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HIV-1 Actively Replicates in Naive CD4⁺ T Cells Residing within Human Lymphoid Tissues

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Summary

Although HIV-1 gene expression is detected in naive, resting T cells in vivo, such cells are resistant to productive infection in vitro. However, we found that the endogenous microenvironment of human lymphoid tissues supports de novo infection and depletion of this population. Cell cycle analysis and DNA labeling experiments established that these cells were definitively quiescent and thus infected de novo. Quantitation of the "burst size" within naive cells further demonstrated that these cells were productively infected and contributed to the local viral burden. These findings demonstrate that lymphoid tissues support active HIV-1 replication in resting, naive T cells. Moreover, these cells are not solely reservoirs of latent virus but are permissive hosts for viral replication that likely targets them for elimination.

Introduction

The hallmark of human immunodeficiency virus type 1 (HIV-1) disease is the progressive loss of CD4$^+$ T cells. In vitro studies with peripheral blood mononuclear cells (PBMC) have suggested that specific cellular characteristics govern the susceptibility of cells to infection by HIV-1, including activation, maturation (i.e., naive versus memory), and proliferation. In particular, cultured memory cells are preferentially infected (Schnittman et al., 1990; Helbert et al., 1997; Spina et al., 1997), while resting and/or naive cells are highly resistant (Zack et al., 1990; Roederer et al., 1997; Chou et al., 1997). Cellular proliferation also regulates HIV-1 infection in PBMC (Bukrinsky et al., 1991; Zack et al., 1992; Spina et al., 1995; Tang et al., 1995).

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Nonetheless, recent work has revealed that nonactivated, naive, and/or nonproliferating lymphocytes within infected patients exhibit active HIV-1 gene expression (Zhang et al., 1999; Ostrowski et al., 1999; Blaak et al., 2000). The basis for these paradoxical observations is not well established, but the HIV-1 life cycle may be regulated differently in the in vitro and in vivo contexts. Furthermore, the cross-sectional design of these recent clinical studies makes it difficult to conclude with certainty whether the cell populations expected to be resistant to HIV-1 infection were infected de novo or rather were infected during an earlier phase of the cellular lifetime.

Culturing human lymphoid tissues ex vivo is a useful method for studying host-virus interactions because the microenvironment is biologically relevant, and experiments are both prospective and longitudinal. In addition, these tissues are permissive for HIV-1 infection independent of exogenous stimulation (Glushakova et al., 1997), thus preserving the endogenous cytokine milieu and cellular heterogeneity. We therefore used human tonsil histocultures to test the hypothesis that the endogenous microenvironment of lymphoid tissues supports the de novo infection of quiescent CD4$^+$ T cells by HIV-1.

Results

Identification of Noncycling and Proliferating Memory and Naive CD4$^+$ T Cells

We used flow cytometry and immunostaining to identify subpopulations of CD3$^+$ T cells within human tonsil histocultures. One population characterized by low forward and side scatter signals (Figure 1A) is termed "lymphocytes." Examination of the proliferative status of this population by both bromodeoxyuridine (BrdU) incorporation (Figure 1B) and propidium iodide (PI) staining (data not shown) detected negligible proliferative activity. A second population characterized by more pronounced forward and side scatter properties (Figure 1A) is termed "blasts" because of the increased size and granularity of the cells; we confirmed that the majority of these larger blasts were indeed single cells (data not shown). BrdU labeling (Figure 1B) and PI staining (data not shown) revealed that a substantial fraction of these cells were cycling. Thus, both morphologic and proliferative characteristics distinguished blasts from lymphocytes. The remaining cells had extremely pronounced side scatter and a broad range of forward scatter signals, and immunostaining revealed a mixture of diverse cell types within this population including dendritic cells, macrophages, and multicellular conjugates of T cells and dendritic cells or macrophages (Figure 1A and data not shown). Therefore, these tissues are heterogeneous and composed of identifiable cell populations.

We next defined the maturation phenotypes of CD4$^+$ T lymphocytes and blasts. The majority of both CD4$^+$ lymphocytes and blasts were found to be memory cells (non-CD45RA$^+$/CD62L$^+$) (Figures 1C and 1D), although the blast fraction contained somewhat fewer memory

