HIV-1, and that T lymphoid differentiation is particularly vulnerable to CXCR4-using viruses. *The Journal of Immunology*, 1998, 161: 3702–3710.

It is generally thought that the CD4⁺ T lymphopenia of HIV-1 disease is due to accelerated destruction of cells in the peripheral lymphoid system. It is also possible that hemopoietic progenitor cells in central hemolymphoid compartments (e.g., bone marrow, fetal liver, and thymus) are affected by HIV-1, resulting in a state of regenerative failure (1). Evidence to support the latter possibility includes the following: multiple abnormalities have been reported in the bone marrow of patients with HIV-1 disease (2, 3), infection of the thymus and thymic dysfunction have been observed in HIV-1-infected fetuses and children (4), and decreases in the absolute number and percentage of naive CD4⁺ and CD8⁺ T cells have been documented in both children and adults infected with HIV-1 (5, 6). In the case of HIV-1-seropositive adults, those with the highest levels of circulating naive CD4⁺ T cells are also found to have abundant thymic mass (as assessed by computed tomography) (7). Finally, recent studies using a novel method for directly measuring the kinetics of CD4⁺ and CD8⁺ T cell subpopulations indicate that regenerative failure plays a quantitatively important role in the T lymphopenia of HIV-1 disease.³

Given the difficulty of obtaining and studying bone marrow and thymic tissue from humans, the heterochimeric SCID-hu mouse was developed (8, 9). This model is constructed upon implantation of either human fetal bone marrow (10) or human fetal liver and fetal thymus (11) into the immunodeficient C.B-17 *scid/scid* mouse. In the case of the more extensively studied SCID-hu Thy/Liv mouse, multilineage human hemopoiesis, including T lymphopoiesis, proceeds for a period of ≥ 12 mo. Human thymopoiesis in the SCID-hu model appears indistinguishable from normal human thymopoiesis, with normal distribution of thymocyte subpopulations, appropriate induction of negative selection, and development of a diverse TCR repertoire (11–13). Perhaps most importantly, relatively large numbers (≥ 50) of SCID-hu Thy/Liv mice can be prepared from the same donor of human tissue, facilitating the design of controlled experiments that investigate the pathogenic properties of HIV-1 in vivo.

Early studies evaluating the effects of HIV-1 on the SCID-hu Thy/Liv organ revealed qualitative interstrain variations in viral pathogenesis: tissue culture-adapted isolates (e.g., HXB2) were noninfectious while primary isolates or molecular clones that had not been extensively passaged in vitro (e.g., JR-CSF and NL4–3) were infectious (14–19). Among the latter HIV-1 isolates, nonsyncytium-inducing (NSI)⁴ (generally, CCR5-using) viruses were observed to slowly replicate in the organ and to be minimally cytopathic. In contrast, syncytium-inducing (SI; generally, CXCR4-using) viruses were observed to rapidly induce thymocyte depletion (20), an effect due to preferential infection and destruction of intrathymic (CD3⁻CD4⁺CD8⁻) T progenitor cells (21). Notably, viruses such as NL4–3 have also been found to deplete multilineage and lineage-restricted CD34⁺ hemopoietic progenitor cells in the Thy/Liv graft.

*Gladstone Institute of Virology and Immunology, San Francisco, CA 94141, and Departments of Microbiology and Immunology and Medicine, University of California, San Francisco, CA 94110; [†]Chemocentryx, Mountain View, CA 94070; and [‡]Department of Obstetrics, Gynecology, and Reproductive Sciences, University of California, San Francisco, CA 94110

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² Address correspondence and reprint requests to Dr. Joseph M. McCune, Gladstone Institute of Virology and Immunology, P.O. Box 419100, San Francisco, CA 94141-9100.

³ M. Hellerstein, M. B. Hanley, D. Cesar, C. Papageorgopoulos, E. Wiedner, D. Schmidt, S. Siler, R. Hoh, R. Neese, D. Macallan, S. Deeks, and J. M. McCune. Direct measurement of T lymphocyte kinetics in humans using a stable isotope-mass spec-

trometric technique: effects of HIV-1 infection and anti-retroviral therapy. *Submitted for publication*.

⁴ Abbreviations used in this paper: NSI, nonsyncytium inducing; SI, syncytium inducing; SDF-1, stromal cell-derived factor-1; PE, phycoerythrin; PerCP, peridinin chlorophyll protein; TC, Tri-color; ECD, phycoerythrin-Texas Red; TN, triple negative; SP8, single-positive CD8; DP, double positive; ITTP, intrathymic T progenitor; SP4, single-positive CD4; MFI, mean fluorescence intensity.

Such depletion occurs even earlier than thymocyte depletion and appears to be effected by indirect means (22).

HIV-1 variants may have differential effects on the hemopoietic system because they are tropic for different cells in the multilineage tree. As has been demonstrated over the past several years, tropism is determined in part by cell surface expression of CD4 and certain chemokine receptors, such as CCR5 and CXCR4 (23). CXCR4 is the receptor for SDF-1 α , an α -chemokine that attracts lymphocytes and monocytes (24–26). CCR5 binds the β -chemokines macrophage inflammatory protein-1 α , macrophage inflammatory protein-1 β , and RANTES, which attract monocytes, T cells, NK cells, basophils, eosinophils, and dendritic cells (23). In the early stages of HIV-1 disease, most virus particles can use only CCR5 as the coreceptor and thus infect only those cells that express CCR5 (27–29). Later, in the symptomatic stages of HIV-1 disease, virus particles often arise that can use both CCR5 and CXCR4, expanding the target cell range to cells that express CCR5 and/or CXCR4 (27–30).

It is still not clear which cell subpopulations in the T cell lineage express CXCR4 or CCR5 and, hence, might be targets for infection with strains of HIV-1 recognizing one or both receptors. While inferences about receptor expression can be made based on transcript analyses (31) or responses to chemokines *in vitro* (23), cell surface CXCR4 and CCR5 proteins can be more directly measured using specific Abs in flow cytometry or immunohistochemistry. Using such Abs, T cells in the bloodstream exhibiting a naive phenotype have been shown to express CXCR4 but not CCR5, while a subset of T cells expressing a memory phenotype expresses CCR5 but not CXCR4 (32). In addition, variations in the levels of CCR5 on peripheral T cells correlate with variations in the infectability of those T cells by a CCR5-using strain of HIV-1 (33), suggesting that the density of surface CCR5 may be a rate-limiting factor for the infection of peripheral T cells. Visualization of CXCR4 or CCR5 on the surface of bone marrow hemopoietic cells has not been reported, but CXCR4 (34) has been found on CD3⁺CD4⁺CD8⁺ thymocytes.

