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A Novel Mechanism of CD4 Lymphocyte Depletion Involves Effects of HIV on Resting Lymphocytes: Induction of Lymph Node Homing and Apoptosis Upon Secondary Signaling Through Homing Receptors

Liqiang Wang,* Jenny J. Y. Chen,† Benjamin B. Gelman,‡ Rolf Konig,* and Miles W. Cloyd¶

Recently, we reported that abortive HIV infection of resting human T lymphocytes up-regulated expression of CD62L, the receptor for homing to lymph nodes (LNs), and enhanced homing of these cells from the blood into the LNs (Wang et al., 1997, Virology 228:141). This suggested that HIV-induced homing of resting lymphocytes (which comprise >98% of all lymphocytes) may be a major mechanism for the reduction of CD4+ lymphocytes in the blood of infected individuals. This mechanism also could be partially responsible for the lymphadenopathy that often develops at the same time that CD4+ lymphocytes are disappearing from the blood. In this study, we show that secondary signaling through the homing receptors (CD62L, CD44, CD11a) of abortively infected resting CD4+ T lymphocytes induced apoptosis. These signals would occur as the cells home into the LNs. Apoptosis did not occur after secondary signaling through some other receptors (CD26, CD4, CD45, and HLA class I) or in HIV-exposed resting CD8+ lymphocytes signaled through the homing receptors. These findings indicate that HIV-induced homing of resting CD4+ lymphocytes to LNs results in death of many of these cells. This was confirmed in the LNs of SCID mice that were i.v. injected with HIV-exposed resting human lymphocytes. Thus, these effects of HIV upon binding to resting CD4+ T lymphocytes, which are not permissive for HIV replication, may significantly contribute to their depletion in vivo. These findings also offer an explanation for the bystander effect observed in the LNs of AIDS patients, whereby cells not making virus are dying.


Loss of CD4+ T lymphocytes in the blood of HIV-infected subjects correlates with increasing levels of plasma virus and decreasing immunocompetence (1–4). It has been thoroughly documented, however, that inversion of the CD4/CD8 ratio does not occur in lymph nodes (LNs)5 when it is occurring in the blood (5, 6). In fact, when blood levels of CD4+ lymphocytes begin to drop significantly, these cells often increase in number in the LNs (lymphadenopathy) (5, 6). This suggests that the loss of CD4+ cells in the blood does not necessarily reflect global depletion of these cells, but rather enhanced homing of CD4+ lymphocytes from the blood into the LNs. Recently, we reported that abortive HIV-1 infection of resting T lymphocytes up-regulated expression of cell surface CD62L (L-selectin), the receptor for homing to LNs, which resulted in a ~12-fold increase in the number of these cells that bound to LN high endothelial venules in an ex vivo homing assay (7). When injected i.v. into SCID mice, HIV-exposed, resting human T lymphocytes rapidly homed from the blood into the LNs (60–70% reduction of cells in the blood by 2 h) (7). This action may help explain why most infected cells are found in the LNs and not in the blood, why CD4+ cells decline in the blood even though very few productively infected cells are found in the blood, and why lymphadenopathy occurs when the numbers of CD4+ cells are falling in the blood (8–14).

A large amount of evidence has indicated that HIV infection causes depletion of CD4+ lymphocytes by an indirect mechanism (15–21). The dying cells in the LNs of AIDS patients are bystander cells (i.e., cells not producing HIV mRNA) (21). This may explain why more CD4+ lymphocytes die than the few at any given time that produce HIV (13, 14). Apoptotic cells are generally not directly detected at higher than normal frequencies in freshly isolated PBLs from HIV-infected individuals (22), but infected subjects display significantly more apoptotic cells in their LNs (23). Lymphocytes, both CD4+ and CD8+, in HIV-infected subjects are in a general state of activation and prone to undergo spontaneous apoptosis when placed into culture or stimulated in vitro by Ags, mitogens, or superantigens (16–19). It is possible that this state results in resting lymphocytes after abortive infection by HIV. The extent of CD4+ lymphocyte depletion via Ag activation is not clear, but any given Ag would only affect a very small percentage of the CD4+ lymphocyte population. Since our previous results demonstrated that resting CD4+ PBLs are profoundly affected by HIV binding in the induction of homing properties, we wondered what happens to these cells as they home into LNs. We found that about one-half of the resting CD4+ lymphocytes that were preexposed to HIV were induced into apoptosis following signaling through their homing receptors (CD62L, CD44, CD11a).