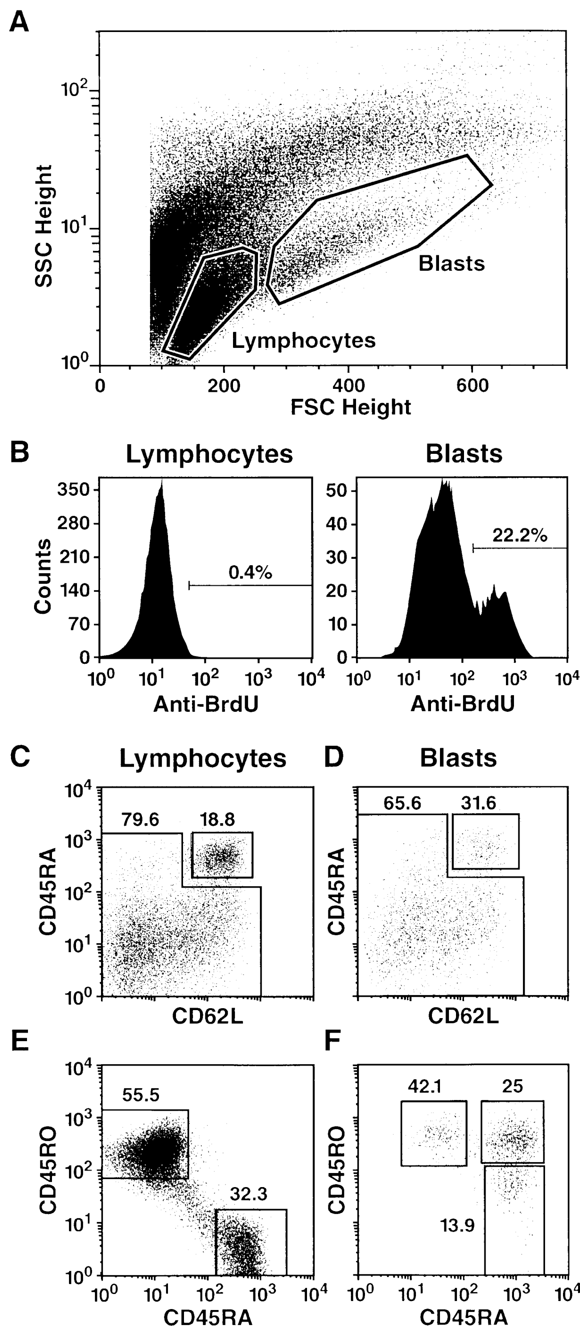


Figure 1. Flow Cytometric Analysis of Lymphocytes and Blasts within Human Lymphoid Histocultures Immediately following Tissue Procurement

(A) Forward and side scatter properties of cells dispersed from human tonsil immediately following tonsillectomy.

(B) Anti-BrdU reactivity of the same tissue.

(C–F) Distribution of memory and naive CD4$^+$ T cell subsets in tonsil tissue. Cells from uncultured tonsil specimens were immunostained with anti-CD45RA and CD62L mAbs (C and D) or anti-CD45RA and CD45RO mAbs (E and F). Shown are dot plots from a representative donor tissue (gated on CD4$^+$ cells). Values indicated for each region represent the mean percentages for each subset derived from a collection of donor tissues analyzed (n = 8–14); SEM for each subset was less than 20% of the mean.

cells (Figure 1D) than did the lymphocyte fraction (Figure 1C). Similarly, the majority of both lymphocytes and blasts had a memory phenotype as determined by reactivity to anti-CD45RO antibodies (Figures 1E and 1F). Interestingly, a population of RA$^+$/RO$^+$ cells was found exclusively within the blast population (Figure 1F), which may represent an intermediate stage in the development of naive T cells into memory cells (LaSalle and Hafler, 1991; Hamann et al., 1996).

We next stained naive and memory CD4$^+$ T lymphocytes and blasts for expression of the HIV-1 coreceptors CXCR4 and CCR5. We found that greater than 95% of all populations examined expressed high levels of CXCR4, whereas the expression of CCR5 was far more restricted (data not shown). Greater than 23% of memory T cells (lymphocytes and blasts) and naive T cell blasts expressed CCR5, but only 10% of naive T lymphocytes expressed detectable levels of CCR5.

Noncycling Naive T Cells Are Permissive for HIV-1 Replication

To determine directly whether maturation phenotype influences susceptibility to productive infection, we measured the specific infection of naive and memory CD4$^+$ lymphocytes following 7 day infections with the CXCR4-dependent (X4) HIV-1 molecular clone NL4-3 (Adachi et al., 1987; Speck et al., 1997; Schols et al., 1998; Penn et al., 1999). We immunostained NL4-3-infected tonsil explants to detect intracellular p24, a marker of productive HIV-1 infection, and expression of CD4, CD45RA, and CD62L. Memory and naive T cells within mock infected tissues demonstrated minimal p24 background staining (Figures 2A and 2D, respectively). In contrast, in cultures inoculated with NL4-3, memory and naive T cells were infected at significant frequencies (Figures 2B and 2E, respectively), although naive T cells exhibited lower rates of infection. Incubation of samples of infected tissue in trypsin prior to staining revealed no reduction in the frequency of infected T cells (Figures 2C and 2F). Treatment with EDTA yielded similar results (data not shown).

To determine if infection could be inhibited by HIV-1 coreceptor antagonists, we infected histocultures in the absence or presence of the CXCR4 antagonist, AMD3100 (250 nM) (Schols et al., 1997; Donzella et al., 1998; Schramm et al., 2000). Mock infected tissues again demonstrated minimal p24 background staining (Figures 2G and 2J), whereas in cultures inoculated with NL4-3, memory and naive T cells were infected at significant frequencies (Figures 2H and 2K, respectively). In contrast, in media containing AMD3100 continuously, no infection was observed in either T cell population (Figures 2I and 2L). We conclude that the intracellular p24 staining method is specific and indicative of productive infection.

We next measured the incorporation of BrdU in uninfected tissue during a 12 hr labeling. Negligible proportions of either naive or memory lymphocytes exhibited significant BrdU incorporation (Figure 3A). In all samples tested, less than 0.2% of naive lymphocytes incorporated BrdU, while significant proportions of naive, memory, and transitional (CD45RA$^+$/RO$^+$) blasts incorporated BrdU (Figure 3A).