To help understand the basis of the differential infectivities of bone marrow cells and thymocytes, we used flow cytometry to determine the surface levels of CXCR4 and CCR5 on cells at different stages of T cell differentiation. This cell lineage included three discrete stages of CD34⁺ cells in the fetal bone marrow, five different subpopulations of fetal thymocytes, and naive and memory T cells in adult and cord blood. We found that the surface levels of CXCR4 and CCR5 vary widely among the cell types analyzed, indicating that the two coreceptors are cyclically up-regulated and down-regulated during the course of T cell differentiation. These findings may have important implications for the roles of chemokines in T cell production and function as well as for our understanding of HIV pathogenesis.

Materials and Methods

Antibodies

A number of fluorescent mAbs were used in these studies. FITC-conjugated anti-human CD4, CD10, CD38, CD45, and CD45RA; phycoerythrin (PE)-conjugated anti-human CD2, CD4, CD8, CD16, CD19, CD20, and CD56; peridinin chlorophyll protein (PerCP)-conjugated anti-human CD3; allophycocyanin-conjugated anti-human CD34; and FITC-conjugated goat anti-mouse Ig were obtained from Becton Dickinson (San Jose, CA). Tricolor (TC)-conjugated anti-human CD8 and allophycocyanin-conjugated anti-human CD3 were obtained from Caltag (Burlingame, CA). PE-Texas Red (ECD)-conjugated anti-human CD8 was obtained from Coulter (Miami, FL). PE-conjugated anti-human glycoporphin A was obtained from Immunotech (Westbrook, ME). PE-conjugated anti-human CXCR4 clone 47718 and unconjugated anti-human CXCR4 clones 47701 and 47712 were gifts from Monica Tsang (R&D Systems, Minneapolis, MN). These three mAbs recognize epitopes on the second extracellular loop of CXCR4,

with contributions from the first extracellular loop (M. Tsang, unpublished observations), and do not stain cells expressing CXCR1, CXCR2, CXCR3, CCR2, or CCR5 (M. Tsang, unpublished observations, and data not shown). The anti-human CXCR4 clone 12G5 (35) was a gift from James Hoxie (University of Pennsylvania, Philadelphia, PA). The anti-human CCR5 clone 3A9 (33) was a gift from LeukoSite (Cambridge, MA); the mAb was conjugated with PE using a commercial kit (Prozyme, San Leandro, CA). Isotype control Abs for the CXCR4 and CCR5 mAbs consisted of unconjugated mouse IgG2a (Dako, Carpinteria, CA) and PE-conjugated mouse IgG2a and IgG2b (Caltag). Isotype control Abs for the anti-CD mAbs consisted of TC-conjugated mouse IgG2a (Caltag), FITC-conjugated mouse IgG1 and IgG2a, PE-conjugated mouse IgG1 and IgG2a, PerCP-conjugated mouse IgG1, and allophycocyanin-conjugated mouse IgG1 (Becton Dickinson).

Cell preparation

Human fetal bone marrow and thymus were obtained from fetuses at approximately 20 wk gestation at the time of elective termination of pregnancy. Thy/Liv implants were surgically removed from SCID-hu Thy/Liv mice, prepared as previously described (11). Thymi or Thy/Liv implants were ground between the membranes of a nylon mesh bag (Tetko, Kansas City, MO) submerged in PBS; the released cells were rinsed with PBS. Femurs were crushed, and the released cells were subjected to Ficol-Hypaque density centrifugation. For depletion of cells expressing markers of mature lineage cells, mononuclear cells at the Ficoll interface were rinsed with PBS and incubated with a mixture of PE-conjugated Abs specific for human CD2, CD4, CD8, CD16, CD19, CD20, CD56, and glycoporphin A for 30 min at 4°C. Cells coated with these Abs were removed with sheep anti-mouse Ig-conjugated magnetic beads (Dynal, Lake Success, NY). PE-negative cells were then purified from the remaining mixture of cells by FACS using a FACSVantage (Becton Dickinson).

SCID-hu Thy/Liv blood was obtained by cardiac puncture. Adult blood (age, 28–34 yr) was obtained by venipuncture. Cord blood was obtained from term deliveries without evidence of maternal or fetal infection. All procedures and practices were approved by the University of California-San Francisco Committee on Human Research or the University of California-San Francisco Committee on Animal Research.

FACS analysis

Staining of cells with the PE-conjugated CXCR4 or CCR5 mAbs was performed concurrently with mAbs against various CD markers using 10⁶ to 10⁷ purified cells or 50 μ l of whole blood in a 100- μ l reaction volume containing PBS and 2% FBS. Anti-CD markers included CD38-FITC and CD34-allophycocyanin mAbs for bone marrow mononuclear cells; CD10-FITC and CD34-allophycocyanin for lineage-depleted bone marrow cells; CD3-allophycocyanin, CD4-FITC, and CD8-TC or -ECD for thymocytes from fetal thymus or SCID-hu Thy/Liv implants; CD3-PerCP and CD45-FITC for whole blood from adult humans or cord blood. The 47718-PE CXCR4 mAb and its IgG2b-PE isotype control mAb were used at 5 μ g/ml, while the 3A9-PE CCR5 mAb and its IgG2a-PE isotype control mAb were used at 1 μ g/ml. After 20 min, the cells were rinsed in PBS-2% FBS, fixed in 0.5% paraformaldehyde, and analyzed on a FACSVantage (bone marrow, thymus) or were exposed to FACS lysing solution (Becton Dickinson) for 5 min, fixed in 0.5% paraformaldehyde, and analyzed on a FACScan (blood samples).

Staining of thymocytes for CXCR4 using the unconjugated mAbs 12G5, 44701, and 44712 was performed in a four-step procedure. First, the cells were incubated with the CXCR4 mAb or the IgG2a isotype control mAb at 2 μ g/ml for 20 min and rinsed with PBS-2% FBS. The cells were then incubated with the FITC-conjugated goat anti-mouse Ig Ab for 20 min, rinsed with PBS-2% FBS, and blocked with normal mouse serum (Dako) for 20 min. After rinsing with PBS-2% FBS, the thymocytes were incubated with the CD4-PE, CD8-TC, and CD3-allophycocyanin mAbs for 20 min, rinsed with PBS-2% FBS, fixed in 0.5% paraformaldehyde, and analyzed on the FACSVantage.