Materials and Methods

Isolation and infection of PBLs

Peripheral blood was obtained by venipuncture from healthy HIV-negative donors (low risk, HIV Ab negative) following informed consent. PBMCs were isolated by centrifugation through lymphocyte separation medium...
(Organon Teknika, Durham, NC) and washed twice with HBSS. Monocytes/macrophages were depleted by plastic adherence at 37°C for 2 h in medium. The nonadherent cells, PBLs, were collected and centrifuged. The cell pellet was resuspended in RPMI 1640 medium (Life Technologies, Gaithersburg, MD) supplemented with 15% FBS, penicillin, streptomycin, 1-glutamine (Sigma, St. Louis, MO), and IL-2 (10 U/ml) at 1 x 10^6 cells/ml and seeded into 48-well tissue culture plates. HIV-1 stocks were prepared as described previously (7). HIV-1 at a multiplicity of infection (MOI) equal to 1 was added, and the PBLs were incubated for 12 h at 37°C. Control cells were mock treated with culture supernatant fluids from uninfected CEM cells, which is the cell line in which the HIV-1 infections were made.

Cross-linking of cell surface receptors

After 12 h of incubation, both HIV-exposed and mock-treated cells were centrifuged, washed, and incubated with one of the mAbs (2 μg/ml) against indicated surface molecules at 4°C for 30 min. Cross-linking of the surface molecules was then achieved by incubating the cells with goat anti-mouse IgG (1 μg/ml) at 37°C for 30 min. Subsequently, cell viability was monitored every day by trypan blue exclusion. The mAbs used were: anti-CD26 (PRREG56), anti-CD44 (J173), anti-CD11a (25.3), anti-CD103 (HML-1, 2G5), anti-CD26 (Tal), anti-CD8 (OKT8), and anti-CD4 (PRAT4 or Sim 4). mAbs were purchased from PharMingen (San Diego, CA), Immunotech (Westbrook, ME), Coulter (Hialeah, FL), or American Type Culture Collection (Manassas, VA). Sim 4 was a gift from Dr. James Hilllreth via the National Institutes of Health AIDS Repository. Each of these mAbs has been shown to be able to signal via cross-linking the receptor to which they bind (24–27). Each experiment was performed in duplicate, and the results were calculated by subtracting the percentage of dead cells in untreated control cells.

Purification of CD4+ or CD8+ T lymphocytes and monitoring of cellular markers

CD8+ or CD4+ T lymphocytes were removed from PBLs by panning, as reported previously (28). Briefly, petri dishes (100 mm in diameter, 1 plate and cultured. The percentages of CD4+ and CD8+ T lymphocytes were then collected, washed with HBSS containing 2% FBS, and incubated by flow cytometry, and were always panning were determined by immunostaining the live cells and analyzing panning.