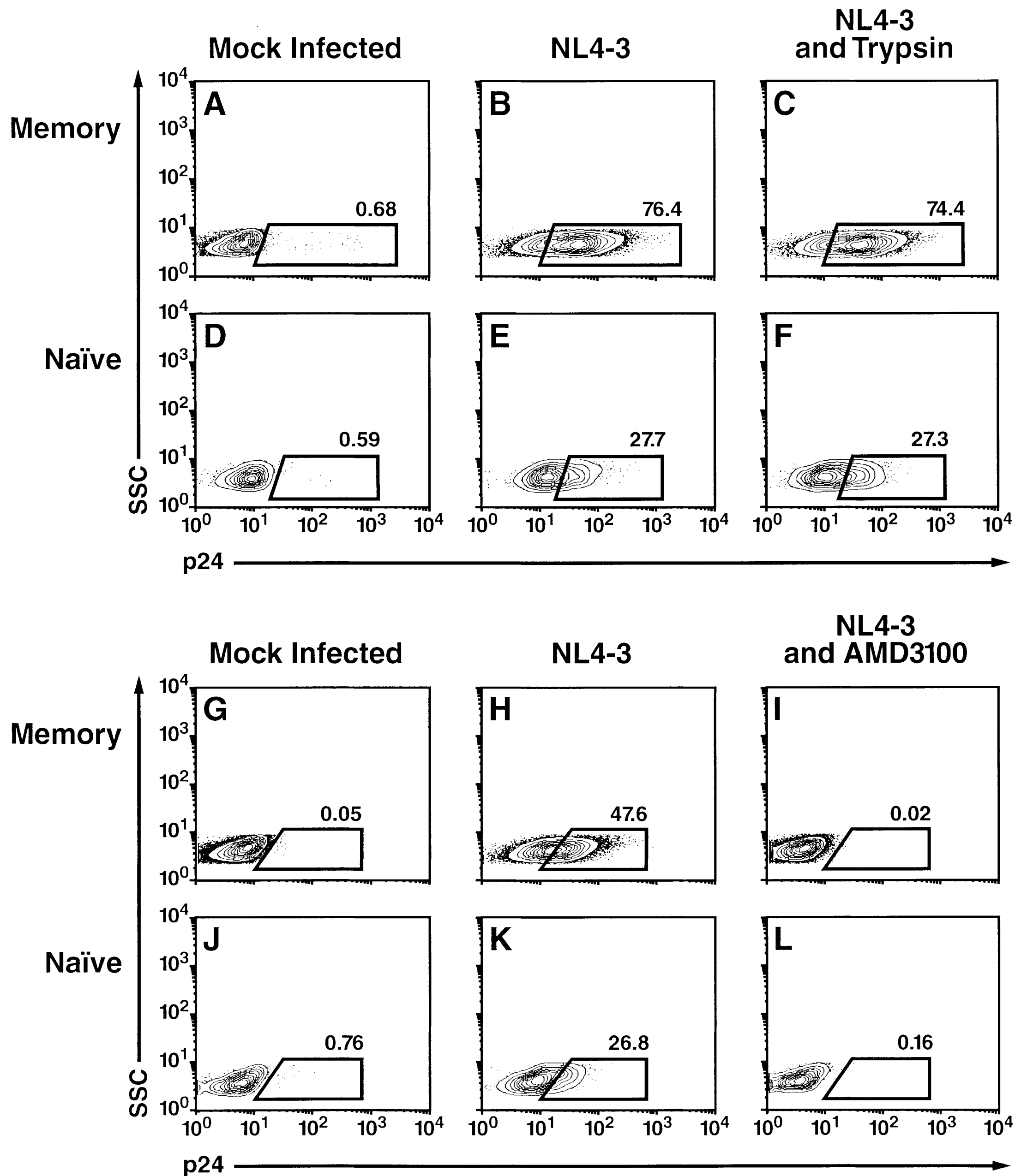


Figure 2. HIV-1 Infection of Naive and Memory CD4$^+$ T Cells

(Top) Intracellular p24 staining of memory (A–C) or naive (D–F) CD4$^+$ T cells from mock infected (A and D), from NL4-3-infected cultures (B and E), or from NL4-3-infected cultures treated with trypsin prior to staining (C and F).

(Bottom) Intracellular p24 staining of memory (G–I) or naive (J–L) CD4$^+$ T cells from mock infected cultures (G and J), from NL4-3-infected cultures (H and K), or from NL4-3-infected cultures incubated with AMD3100 for the duration of the infection (I and L). Presented are typical experiments from among three with indistinguishable results.

To compare the permissiveness of lymphocytes and blasts, we next measured the specific infection of naive and memory CD4$^+$ T cells following infection of parallel tissue samples in media free of BrdU. Cultures were inoculated with NL4-3 or the CCR5-dependent (R5) molecular clone 49-5 (Chesebro et al., 1992; Penn et al., 1999), and following 7 day infections were immunostained to detect intracellular p24 and various surface markers. Naive lymphocytes (CD45RA$^+$/RO$^-$) were infected at significant frequencies (Figure 3B) despite their minimal proliferative activity (Figure 3A). Identical results were obtained when CD45RA and CD62L were used to define naive cells (data not shown). Memory lymphocytes were also productively infected by NL4-3 at very high levels (Figure 3B and data not shown) despite low proliferative activity (Figure 3A). In addition, blasts of all maturation phenotypes were also substantially infected by NL4-3, with the highest levels being observed in the