Results

Expression of CXCR4 in thymus

To determine the expression levels of CXCR4 during discrete stages of human thymocyte differentiation (Fig. 1), thymocytes from SCID-hu Thy/Liv implants were analyzed by flow cytometry. The implants were derived from different donors of fetal thymus

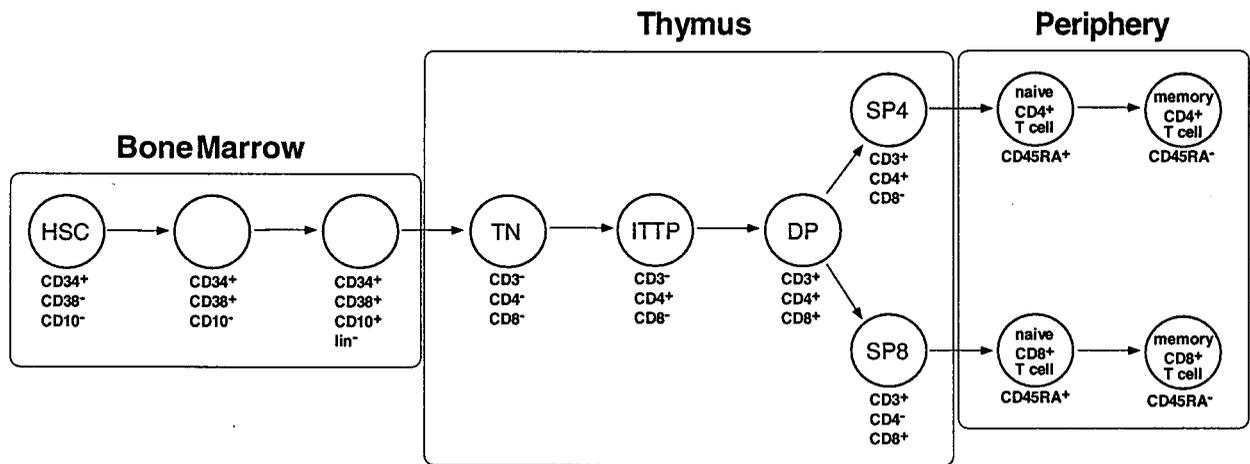


FIGURE 1. Pathway of T cell differentiation. Cell types are described in the text. HSC, hemopoietic stem cell. CD markers used in this study to identify each cell type are listed under each cell.

tissue to assess the variability in CXCR4 expression among individuals. Thymocytes were isolated from the implants and incubated with Abs specific for human CD3, CD4, and CD8 in combination with one of four different mAbs specific for CXCR4: 44701, 44712, 44718-PE (see *Materials and Methods*), and 12G5

(35). A CD4 vs CD8 plot discriminates four subpopulations of thymocytes (Fig. 2A): $CD4^-CD8^-$, $CD4^+CD8^-$, $CD4^-CD8^+$ (single-positive CD8, or SP8), and $CD4^+CD8^+$ (double-positive, or DP). Cells within the $CD4^-CD8^-$ and $CD4^+CD8^-$ subpopulations were further subdivided based on their CD3 expression into

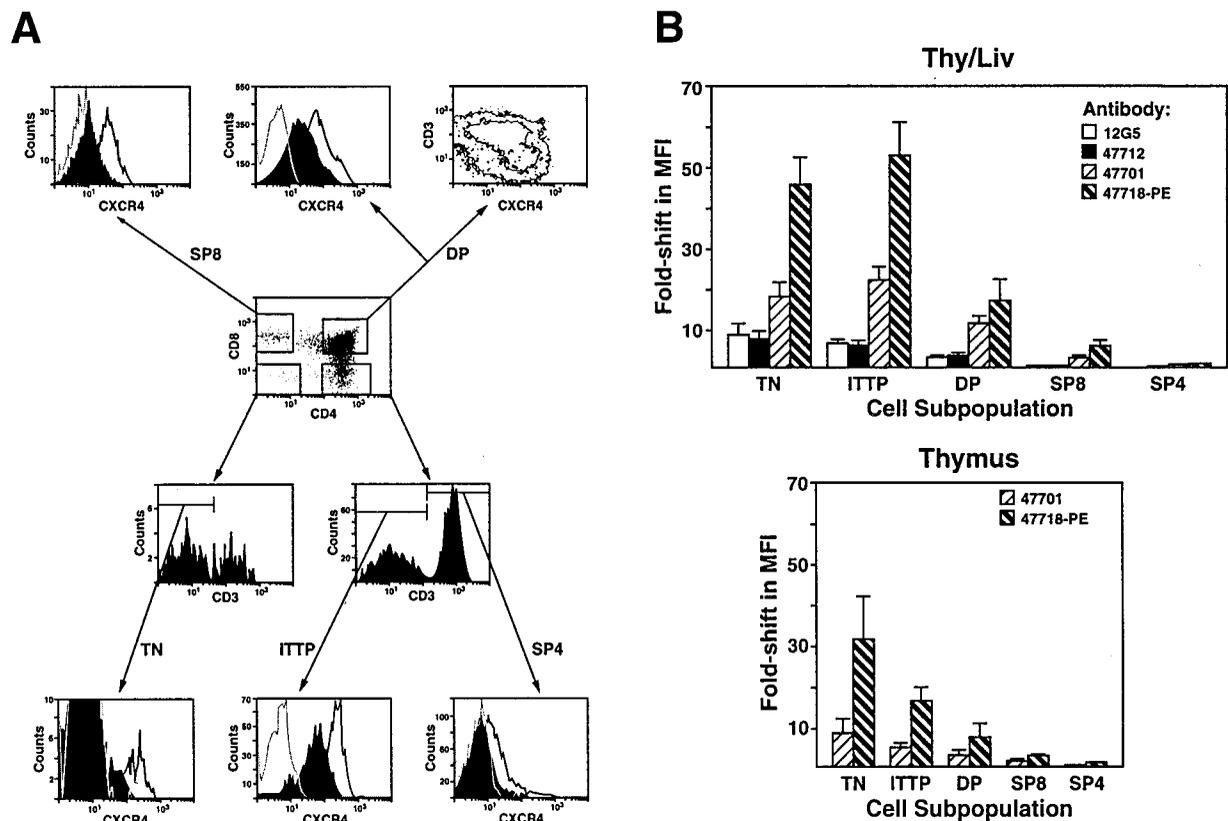


FIGURE 2. Flow cytometric analysis of CXCR4 expression on SCID-hu Thy/Liv and fetal thymocyte subpopulations. *A Middle*, Distribution of subpopulations using a CD4 vs CD8 plot and CD3 histograms on $CD4^-CD8^-$ and $CD4^+CD8^-$ cells. The five major subpopulations, TN, ITTP, DP, SP8, and SP4, are described in the text. *Top and bottom*, CXCR4 histograms for the five subpopulations of a representative SCID-hu Thy/Liv implant, detected by the 12G5 mAb (shaded), the 44701 mAb (black line), and the isotype control mAb (gray line) in combination with an FITC-conjugated goat anti-mouse Ig (F-GAM) secondary Ab. At the *top right* is a CD3 vs CXCR4 contour plot of DP cells. *B*, Average fold shifts in MFI (quotient of the CXCR4 mAb MFI divided by the isotype mAb MFI) for each thymocyte subpopulation in eight (12G5, 44701, and 44712 mAbs) or five (44718-PE mAb) SCID-hu Thy/Liv implants (*top*) and in three (47701) or four (47718-PE) fetal thymi (*bottom*). For TN cells, the MFI of the $CXCR4^+$ subset was divided by the isotype mAb MFI for all TNs. Error bars represent SEs.