Control cells were mock treated with culture supernatant fluids for 1 h at 4°C with 2% FBS (10^7 cells/10 ml), and placed onto the goat anti-mouse IgG incubated with OKT8 or Sim 4 hybridoma culture supernatant fluids for 1 h at 4°C with 20 ml of the same solution. PBLs were incubated with OKT8 or Sim 4 hybridoma culture supernatant fluids for 1 h at 4°C with constant mixing, washed twice, resuspended in HBSS containing 2% FBS five times and incubated for 1 h at 4°C with 20 ml of the same solution. PBLs were incubated with OKT8 or Sim 4 hybridoma culture supernatant fluids for 1 h at 4°C with constant mixing, washed twice, resuspended in HBSS containing 2% FBS (5 μg/ml) on a plate overnight at 4°C. The dishes were washed with 10 ml of HBSS containing 2% FBS five times and incubated for 1 h at 4°C with 20 ml of the same solution. PBLs were incubated with OKT8 or Sim 4 hybridoma culture supernatant fluids for 1 h at 4°C with constant mixing, washed twice, resuspended in HBSS containing 2% FBS (10 cells/10 ml), and placed onto the goat anti-mouse IgG (Sigma)-coated plates for 3 h at 4°C, with gentle tilting at 1.5 h. Nonadherent cells were then collected, washed with HBSS containing 2% FBS, and cultured. The percentages of CD4+ and CD8+ T lymphocytes following panning were determined by immunostaining the live cells and analyzing them by flow cytometry, and were always ≥95% enriched. The percentages and fluorescence intensity of cells expressing CD62L, CD44, and CD11a were monitored by flow cytometry of immunostained live cells.

Purification of memory and naive CD4+ T lymphocytes

Memory and naive CD4+ T lymphocytes were obtained by panning using anti-CD45RA or anti-CD45RO mAbs in addition to OKT8. The resulting CD4+ populations were ≥95% positive for CD45RO or CD45RA, respectively.

Coating plates with various reagents

As reported by others (29–31), 48-well plates were coated with mannos-6-phosphate (M6P), hyaluronic acid (HA), mannose-1-phosphate (M1P), or chondroitin sulfate A (CHA) at concentrations of 5 mg/ml in PBS at 4°C overnight. The plates were washed six times with cold PBS to remove any reagents that did not attach to the wells.

In vivo assessment of the fate of HIV-1-infected human PBLs that have homed in SCID mice

Fresh human PBLs were isolated and exposed to HIV-1 without stimulation, as described above. After 40 h of culture, the infected and mock-treated cells were centrifuged and then resuspended in culture medium at 2 or 3 x 10^7 cells/300 μl for i.v. (retroorbital sinus) injection into SCID mice (Taconic Farms, Germantown, NY). Before injection, SCID mice were transferred from University of Texas Medical Branch Animal Care Center (Galveston, TX) to the P3 lab and anesthetized with Nembutal (40 mg/kg of body weight). At 2, 4, and 6 days postinjection, the mice were anesthetized again and sacrificed; peripheral LN were removed, frozen in liquid nitrogen, and subsequently stored at −70°C.

Detection of human T lymphocytes in SCID mice

Frozen sections of the LN were rehydrated in glass-distilled water and then PBS for 20 min each. For total human T lymphocytes, a polyclonal rabbit anti-human CD3 Ab (Biomed, Fullerton, CA) was used for 1 h at room temperature, followed by extensive washing in PBS. Sections were incubated with 1% normal goat serum (Vector, Burlingame, CA), 1% non-fat milk, and 0.2 M ammonium acetate to block nonspecific binding. The sections were then incubated with goat anti-rabbit horseradish peroxidase (Bio-Rad, Richmond, CA) at room temperature for 30 min. After extensive washing in PBS, sections were incubated in diaminobenzidine (Research Genetics, Huntsville, AL), according to the manufacturer’s instructions. After extensive rinsing in water, sections were counterstained for 2 min with Mayer’s hematoxylin solution, followed by 2 min in Blueing solution. After extensive washing in water, sections were mounted under coverslips. For human CD4+ or CD8+ T lymphocyte subsets, biotin-labeled anti-human CD4 or CD8 mAbs (Catlug, South San Francisco, CA) were used, followed by avidin-horseradish peroxidase (Vector) and diaminobenzidine as substrate.