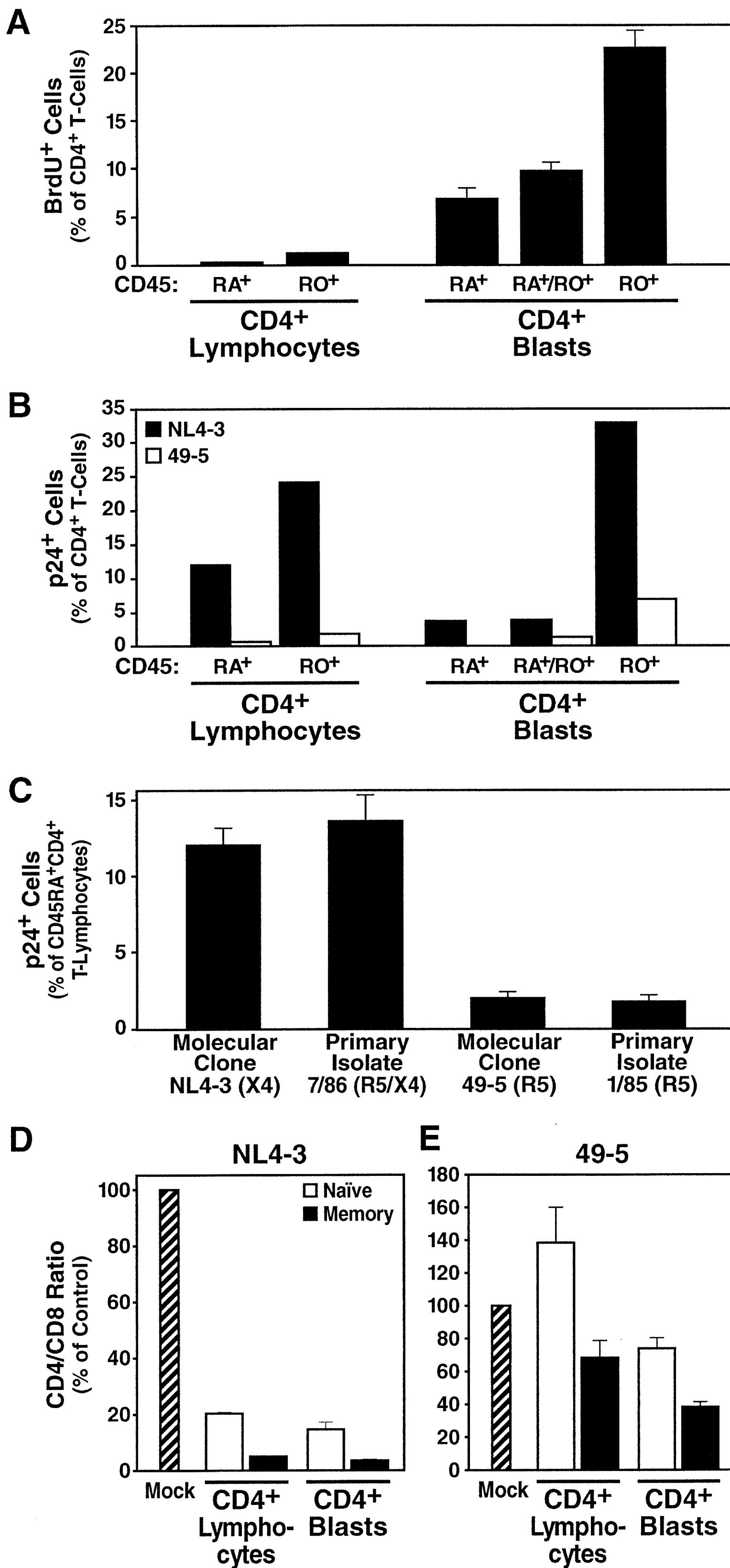


Figure 3. Cellular Proliferation and HIV-1 Infection and Depletion of Naive and Memory $CD4^+$ T Cell Subsets

(A) BrdU incorporation in naive and memory (identified by anti-CD45RA and -CD45RO mAbs) lymphocytes and blasts from tonsil histocultures following a 12 hr labeling with BrdU.

(B) Productively infected T cell subsets in these tissues following an 8 day infection with NL4-3 (closed bars) or 49-5 (open bars).

(C) Productively infected naive T cells following an 8 day infection with NL4-3, 7/86, 49-5, or 1/85.

(D) Depletion of T cell subsets in tonsil tissue following a 12 day infection with NL4-3.

(E) Depletion of T cell subsets in tonsil tissue following a 12 day infection with 49-5. Shown are mean values with SEM (n = 3) of a representative experiment from among experiments performed with three to seven different donor tissues.

memory fraction (Figure 3B). Interestingly, blasts with a naive ($CD45RA^+$) or intermediate phenotype ($CD45RA^+$/$CD45RO^+$) were consistently infected at lower levels than were noncycling lymphocytes (Figures 3A and 3B). The R5 virus 49-5 also demonstrated a strong preference for memory cells within both the lymphocyte and

blast T cell populations (Figure 3B). Nevertheless, productive infection of naive cells by 49-5 was detected, although at a low frequency consistent with the limited expression of CCR5 (Penn et al., 1999; Schramm et al., 2000; Grivel et al., 2000). The percentage of productively infected subsets by 49-5 was comparable to the frequency of cycling cells within these populations (Figure 3A). Because CCR5 identifies not only cells that are permissive for R5 HIV-1 but also activated and proliferative cells, usage of CCR5 by these viruses coincidentally restricts them to proliferating host cells. Thus, although it appears that X4 viruses may infect T cells independent of cellular proliferation, we cannot resolve whether R5 viruses do so as well.

To confirm that the ability to infect nonproliferating, naive T cells productively was also a feature of X4 primary virus isolates, we measured intracellular p24 expression in tissue inoculated with the dual-tropic (X4/R5) primary HIV-1 isolate 7/86 or the mono-tropic R5 primary isolate 1/85 (Connor et al., 1997). The X4 strains NL4-3 and 7/86 infected a substantial proportion of all $CD4^+$ T cell subsets, while the R5 strains 49-5 and 1/85 infected a smaller fraction of cells (data not shown). Most importantly, we detected substantial infection of naive lymphocytes by 7/86 (Figure 3C), confirming that X4 primary isolates are also capable of infecting noncycling T cells within lymphoid tissue. These results provide strong evidence that cellular proliferation is not an absolute requirement for infection of T cells within lymphoid tissues.