Table I. Expression of CXCR4 and CCR5 in T lineage cells^a

Cell Type	Fetal, Cord		SCID-hu Thy/Liv		Adult	
	CXCR4 47718-PE	CCR5 3A9-PE	CXCR4 47718-PE	CCR5 3A9-PE	CXCR4 47718-PE	CCR5 3A9-PE
CD34 ⁺ CD38 ⁻	4.7 ± 1.4 (3)	1.3 ± 0.1, 12.5 ± 2.3 (3) ^d	ND	ND	ND	ND
CD34 ⁺ CD38 ⁺	2.9 ± 0.6 (3)	1.4 ± 0.2 (3)	ND	ND	ND	ND
CD34 ⁺ CD10 ⁺ lin ⁻	2.1 ± 0.4 (2)	1.1 ± 0 (2)	ND	ND	ND	ND
TN ^b	31.3 ± 10.3 (4)	1.2 ± 0.1 (2)	45.3 ± 6.9 (5)	1.4 ± 0.3 (4)	ND	ND
ITTP	16.4 ± 3.6 (4)	1.1 ± 0 (2)	52.4 ± 9.0 (5)	1.4 ± 0.1 (4)	ND	ND
DP	7.9 ± 3.3 (4)	3.2 ± 1.1 (2)	17.5 ± 5.3 (5)	3.6 ± 0.8 (4)	ND	ND
SP8	3.6 ± 0.4 (4)	3.6 ± 0.8 (2)	6.5 ± 1.7 (5)	5.4 ± 1.6 (4)	ND	ND
SP4	2.0 ± 0.2 (4)	1.1 ± 0 (2)	1.9 ± 0.2 (5)	1.1 ± 0 (4)	ND	ND
CD45RA ⁺ ^c	44.7 ± 1.8 (4)	1.9 ± 0 (4)	21.9 ± 6.8 (3)	1.2 ± 0.2 (2)	28.8 ± 4.3 (6)	2.1 ± 0.1 (6)
CD45RA ⁻	34.0 ± 2.0 (4)	2.2 ± 0.1 (4)	ND	ND	11.7 ± 1.1 (6)	2.1 ± 0.1, 24.4 ± 3.8 (6) ^d

^a Numbers represent average fold-shifts in mean fluorescence intensity (MFI) of each Ab over an isotype control mAb. Numbers in parentheses represent the number of specimens analyzed.

^b For TN cells, the average fold-shifts for the CXCR4 mAb are for the CXCR4⁺ subpopulation only.

^c CD45RA⁺ cells in SCID-hu Thy/Liv include all human T cells in the peripheral blood.

^d The first number represents the CCR5⁻ subset and the second represents the CCR5⁺ subset (see Figs. 4A and 5A).

CD3⁻CD4⁻CD8⁻ (triple-negative, or TN), CD3⁺CD4⁻CD8⁻, CD3⁻CD4⁺CD8⁻ (intrathymic T cell progenitor, or ITTP) (36), and CD3⁺CD4⁺CD8⁻ (single-positive CD4, or SP4) cells.

Figure 2A shows CXCR4 histograms for the TN, ITTP, DP, SP4, and SP8 thymocyte subpopulations from a representative SCID-hu Thy/Liv implant. CXCR4 was expressed at high levels, relative to an isotype control mAb, on the ITTPs and on a subset of the TN cells. CXCR4 levels on the DP cells varied widely, from high levels on those that are CD3⁻ to lower levels as CD3 is turned on (see contour plot, top right of Fig. 2A). SP4s and SP8s expressed relatively low levels of CXCR4, but SP8 cells expressed higher levels than did SP4 cells in each implant.

Figure 2B shows the fold shifts in average mean fluorescence intensity (MFI) for each of the Abs over the isotype mAb on each of the Thy/Liv thymocyte subpopulations. The average percentages of TN cells that were positive for CXCR4, as measured by the 12G5, 47701, and 47718-PE mAbs, were 24, 36, and 26% respectively. Each CXCR4 Ab revealed that the expression of CXCR4 diminishes during thymocyte maturation. In addition, the 44701 and 44718-PE Abs consistently bound to the CXCR4⁺ cells to a greater extent than did the 44712 and 12G5 Abs, with the 44718-PE mAb consistently exhibiting the highest MFIs.

To determine whether the pattern of expression of CXCR4 observed in the SCID-hu Thy/Liv implants reflected the pattern of CXCR4 expression in normal human fetal thymus, the 44701 or 44718-PE mAbs were used in combination with the CD3, CD4, and CD8 mAbs to stain thymocytes from three or four fetal thymus specimens, respectively. As seen in SCID-hu Thy/Liv, CXCR4 levels in the fetal thymus were highest on a TN subset (roughly 37%) and ITTPs, widely ranging but mostly intermediate on DPs, lower in SP8s, and at background levels on SP4s (Fig. 2B). In the case of all the CXCR4⁺ subpopulations, MFI fold-shifts in fetal thymus were 1.5- to 3.6-fold lower than those on their counterparts in the SCID-hu Thy/Liv implants (Table I).

Expression of CCR5 in thymus

CCR5 expression on the thymocyte subsets of four SCID-hu Thy/Liv implants and two fetal thymus specimens was analyzed using a PE-conjugated form of the anti-human CCR5 mAb 3A9 (33). In contrast to the pattern of expression of CXCR4, CCR5 was undetectable on TNs and ITTPs, was expressed at low levels on DPs and SP8s, and was undetectable on SP4s (Fig. 3A). On DPs and SP8s from Thy/Liv implants and from fetal thymus, the MFI fold-shifts were roughly equivalent (Fig. 3B and Table I).