Immunocytochemical colocalization of DNA fragmentation (apoptosis) and surface markers of human T lymphocytes in SCID mice

Frozen sections of tissues were prepared as described above and immunostained with either CD4 or CD8 Ab. Detection of DNA fragmentation was performed by the terminal deoxynucleotidyltransferase (TdT)-mediated 5'-uridine triphosphate (UTP) nick-end labeling (TUNEL) method (32) with some modifications. Briefly, sections were treated with Autozyne (Biomed) for 60 min at room temperature, according to the manufacturer’s instructions. After washing with PBS, sections were soaked in Tris-EDTA (pH 8) for 10 min and then in TdT buffer (25 mM Tris chloride, 200 mM sodium cacodylate, 5 mM cobalt chloride) for 10 min. TdT-mediated uptake of deoxyUTP-digoxigenin (Boehringer Mannheim, Indianapolis, IN) was performed at 37°C for 1 h, followed by washing in TBS (50 mM Tris HCl, 138 mM NaCl, 3 mM KCl (pH 8)) three times for 10 min each. Sections were blocked for 30 min at room temperature in TBS containing 2% BSA and 0.1% Triton, followed by incubation with alkaline phosphatase-conjugated anti-digoxigenin Fab', fragment (Boehringer Mannheim) diluted 1/500 in TBS plus BSA and Triton for 30 min at room temperature. After extensive washing with TBS, phosphatase activity was developed using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazo- lium solution (Sigma). After 5 min, the sections were washed with distilled water, covered with water-soluble media, and coverslipped. Sections of a previously tested LN with intense DNA fragmentation signal with or without TdT treatment were run in parallel as positive and negative controls, respectively. A total of 5–10 frozen sections of LNs from two to five mice sacrificed at 2, 4, and 6 days postinjection were photographed at ≥400 magnification, and the numbers of total human cells and TUNEL-positive human cells were visually counted.

Results

We first explored the fate of resting T lymphocytes abortively infected with HIV and induced to home into the LNs by signaling through their homing receptors. Cross-linking of the homing receptors CD62L, CD44, or CD11a with mAbs induced 25–38% of the HIV-exposed PBLs to die by 5 days, whereas mock-exposed resting PBLs did not significantly die when they were cross-linked (Fig. 1, A and B). Cross-linking of CD103 (a receptor involved in homing of a subset of T lymphocytes to mucosal lymphoid tissues), CD26, CD4, or CD8 with mAbs also did not affect viability of HIV-exposed PBLs (Fig. 1), demonstrating specificity of this effect following signaling through homing receptors. Since CD26 expression is low on resting lymphocytes, we examined cell death following cross-linking of some highly expressed receptors on resting lymphocytes, uninfected or abortively infected with HIV (Fig. 1C). Cross-linking of HLA class I or CD45RO and RA did not induce death of HIV-exposed PBLs. This is in contrast to cross-linking of CD62L on PBLs preexposed to HIV1, or HIVmck (both T cell-tropic strains) or HIVBallo (a macrophage-tropic HIV), which induced 30–50% of the PBLs to die by 4 days postexposure (Fig. 1C). CD62L cross-linking of mock-infected PBLs or PBLs pretreated with supernatant from ultracentrifuged

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HIV 213 stocks (to remove HIV particles) did not induce death (Fig. 1C). Our previous study showed that supernatant from ultracentrifuged HIV stocks could not up-regulate homing receptors, but UV- or heat-inactivated HIV could induce CD62L expression (7). Similarly, heat-inactivated HIV213-exposed PBLs still died following CD62L cross-linking (Fig. 1C). This effect followed a dose response, for if lower doses of infectious or inactivated HIV were used, reduced numbers of apoptotic cells following homing receptor cross-linking were observed (data not shown). Thus, binding of HIV particles to the cells appeared to induce a state in which second signals through homing receptors resulted in apoptosis.