To determine if the differential infection of naive and memory T cells correlated with sensitivity to HIV-1-induced cytopathic effects, we next measured the specific depletion of naive and memory T cell subsets following inoculation. NL4-3 induced severe depletion of both naive and memory $CD4^+$ lymphocytes by 12 days postinfection, but preferential depletion of memory cells was nonetheless evident (Figure 3D). Similarly, memory lymphocytes were significantly and preferentially depleted by 49-5 relative to naive cells (Figure 3E), but the overall degree of depletion was lower than that caused by the X4 virus. Because of the low expression of CCR5 on naive T cells and possible requirement for cellular division, the infection and depletion of this subset by R5 viruses typically was minimal. Nevertheless, for both NL4-3 and 49-5, blasts typically exhibited overall greater susceptibility to depletion than did lymphocytes, but the preferential depletion of memory cells within either the lymphocyte or blast populations was most striking and consistent (Figures 3D and 3E). Thus, diverse subsets within the lymphoid tissue environment are highly susceptible to the cytopathic effects of HIV-1, and no subset examined here is invulnerable to elimination by X4 HIV-1. We utilized X4 viruses for subsequent examination of the infection of these diverse cells.

Naive $CD4^+$ T Cells Are Productively Infected by HIV-1 De Novo

The infection of naive lymphocytes is particularly striking since naive cells isolated from peripheral blood are resistant to de novo infection in vitro (Roederer et al., 1997). Since proliferating effector cells occasionally return to a noncycling state that is accompanied by reacquisition of naive surface markers (Lee and Pelletier, 1998), we performed pulse-chase experiments to determine the fraction of proliferating blasts that return to nonproliferating status. Histocultures were labeled for 12 hr with BrdU and then chased in the absence of BrdU for an additional 16–24 hr. Following the initial labeling period, approximately 19% of $CD4^+$ blasts but only 1% of $CD4^+$ lymphocytes had incorporated BrdU (Figure 4A). After the chase intervals, no significant change in the percentage of $BrdU^+$ blasts was observed (Figure 4A), suggesting that residual labeling during the chase period was minimal. In contrast, the percentage of labeled total lymphocytes increased modestly (3.5-fold) during the chase period to a maximum of nearly 4% (Figure 4A). Importantly, nearly all of these $BrdU^+$ lymphocytes were memory cells, while the fraction of BrdU-labeled naive T cells increased by only 0.1% during the chase period (Figure 4A). These results indicate that only a very small minority of nonproliferating, naive lymphocytes productively infected by HIV-1 may be derived from cells that were infected as blasts. Thus, cells within this population are likely infected de novo.

To determine directly whether the proliferating fraction of naive lymphocytes can account for the surprisingly high frequency of productively infected cells in this population, the cumulative fraction of proliferating naive T cells and blasts trafficking into this population was measured throughout a typical culture period. First, parallel histocultures were either infected with NL4-3 or cultured continuously in BrdU for 7 days. Cells were then immunostained for BrdU incorporation or intracellular p24. 30% of blasts within the infected samples were p24-positive, and 20% of blasts within the parallel BrdU-labeled samples had incorporated BrdU over the same period (Figure 4B). In contrast, approximately 14% of naive lymphocytes were p24-positive, while only 2% of naive lymphocytes within the parallel samples had incorporated BrdU, representing a nearly 7-fold excess of infected cells relative to BrdU-labeled cells (Figure 4B).

Second, to determine if the presence of virus itself may have altered the proliferative status of T cells, we inoculated histocultures with NL4-3 and cultured the tissue continuously in BrdU. Following a 7 day incubation, cells were coimmunostained for both BrdU and intracellular p24. Approximately 30% of productively infected blasts had incorporated BrdU, while only 8% of productively infected $CD45RA^+$ lymphocytes had incorporated BrdU during the same period. These data provide direct evidence that nonproliferating, naive $CD4^+$ lymphocytes are infected de novo by X4 HIV-1 within histocultures.

Productive Infection of Naive T Cells Is Not Restricted by Cell Cycle

Recent studies have suggested that productive HIV-1 infection of peripheral $CD4^+$ T cells depends upon a particular stage of the cell cycle that precedes cell division, a period termed G1b (Korin and Zack, 1998). In contrast to cells in G0 and G1a, cells in G1b display very high RNA content but have not yet begun synthesizing DNA (Darzynkiewicz et al., 1980; Gilbert et al., 1992). A combination of the nucleic acid dyes 7-aminoactinomycin D for DNA content and pyronin Y for RNA content

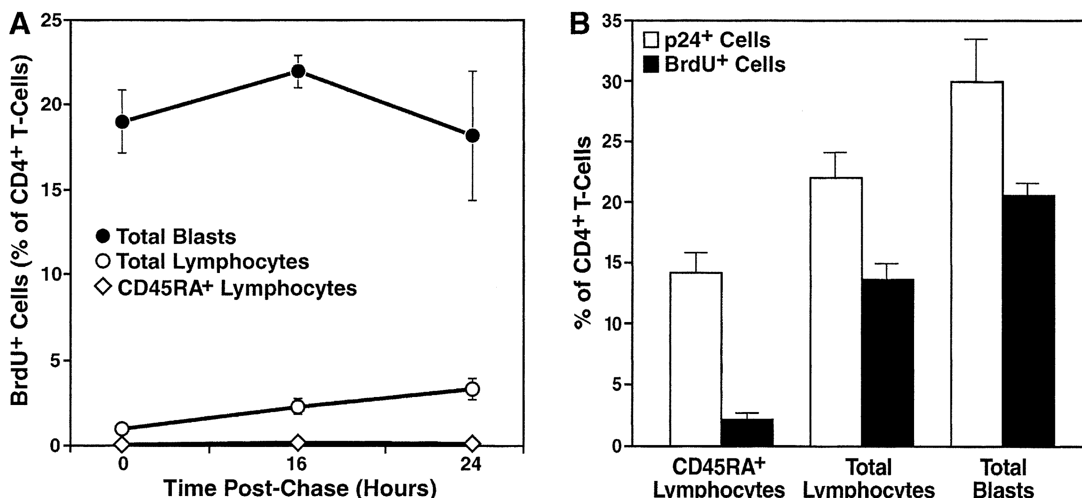


Figure 4. Kinetic Analysis of BrdU Incorporation in Tonsil Histoculture Lymphocytes and Blasts

(A) Histocultures were labeled with BrdU for 12 hr and washed extensively. Tissues were cultured for an additional 16 or 24 hr, dispersed, immunostained with mAbs to CD4 and BrdU, and analyzed by flow cytometry.