Expression of CXCR4 and CCR5 in bone marrow

To determine whether CXCR4 and/or CCR5 are expressed on extrathymic subpopulations thought to include T cell progenitors, three fetal bone marrow specimens (each about 20 wk gestation) were analyzed by flow cytometry using 44718-PE or 3A9-PE mAb in combination with Abs specific for human CD34 and CD38

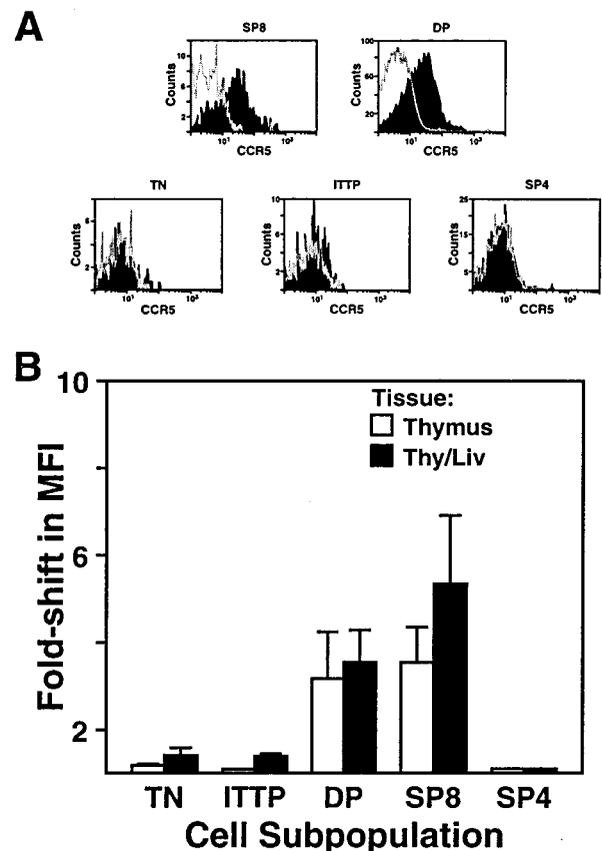


FIGURE 3. Flow cytometric analysis of CCR5 expression on SCID-hu Thy/Liv and fetal thymocyte subpopulations. *A*, CCR5 histograms for the five thymocyte subpopulations of a representative SCID-hu Thy/Liv implant (see Figs. 1 and 2), detected by the 3A9-PE mAb (shaded) or the isotype control mAb (gray line). *B*, Average fold shifts in MFI for each thymocyte subpopulation in two fetal thymi and four SCID-hu Thy/Liv implants, using the 3A9-PE mAb. Error bars represent SEs.