To determine which subset(s) of lymphocytes was dying, we tested purified CD4+ or CD8+ T lymphocytes in the same assay system (Fig. 2). Purified CD4+, but not CD8+, resting PBLs abortively infected with HIV died after cross-linking of CD62L or CD44. Up to 60% of the CD4+ lymphocytes died by 5 days post-cross-linking. Cross-linking of CD103 did not induce death of these cells, serving as a control, and mock-treated CD4+ or CD8+ lymphocytes did not die following cross-linking of the homing receptors (Fig. 2). There was no correlation between the levels of expression of these homing receptors and whether the CD4 cells died or not, since cells not dying expressed high levels of these markers (data not shown). Thus, many of the resting CD4+ lymphocytes abortively infected with HIV apparently will die after signaling through the LN homing receptors.

It is possible that signaling the homing receptors of HIV-exposed, resting CD4+ T lymphocytes activated the cells to the extent that the virus completed its replication cycle and began to produce progeny virus (33). This, in turn, could potentially injure and/or kill many of the cells. We therefore determined whether HIV was produced by the cross-linked cells. Supernatants from two cultures of HIV-exposed resting CD4+ T lymphocytes cross-linked by anti-CD62L or anti-CD44 were harvested and tested for HIV p24 by Ag capture enzyme immunoassay (Coulter). All samples were found to be negative (a representative experiment shown in Fig. 3). Therefore, the death of HIV-exposed, resting CD4+ T lymphocytes signaled through the homing receptors does not involve any, or any significant amount, of HIV production.

FIGURE 1. Signaling through homing receptors CD62L, CD44, or CD11a induced death of HIV-exposed PBLs. Resting PBLs were exposed to HIV for 12 h in culture and then signaled through the indicated cell surface receptors by mAb cross-linking. Cell death was monitored by trypan blue exclusion. A, Kinetics of cell death following HIV exposure and mAb cross-linking. Data shown are the mean and SE of five separate experiments. B, Kinetics of cell death following mAb cross-linking of mock-treated PBLs. C, Summation of maximum cell death by 4 days for PBLs pretreated with CEM culture fluid (mock), supernatant from ultracentrifuged (30,000 rpm, 2 h) HIV213 stock (Sup), heat-inactivated (56°C, 1 h) HIV213 (HI-213), or stocks of HIV213, HIVmck, or HIVbal (at an MOI of ~1) and cross-linked by anti-CD62L, anti-CD45, or anti-HLA class I mAbs.
We next tested whether signaling the homing receptors with their natural ligands induced the death of resting CD4⁺ lymphocytes abortively infected with HIV. The natural ligands for L-selectin and CD44 are highly glycosylated molecules expressed on endothelial cells (29–31). M6P has been used as a natural ligand for L-selectin (31), and HA has been used as a natural ligand for CD44 (29, 34). Coating plates with these molecules and allowing HIV-exposed, resting PBLs to settle onto the plates specifically induced death of up to 30% of the CD4⁺ T lymphocytes, but not the CD8⁺ T lymphocytes (Fig. 4). Serving as negative controls, M1P and CHA did not induce cell death of either HIV-exposed CD4⁺ or CD8⁺ T lymphocytes (Fig. 4).

The above results showed that signaling through the homing receptors on resting CD4⁺ T lymphocytes abortively infected with HIV-1 caused 30–50% of the cells to specifically die over a 5-day period. Obviously, many of the cells did not die within the 5-day observation period. However, we did not extend this period, because thereafter the extent of spontaneous death in untreated control PBLs significantly increased. In addition, shorter (8 h) or longer (24 and 48 h) HIV exposure times before receptor cross-linking did not significantly alter the rate or proportion of PBL dying (data not shown).

The earliest detectable problem of the immune system in HIV-infected subjects is a poor Th response to recall Ags, which is known to be mediated by memory T cells (CD45RO⁺) (35, 36). One explanation may be that memory cells are eliminated earlier in the course of infection, perhaps because these cells express reduced amounts of Bcl-2 (37). We, therefore, asked whether CD4⁺ memory cells were more susceptible to induction of death than were naive cells (CD45RA⁺). We found both memory and naive CD4⁺ T lymphocytes preexposed to HIV were equally susceptible to induction of death by cross-linking of the homing receptors (Fig. 5). Thus, whether or not the cell dies following signaling through its homing receptors does not depend upon whether it is a naive or...
memory CD4+ T cell. Furthermore, the levels of expression of the homing receptors did not correlate to whether or not the cells died (data not shown).