(B) Parallel histocultures were infected with NL4-3 or cultured continuously in medium containing BrdU for 7 days. Cells were dispersed and immunostained for CD4, CD45RA, and either intracellular p24 (open bars) or intracellular BrdU (solid bars). Shown are mean values with SEM (n = 3) from a typical experiment from among three experiments with different tissue specimens.

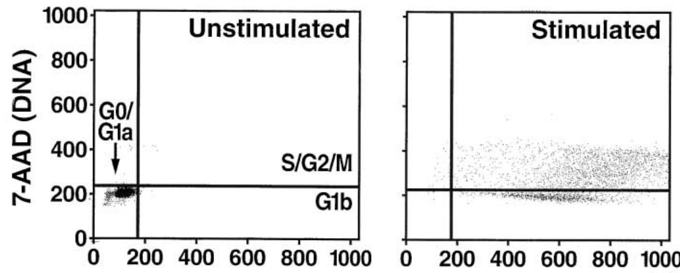
may distinguish between these phases of the cell cycle by flow cytometry. The majority of unstimulated, noncycling PBMC reside within G0/G1a (Figure 5A) and produce only partial reverse transcripts upon HIV-1 infection. In contrast, stimulated cells progress to G1b or beyond (Figure 5A) and support the completion of reverse transcription (Korin and Zack, 1998). To determine whether T cells that are permissive to HIV-1 infection in lymphoid tissues have progressed to G1b, we stained these cells with the nucleic acid dyes and various antibodies. To examine the relationship between productive HIV-1 infection and cell cycle progression, we performed these analyses using tissue that had been inoculated with NL4-3 and cultured for 7 days. Nearly all $CD4^+$ lymphocytes were in G0/G1a, whereas a substantial fraction of the blasts resided in either G1b or S/G2/M (Figures 5B and 5C). The lymphocyte and blast populations within lymphoid tissues are thus analogous to the unstimulated and stimulated PBMC, respectively. Examination of the naive T lymphocyte subset revealed that greater than 99% of cells in this population were in G0/G1a, and no cells were found in S/G2/M (Figure 5C). An identical cell cycle profile was observed in uninfected tissues (data not shown). These results demonstrate that the resting, noncycling lymphocytes have not progressed beyond G0/G1a and substantiate that the overall proliferative status of the tissue is unaffected by infection. Therefore, in contrast to PBMC, de novo HIV-1 infection of resting and naive lymphocytes within tonsil tissues is G1b independent.

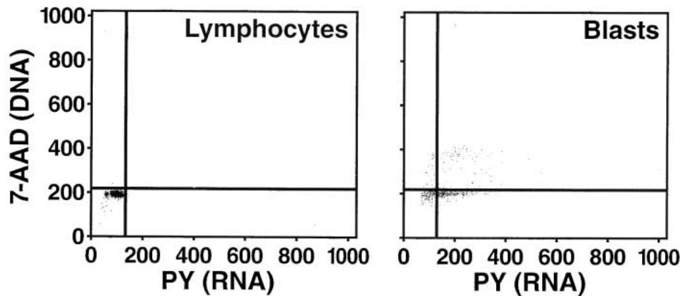
Productively Infected Naive T Cells Demonstrate Reduced Burst Size

Finally, we sought to quantitate the burst size of naive cells that had been productively infected. We employed a modified culture method, termed human lymphoid aggregate culture (HLAC), which offers added experimental flexibility while preserving the biology of human lymphoid tissue. In particular, cellular activation and proliferation, cell diversity, and HIV-1 infection, replication, and depletion within HLAC are nearly indistinguishable from that observed in the histoculture system (data not shown). We inoculated HLAC with NL4-3, and following 5 day incubation, the cultures were harvested and negatively selected using antibodies to CD8, CD14, and CD19 and magnetic bead separation. The remaining $CD4^+$ T lymphocytes were sorted by FACS into naive and memory T cell fractions using CD45RA/CD62L staining; such positive selection by CD45 isoform expression and isolation by flow cytometry does not alter the cellular phenotype or proliferative response (Spina et al., 1997). To determine the fraction of sorted lymphocytes that were productively infected by HIV-1, an aliquot of each fraction was stained for intracellular p24. The remainder of each fraction was then analyzed by several parallel approaches to quantitate viral output per cell, as described below.

First, a portion of each fraction was lysed and the "cell-associated p24" content was quantitated by ELISA. To represent the average p24 content as "virion equivalents" per infected cell, we divided this measured p24 value by the number of infected cells (as determined earlier), the molecular mass of p24, and the p24 content of a typical HIV-1 virion (estimated 1800 copies per virion) (Luftig et al., 1990; Piatak et al., 1993; Frankel and Young, 1998). We found that productively infected naive cells contained an average of 5.5-fold (range 2.9 to 6.4) less p24 antigen per cell than did infected memory cells (Figure 6A).