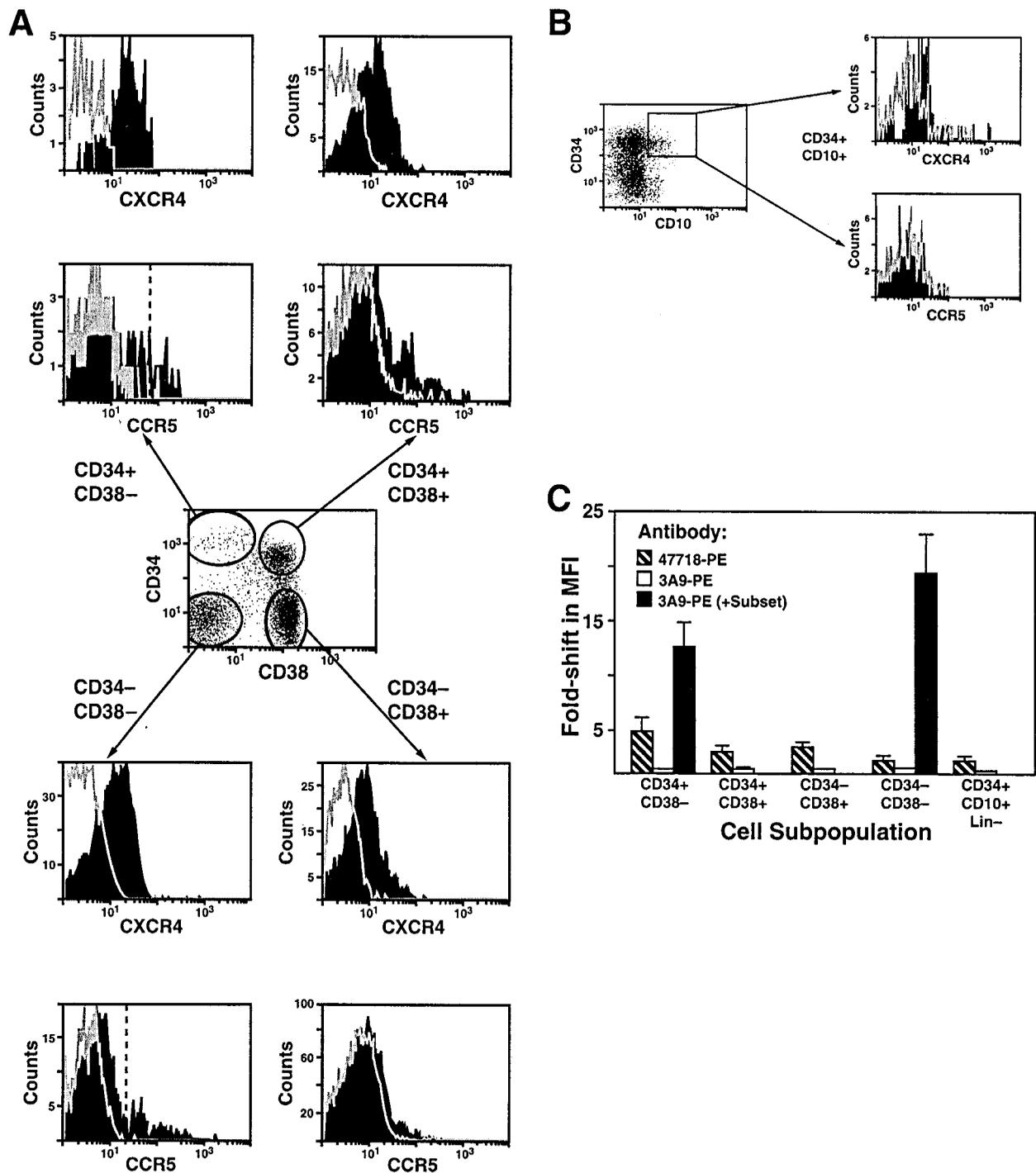


FIGURE 4. Flow cytometric analysis of CXCR4 and CCR5 expression on fetal bone marrow cells. **A**, A representative fetal bone marrow sample was divided into four subpopulations based on the expression of CD34 and CD38 (middle). CXCR4 and CCR5 histograms for each of these four subpopulations are shown, using the 47718-PE or 3A9-PE mAb (shaded) or the appropriate isotype control mAb (gray line). Minor subpopulations of CD34⁺CD38⁻ and CD34⁻CD38⁻ cells that are positive for CCR5 are identified at MFIs higher than the dotted line in the respective histograms. **B**, Mononuclear cells from a representative fetal bone marrow sample depleted of cells positive for CD2, CD4, CD8, CD16, CD19, CD20, CD56, and glycoprotein A were stained with Abs specific for CD34 and CD10 (left) in combination with either the 47718-PE mAb or the 3A9-PE mAb. Lin⁻ cells positive for CD34 and CD10 were gated and analyzed for binding of the 47718-PE (top) or 3A9-PE (bottom) mAbs (shaded) and their isotype control mAbs (gray lines). **C**, Average fold shifts in MFI for the four CD34/CD38 subpopulations in three fetal bone marrow samples and for the CD34⁺CD10⁺lin⁻ subpopulation in two lin-depleted fetal bone marrow samples, using the 47718-PE or 3A9-PE mAbs. CCR5 expression on the CCR5⁺ subset of the CD34⁺CD38⁻ and CD34⁻CD38⁻ subpopulations is shown separately from CCR5 expression on the CCR5⁻ subset. Error bars represent SEs.

(Fig. 4, A and C). Bone marrow mononuclear cells were divided into four subpopulations based on CD34 and CD38 staining, with cells in the CD34⁺CD38⁻ gate containing primitive multilineage hemopoietic progenitors and cells in the CD34⁺CD38⁺ gate containing more

mature lineage-restricted progenitors (37). The 47718-PE CXCR4 mAb stained each subpopulation at levels higher than those with the isotype control mAb but lower than those exhibited on fetal thymus TNs and ITTPs (Table I). In contrast, the 3A9-PE CCR5 mAb stained

subsets of the $CD34^+CD38^-$ (10–13%) and $CD34^-CD38^-$ (6–14%) subpopulations in each of the three marrow specimens but failed to stain the remaining bone marrow mononuclear cells at levels above that of the isotype control mAb.

Recently a progenitor population has been identified in bone marrow that contains T, B, NK, and dendritic cell potential but not myeloid or erythroid potential (38). This population may be analogous to or a progenitor of those cells that migrate to the thymus, since primitive $CD34^+CD3^-CD4^-CD8^-$ thymocytes include cells capable of T, B, NK, and/or DC differentiation (39–45). The population is negative for the lineage markers CD2, CD4, CD8, CD16, CD19, CD20, CD56, and glycophorin A, and is positive for CD34 and CD10 (38). It is also negative for CXCR4 and CCR5 (Fig. 4, B and C).

Expression of CXCR4 and CCR5 in the bloodstream

The 12G5 and 3A9 Abs have been used to show that naive ($CD45RA^+$) peripheral T cells, which are thought to be thymic emigrants, express CXCR4 but not CCR5 (32, 33). To determine whether CXCR4 is up-regulated and CCR5 is down-regulated upon transit from the thymus to the bloodstream, we assayed the CXCR4 and CCR5 expression levels in peripheral T cells from three different sources: SCID-hu Thy/Liv blood, in which many (about 74%) of the T cells are naive ($CD45RA^+$) in phenotype (46); cord blood isolated at term, where peripheral T cells have had limited exposure to foreign Ags and are mostly naive in phenotype; and blood from healthy adult humans, where a mixture of naive and nonnaive (activated or memory) T cells exists (Fig. 5 and Table I).

Whole blood from three SCID-hu Thy/Liv mice was stained with the 44718-PE CXCR4 mAb or the 3A9-PE CCR5 mAb in combination with Abs specific for human CD45 and CD3 (Fig. 5A, top panel). The 44718-PE CXCR4 mAb stained $CD45^+CD3^+$ cells in SCID-hu Thy/Liv blood at relatively high levels. In contrast, the 3A9-PE CCR5 mAb did not stain peripheral T cells over the isotype Ab.

Four cord blood specimens were stained with the 44718-PE or 3A9-PE mAb in combination with Abs specific for human CD45RA and CD3 (Fig. 5A, middle panel). As in the case of SCID-hu Thy/Liv blood, all $CD45RA^+$ cells were $CXCR4^+$. Few if any $CCR5^+$ T cells were detected, perhaps due to the low numbers of truly $CD45RA^-$ cells.

Six age-matched human adult blood specimens were similarly stained with 44718-PE or 3A9-PE mAb in combination with CD45RA and CD3 mAbs (Fig. 5A, bottom panel). As in the case of cord blood and SCID-hu Thy/Liv peripheral T cells, adult human peripheral $CD45RA^+$ T cells were found to bind the 44718-PE CXCR4 mAb but not the 3A9-PE CCR5 mAb. In addition, the $CD45RA^-$ T cell subset was $CXCR4^+$, although the levels of CXCR4 expressed on these cells were approximately threefold lower than those on $CD45RA^+$ cells. The 3A9-PE CCR5 mAb stained only a subset (3–23%) of $CD45RA^-$ adult human T cells.

Discussion

These studies demonstrate that discrete stages of hemopoietic differentiation are marked by differential display of important coreceptors for HIV-1 infection, CXCR4 and CCR5. Thus, CXCR4 is expressed at low levels on $CD34^+CD38^-$ hemopoietic progenitor cells in the fetal bone marrow, is up-regulated on immature ($CD3^-CD4^+CD8^-$ and $CD3^-CD4^+CD8^+$) thymocytes, is down-regulated on more mature ($CD3^+CD4^+$ and $CD3^+CD8^+$) thymocytes, and is up-regulated again upon exit of cells from the thymus.