We next asked whether the cells were dying via apoptosis or not. As shown in Fig. 6A, cross-linking of CD62L appeared to induce apoptosis of HIV-exposed CD4+ T lymphocytes, as indicated by TUNEL labeling. Similar data were obtained with cross-linking of CD44 (data not shown). Apoptotic death of these cells was confirmed by electron microscopy studies (Fig. 6B), which demonstrated that the cells exhibited the chromatin condensation and cell shrinkage characteristics of apoptosis. Similar data were obtained after cross-linking of CD62L or CD44. Again, cross-linking of CD103 did not induce death of HIV-exposed CD4+ T lymphocytes and served as a negative control (Fig. 6C).

Finally, we wanted to determine whether HIV-exposed human CD4+ lymphocytes that home to LNs in vivo also die. We demonstrated previously that HIV-exposed resting human T lymphocytes exhibited enhanced homing into LNs after i.v. injection into SCID mice (7). This included CD8 cells, too, which had slightly enhanced L-selectin expression, but more CD4 cells homed than CD8 (7). At 2, 4, and 6 days after injecting mock (control)- or HIV-exposed (for 12 h) resting human PBLs i.v. into SCID mice, cervical and inguinal LNs were taken, frozen, and sectioned for immuno- and TUNEL staining. Fig. 7, A and B, demonstrates examples of human CD3+ T lymphocytes (brown) in the LNs of SCID mice injected with either mock (A) or HIV-exposed (B) human PBLs. The slides were lightly counterstained with hematoxylin to illustrate cell morphology. We detected a much higher number of human PBLs in the LNs of mice injected with HIV-exposed PBLs in comparison with mice injected with mock-exposed PBLs. To test whether HIV-exposed T lymphocytes were signaled into apoptosis inside SCID mouse LNs, we performed dual staining for apoptosis (TUNEL) and for T lymphocyte markers (CD8 or CD4). Fig. 7, C and E are stained for human CD8, and Fig. 7, D and F are stained for human CD4. About 30% of the CD4+ and only 2–5% of the CD8+ T lymphocytes were double labeled. The results of quantitating TUNEL-positive human cells in frozen LN sections from multiple mice injected i.v. with mock- or HIV-exposed resting human PBLs are summarized in Table I. This shows that 29 ± 17% of the HIV-preexposed CD4 T cells were dying in the LNs by 6 days postinjection, whereas only 3–5% of mock-exposed CD4 or HIV-exposed CD8 cells were dying. Thus, it appears...
that similar to the in vitro studies above, the HIV-exposed resting CD4 PBLs actually die after homing into the LNs.

**Discussion**

These studies demonstrate that signaling through LN homing receptors of resting CD4 T lymphocytes that were abortively infected with HIV induced apoptosis both in vitro and in vivo. This was specific for CD4+ T lymphocytes and not CD8 cells, and for HIV-exposed cells and not mock-exposed cells. Furthermore, signaling through CD4, CD45, HLA class I, or CD103 (receptor for homing to Peyer’s patches) did not induce apoptosis in HIV-exposed resting CD4+ lymphocytes, demonstrating specificity of this effect. Various studies have shown that both CD4+ and CD8+ lymphocytes from HIV-infected subjects undergo spontaneous apoptosis at a higher than normal frequency when placed into culture without stimulation, and it is almost certain that many of those apoptotic cells would be resting lymphocytes that had been exposed to HIV and were not replicating the virus. Other studies have shown higher than normal frequencies of apoptotic cells in PBLs from infected individuals following mitogenic, antigenic, or superantigenic stimulation in vitro (17, 19, 22). Approximately 20% of the lymphocytes underwent apoptosis following stimulation with Ags or superantigens (19), whereas stimulation with mitogen (PWM) induced a higher frequency of cell death (~40–50%). Mitogenic stimulation probably does not occur in vivo, but antigenic and superantigenic stimulations may account for some lymphocyte depletion. However, both CD4+ and CD8+ cells should be involved. Induction of apoptosis in CD4+ lymphocytes upon homing into the LNs may be more relevant to specific in vivo depletion of CD4+ T lymphocytes in HIV patients.