Second, to distinguish whether this difference in the intracellular p24 content of these cell types resulted from either reduced capacity for HIV-1 replication in naive cells or reduced viral egress from memory cells (thereby leading to its intracellular accumulation), the





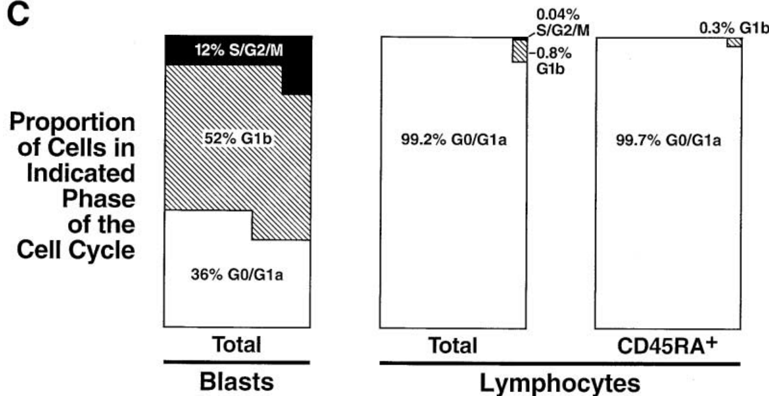


Figure 5. Cell Cycle Analysis of CD4$^+$ Lymphocytes and Blasts in PBMC and HIV-1-Infected Tonsil Histocultures

Cells were stained with 7-AAD (for DNA content), pyronin Y (for RNA content), and anti-CD45RA and anti-CD4 mAbs and analyzed by flow cytometry. Cell cycle profiles of unstimulated and anti-CD3, anti-CD28-stimulated PBMC (A), and histoculture lymphocytes and blasts (B). Frequency of histoculture total blasts, total lymphocytes, and CD45RA$^+$ lymphocytes in indicated phases of the cell cycle (C). Presented is a typical experiment from among three with indistinguishable results.

remaining fractions of purified naive and memory T cells were returned to culture, and we measured the kinetics of HIV-1 replication within the samples. We cultured one aliquot in normal media for an additional 1.5 days, the estimated generation time for HIV-1 within lymphocytes in vivo (Perelson et al., 1997). Supernatants were harvested at the end of the culture, and the concentration of p24 was measured by ELISA. Consistent with the earlier finding of differential cell-associated p24 content, we found that naive cells produced an average of 14-fold (range 6.4–28.4) less cell-released p24 than did memory cells during this culture period (Figure 6B). Likewise, a sample of the harvested supernatant was also used to determine the $TCID_{50}$ of the virus produced by naive and memory cells. This analysis revealed an average productive capacity of memory cells of 0.0045 infectious units per infected cell (range 0.00433–0.00473), while the average burst size of naive cells was at least 4-fold lower than this value and frequently below the resolution of our assay (Figure 6C). This pattern of enhanced viral replication within memory T cells compared to naive T cells correlates well with our earlier findings of higher quantities of cell-associated p24 within memory cells, and we conclude that the accumulation of p24 in these cells is not due to a block in the egress of mature virions from the cell membrane. Thus, the ratios of viral antigen production to virion release are similar between memory and naive T cells.

Third, to compare the long-term production of soluble HIV-1 antigens by different cell subsets, we cultured another fraction of the sorted naive and memory cells for 3.5 days in media containing inhibitory concentrations of AMD3100, thereby preventing further viral spread. While both naive and memory cells continued to secrete p24 beyond the initial 1.5 day culture described earlier, naive cells released an average of 3.1 (range 2.67–3.62) times less p24 per cell compared to memory cells (Figure 6D). Therefore, kinetic differences in the rate of HIV-1 replication within naive and memory T cells do not substantially contribute to the observed differences in the estimated burst sizes of these cells. Thus, productively infected naive T cells exhibit a reduced burst size compared to productively infected memory T cells. Importantly, for all donors examined, the cumulative p24 released by