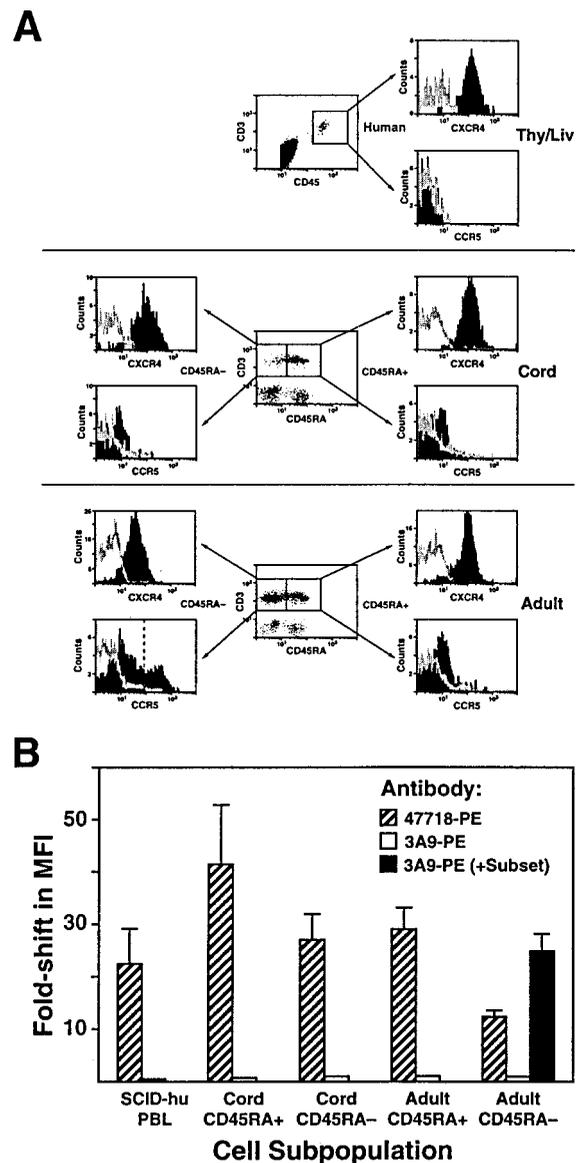


FIGURE 5. Flow cytometric analysis of CXCR4 and CCR5 expression on peripheral T cells. *A Top*, Whole blood from a representative SCID-hu Thy/Liv mouse was analyzed with Abs to human CD45 and CD3 in combination with either the 44718-PE CXCR4 or the 3A9-PE CCR5 mAb. Cells positive for CD45 and CD3 (left) were gated and analyzed for binding of the 44718-PE (top) or 3A9-PE (bottom) mAbs (shaded) and their isotype control mAbs (gray lines). *Middle*, Whole blood from a representative fetal cord was analyzed with Abs to CD3 and CD45RA, in combination with either the 44718-PE or the 3A9-PE mAb. $CD45RA^{-/lo}$ (left) and $CD45RA^+$ (right) $CD3^+$ T cells were gated and analyzed for binding of the 44718-PE (top) or 3A9-PE (bottom) mAbs (shaded) and their isotype control mAbs (gray lines). *Bottom*, Same as middle, but with a representative adult human blood specimen. A subpopulation of $CD45RA^-$ cells that is positive for CCR5 is identified at an MFI higher than the dotted line in the lower left histogram. *B*, Average fold shifts in MFI for the human T cells in three (CXCR4) or two (CCR5) SCID-hu Thy/Liv blood samples and for the $CD45RA^+$ and $CD45RA^-$ T cells in four fetal cord and six adult blood samples, using the 44718-PE or 3A9-PE mAbs. CCR5 expression on the $CCR5^+$ subset of adult T cells (+ subset) is shown separately from CCR5 expression on the $CCR5^-$ subset. Error bars represent SEs.

CCR5 is expressed on a subpopulation of $CD34^+CD38^-$ bone marrow progenitor cells and at low levels on immature $CD4^+CD8^+$ and more mature $CD3^+CD8^+$ thymocytes (Fig. 6 and

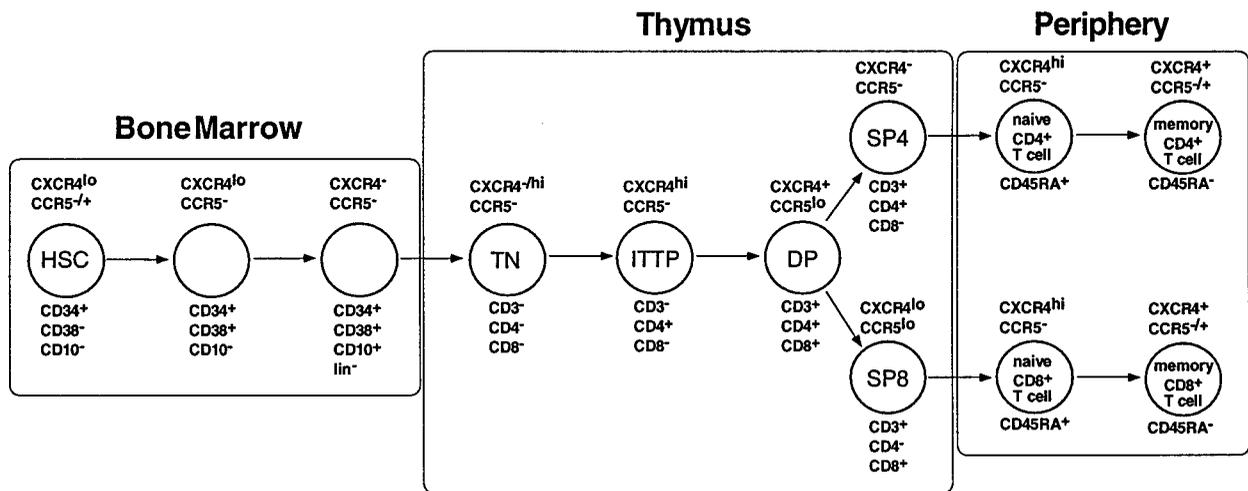


FIGURE 6. Expression of CXCR4 and CCR5 on T-lineage cells. HSC, hemopoietic stem cell.

Table I). Qualitatively similar patterns of expression were observed both in samples derived from human subjects and in samples analyzed in the context of the SCID-hu Thy/Liv mouse. To the extent that HIV-1 pathogenesis can be studied in this animal model, these results serve to illuminate the relationship between viral tropism and pathogenic effects on hemopoietic, including T-lineage, differentiation.

The observation that ITTPs express significantly higher levels of CXCR4 than do SP4s provides evidence that the level of coreceptor expressed by a cell may determine the infectability of that cell, since ITTPs appear to be more permissive to infection than SP4s by a CXCR4-using HIV-1 isolate in vivo (21, 22). Coreceptor regulation of infection has been observed previously for CCR5 expression in peripheral T cells (33). The fact that ITTPs and DPs express relatively high levels of CXCR4 has important consequences for HIV-1 pathogenesis, since these two CD4⁺ subpopulations should be infectable by naturally occurring, SI, CXCR4-using strains of HIV-1. DP thymocytes typically account for 80 to 90% of T progenitor cells in the thymus; infection and destruction of these cells by SI HIV-1 might be responsible for the significant lymphoid depletion observed in thymi (4) from patients in the symptomatic stages of HIV disease, when SI strains of HIV predominate (27–30). Perhaps more importantly, infection and destruction of ITTPs would block the production of new DPs and, eventually, new T cells.