Normally, T lymphocytes continuously circulate from the blood into lymphoid organs and then back to the blood (38). Exposure to HIV enhances homing of these cells from the blood into the LNs (7). These are resting lymphocytes that are not permissive for productive infection by HIV and comprise most (98–99%) of the total lymphocytes. Our results show that certain signals will induce apoptosis in the resting cells that were exposed previously to HIV, and that these dying cells are not producing virus. The high frequency of apoptosis following signaling through the homing receptors can help explain the bystander effect observed in the LNs of AIDS patients, in which cells not producing HIV are dying (21). Since most of the lymphocytes in vivo are resting and not permissive for productive HIV infection (33, 39, 40), any detrimental effect of HIV on these cells could have profound consequences.

Our findings that resting CD4+ lymphocytes preexposed to heat-inactivated HIV also underwent apoptosis certainly implicates that the virus is causing this cellular state via its binding to a receptor and inducing signals. It also could further implicate pathogenic roles for noninfectious virus. In the blood, most viral particles are noninfectious (41). However, most virus is produced from cells in the LNs, and by the time newly produced virus appears in the blood, it is most likely coated with neutralizing Abs. Thus, virus binding to resting lymphocytes is probably rare in the blood but frequent in the LNs. Many lymphocytes in the LNs recirculate back into the blood within a 24-h period, in numbers equal to those homing out of the blood into the LNs over the same period. There are data indicating that ~1.5% of the blood lymphocytes home out per hour (42), and thus ~35–40% of the blood lymphocytes (which in turn comprise 2% of the total lymphocytes in the body) circulate through the LNs and back into the blood per day (43). It has been shown that ~7500 lymphocytes per million in the LNs at any given time are harboring unintegrated HIV DNA (14), and the vast majority of these are likely to be resting CD4+

**FIGURE 6.** Cross-linking of the homing receptors of HIV-exposed CD4+ T lymphocytes induced apoptosis. Apoptosis was evaluated by the TUNEL (TdT-mediated biotin-deoxyUTP-end labeling) method (A) and electron microscopy (EM) (B), as described in Materials and Methods. A. At the indicated time points after mAb cross-linking of HIV-exposed or mock-treated resting CD4+ T lymphocytes, the cells were pelleted and washed twice in cold PBS, fixed in 2% paraformaldehyde, permeabilized in acetone, labeled by the TUNEL method (32), and analyzed by flow cytometry. B and C. At 3 days after HIV exposure and cross-linking with indicated mAbs, the CD4+ PBLs were pelleted by centrifugation into Beam capsules (1000 x g), fixed immediately in a cacodylate-buffered 2% paraformaldehyde and 2% glutaraldehyde, and postfixed in 1% osmium tetroxide. After dehydration with a series of graded ethanol concentrations, the specimens were embedded in Polybed 812 resin. Thin sections were cut with a Reichert Ultracut ultramicrotome and poststained with uranyl acetate and lead citrate. The specimens were examined and photographed on a Philips 201 electron microscope.
lymphocytes abortively infected with HIV (33). Many of these cells will recirculate back into the blood, and since HIV-induced up-regulation of L-selectin reaches a maximum at 48 h, there is time for many of these cells to return to the blood before maximum expression of L-selectin. These cells, in turn, will possess an even greater propensity to home back into LNs, and this time, many of