Detection of CXCR4 on CD34⁺ bone marrow cells is not surprising, since these cells are attracted by SDF-1 α in vitro (47) and since CXCR4 mRNA was detected by RT-PCR in purified CD34⁺ bone marrow cells from all nine persons analyzed (31). We observed only low levels of CXCR4 on these cells despite the fact that we used an Ab that exhibits higher MFIs than those of the previously reported anti-CXCR4 mAb 12G5 (35). The low level of CXCR4 in CD34⁺ bone marrow cells, which have been shown to express low levels of CD4 (48, 49), may explain why they are poorly infected by HIV-1 in vitro and in vivo (2, 50, 51). However, this level of expression may be sufficient to trigger indirect effects of HIV-1, e.g., those mediated by viral envelope proteins produced in adjacent target cells.

The observation that CXCR4 expression was higher in ITTPs than in CD34⁺CD10⁺lin⁻ bone marrow cells implies that CXCR4 expression is up-regulated during or after the T progenitor cell has moved to the thymus. The exact timing of CXCR4 up-regulation may be revealed by further analysis of the TN compartment, which we found to contain both CXCR4⁺ and CXCR4⁻ cells. If the

CXCR4⁻ cells are the immediate predecessors of the CXCR4⁺ cells (as determined by lineage analysis), CXCR4 up-regulation must be occurring in TNs after, not during, thymus homing.

The data also suggest that CXCR4 is down-regulated during positive and negative selection in the thymus. CXCR4 down-regulation begins only after the thymocyte has become CD4⁺CD8⁺; from that point on, CXCR4 is down-regulated in a coordinated fashion with CD3 up-regulation. It is interesting that CXCR4 is down-regulated to a greater degree in SP4s than SP8s. The reason for this disparity is not obvious in light of the observation that both SP4s and SP8s up-regulate CXCR4 upon exit from the thymus.

Our data are in agreement with a previously published flow cytometric analysis of CXCR4 on fetal thymocytes (34). In that study, CXCR4 expression was found to be modulated during thymocyte maturation, with DP thymocytes showing higher levels than most SP4 and SP8 thymocytes. Of note, some SP4 thymocytes were observed to be CXCR4^{high}. Based on our results, these are probably CD3⁻CD4⁺CD8⁻ ITTPs, and not more mature SP4 thymocytes.

Our observation that CXCR4 is up-regulated and CCR5 is down-regulated upon transit of mature thymocytes into the bloodstream is in agreement with findings concerning CXCR4/CCR5 expression of human naive peripheral T cells (32). However, our observation that CD45RA⁻ peripheral T cells express moderately high levels of CXCR4 is not consistent with that report, in which the memory T cell subset was found to be CXCR4⁻ with the 12G5 mAb (32). However, >80% of human T cells, including both CD45RA⁺ and CD45RO⁺ cells, undergo chemotaxis in response to SDF-1 α in vitro (24), implying that memory T cells do indeed express CXCR4. The observed differences in CXCR4 expression levels between the prior study and this one are probably due to differences in sensitivity between the 12G5 and 44718-PE mAbs (see Fig. 2A).

The data also indicate that CCR5 is expressed minimally or not at all in the CD34⁺CD38⁺ bone marrow subpopulation, which typically accounts for >99% of all CD34⁺ bone marrow cells, and is expressed at low levels on only 10 to 13% of the cells in the CD34⁺CD38⁻ subpopulation. These results are in line with those of previous related studies. First, >99% of purified CD34⁺ bone marrow cells were not significantly attracted to CCR5 ligands in in vitro chemotaxis assays (47). Second, CCR5 mRNA was detected by RT-PCR of purified CD34⁺ bone marrow cells from only a minor percentage of donors (31). Third, HIV-1 proviral DNA was detected only rarely in CD34⁺ bone marrow cells from subjects in

the asymptomatic stage of HIV-1 disease (52, 53), when virus particles often use only CCR5 (27–29). The identity of the CD34⁺CD38⁻CCR5⁺ subset is not known but warrants further investigation.

Lastly, we observed that CCR5 is expressed at low but significant levels on SP8 and DP thymocytes, which comprise most of the T progenitor cells in the thymus. This observation implies that CCR5-using, NSI strains of HIV-1 might be able to infect the thymus during the asymptomatic stage of HIV-1 disease, when these strains of HIV-1 predominate (27–29). Studies are currently in progress to determine the pathogenic outcomes of infection of DP thymocytes with CXCR4- or CCR5-using viruses.

The relevance of these data to HIV-1 pathogenesis in humans must be considered with two caveats in mind. First, data obtained in SCID-hu Thy/Liv mice are correlative and subject to unknown perturbations specific to the animal model itself. Secondly, it is not known whether the levels of coreceptor expression observed in fetal organs of hemopoiesis (e.g., bone marrow and thymus) are similar or pertinent to those found in their adult counterparts. It is notable, however, that SI, CXCR4-using variants of HIV-1 often predominate in late stages of both pediatric and adult HIV-1 disease, whereas NSI, CCR5-using variants are encountered more frequently in early stages of disease (27–30). In parallel, the former, but not the latter, types of viruses destroy the thymus in the SCID-hu Thy/Liv mouse (20). Furthermore, thymic function has been observed in a surprisingly large fraction of HIV-1-infected adults, but not in those in later stages of disease (CD4 counts of <200 cells/ μ l) (7). Together, these observations suggest that the switch to CXCR4-using variants in later stages of HIV-1 disease may presage and result in regenerative failure, precisely because such viruses abrogate the function of hemopoietic progenitor cells more efficiently than do CCR5-using viruses. Infection of these progenitor populations could thus be a pivotal event in the disruption of T lymphopoiesis during the course of HIV-1 disease in both children and adults.

Note added in proof. We have found in subsequent experiments that, unlike the CCR5 mAb 3A9 used in this study, the CCR5-specific mAb 2D7 (PharMingen, San Diego, CA) does not stain DP or SP8 thymocytes by flow cytometry. However, in a recently published study (54), a novel CCR5-specific mAb (clone 45502.111; R&D Systems) stained DP and SP8, but not DN or SP4, thymocytes by flow cytometry, confirming our results with the CCR5 mAb 3A9.

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