**FIGURE 7.** Immunocytochemical staining of human T lymphocytes in SCID mouse LNs. Frozen sections of LNs were obtained 4 days after i.v. injection of resting human PBLs. A and B were stained with anti-human CD3 Ab (brown) and counterstained with hematoxylin (blue). A is a LN section from a mouse i.v. injected with mock-treated resting human PBLs, and B is from a mouse given HIV-exposed PBLs. C, D, E, and F are immunocytochemical colocalization of DNA fragmentation and lymphocyte surface markers. DNA fragmentation (apoptotic cells) was labeled by the TUNEL method (dark blue/black). Human T lymphocytes were stained by anti-human CD8 (C, E) or CD4 (D, F) Abs (brown). Double-labeled cells are dark brown or with dark nuclei (arrows). A and B, ×400 magnification; C and D, ×650; and E and F, ×1000.
Thus, only make HIV (hence, a bystander effect). A few of the CD4* cells produced per day (10^9 –10^10 virions) (48), since one productively infected cell is needed to account for the estimated amounts of HIV blood into the LNs and die. Only a small fraction of virus-producing infected bystander CD4* SCID mice (7). We suspect that CD4* in vitro experiments exposed each lymphocyte to between 10 –100; following exposure of resting lymphocytes to a high MOI (21), these lymphocytes were signaled similarly to what we achieved in vitro, and that many of these cells that appear in the blood within the ensuing 24–72 h may be destined for homing-induced death.

Our data also indicate that both macrophage-tropic and T cell-tropic HIV strains similarly induced this state in resting lymphocytes, whereby second signals through the homing receptors induced apoptosis. Studies using the SCID/hu model system have shown that the macrophage-tropic strains are more effective in depleting CD4 lymphocytes in comparison with T cell-tropic strains (44, 45). It is possible that these differential effects are not due to inherent differences in the abilities of the viruses to induce the state we are studying, but rather may be due to differences in the extent or degree of virus spread and replication in this mouse system (46). Studies have indicated that infected humans possessing either macrophage-tropic or T cell-tropic HIVs progress in disease (47).

We thus propose the following scheme as a major mechanism of HIV pathogenesis. HIV is produced in the LNs, as this is where most of the productively infected cells reside, and is binding to surrounding resting CD4* lymphocytes. Many of these abortively infected bystander CD4* cells will recirculate back into the blood within 1 or 2 days. These T lymphocytes then home back into the LNs at an enhanced rate, accounting for disappearance of the CD4* lymphocytes in the blood. As they home in, signals are induced through the homing receptors that trigger induction of apoptosis in about one-half of the CD4* T lymphocytes, but not in the CD8* T lymphocytes. These dying CD4* lymphocytes never make HIV (hence, a bystander effect). A few of the CD4* T lymphocytes that survive may enter the cell cycle if signaled by Ag in the LNs, and then they will produce virus. Thus, as more of these cells home into the LNs, more productively infected cells result and HIV levels in the body should continuously rise. This in turn will induce more CD4* lymphocytes to eventually home from the blood into the LNs and die. Only a small fraction of virus-producing cells is needed to account for the estimated amounts of HIV produced per day (10^9–10^10 virions) (48), since one productively infected cell produces 10^2–10^3 virions per 24-h period (49, 50). Thus, only ~10^7 productively infected cells of 10^12 total lymphocytes in the whole body would suffice to produce the observed amounts of virus, and most of these virions would be interacting with resting lymphocytes.

These effects of HIV (induction of LN homing and apoptosis after signaling through homing receptors) on resting T lymphocytes may explain the depletion of CD4* T lymphocytes from the peripheral blood when very few blood CD4* lymphocytes are productively infected, and the development of lymphadenopathy at a time when CD4* T cell numbers are falling in the blood. It also can help explain some of the depletion of CD4* T lymphocytes from the LN. Therapeutic approaches involving inhibition of viral-induced homing and/or homing-induced apoptosis, together with inhibition of viral replication, may prove beneficial for HIV-infected subjects.

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