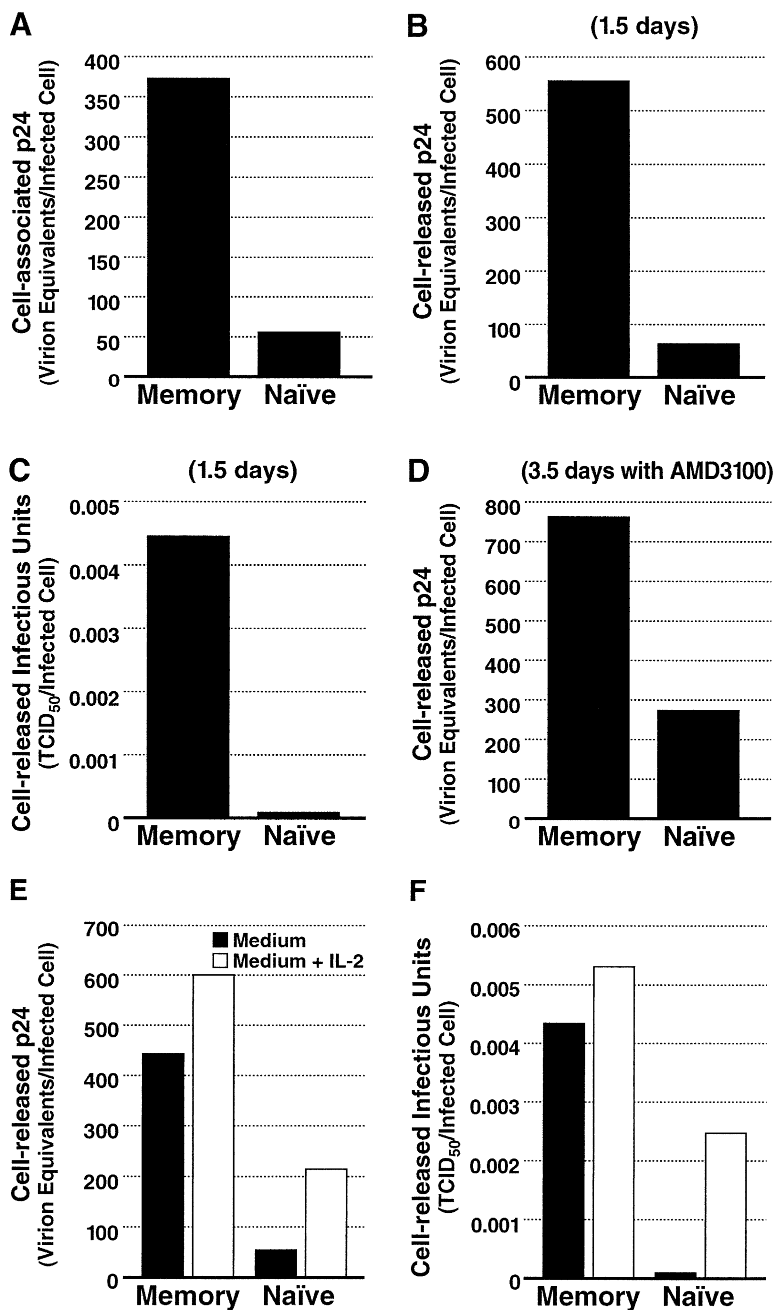


Figure 6. Burst Size of NL4-3-Infected Memory and Naive T Cells Isolated from Tonsil Cultures

Cells from tonsil tissues were stained with anti-CD45RA and anti-CD62L mAbs and sorted by flow cytometry.

(A) p24 content of memory and naive T cells.

(B) Soluble p24 and (C) infectious units released by infected memory and naive T cells during 1.5 days of culture in normal media.

(D) Soluble p24 released by infected memory and naive T cells during 3.5 days of culture in the presence of AMD3100 to prevent viral spread.

(E) Soluble p24 and (F) infectious units released by infected memory and naive T cells during 1.5 days of culture in media containing IL-2. Shown is a typical experiment from among four with indistinguishable results.

naive cells during extended culture in media containing AMD3100 exceeded the total mass of cell-associated p24 measured immediately following sorting (data not shown), confirming that naive cells within lymphoid tissues are permissive hosts for productive infection by HIV-1.

Fourth, to evaluate the effect of a prototypical growth factor on the relative burst sizes of naive and memory T cells, an additional aliquot of the sorted cell populations was cultured for 1.5 days in media containing recombinant human IL-2 (200 IU/ml). IL-2 was found to increase the quantity of cell-released p24 from memory cells by approximately 33%. Strikingly, IL-2 increased the cell-released p24 from naive cells by greater than 200% (Figure 6E). Likewise, IL-2 increased the number of cell-released infectious units from memory cells by 21%, but that from naive cells by at least 200% (Figure 6F). These profound effects of IL-2 on the productivity of naive cells confirm our conclusion that naive T cells may be productively infected de novo by HIV-1 and emphasize that they can serve as viral reservoirs that may be induced upon exposure to cytokines and other activating stimuli.

Such stimuli are typically found in abundance within lymphoid tissues in vivo. To examine their effects, we sought to quantitate the burst sizes of infected memory and naive T cells within the lymphoid microenvironment. We again sorted memory and naive CD4^{+} T cell lympho-

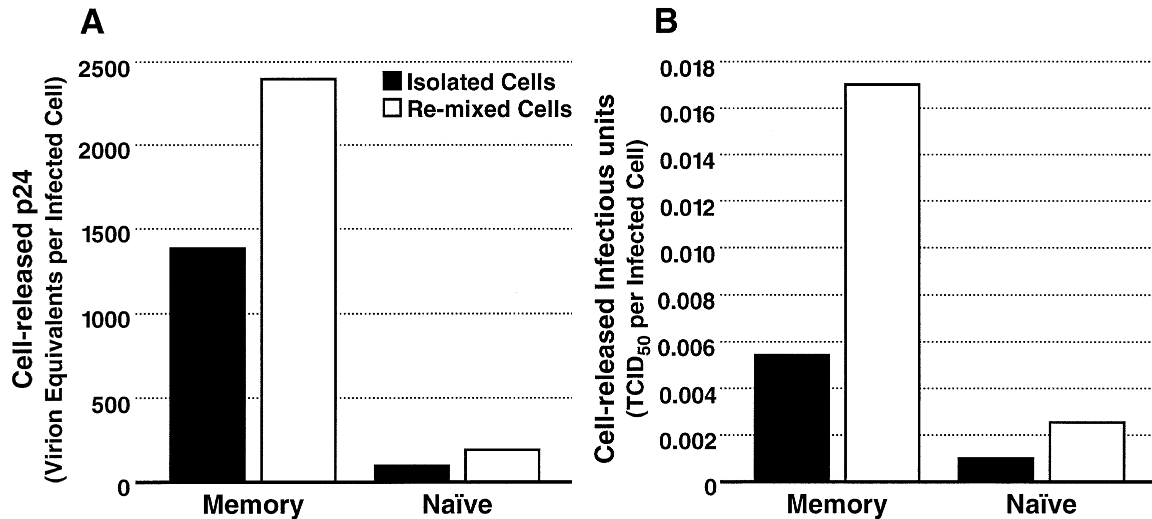


Figure 7. Burst Size of NL4-3-Infected Memory and Naive T Cells Reincorporated into Tonsil Cultures

Cells from tonsil tissues were stained with anti-CD45RA and anti-CD62L mAbs and sorted by flow cytometry. Sorted memory or naive T cells were cultured as isolated cells or in the presence of donor-matched lymphoid tissue.

(A) Soluble p24 released by isolated and remixed infected memory or naive T cells during 3.5 days of culture in the presence of AMD3100.

(B) Infectious units released by isolated and remixed infected memory or naive T cells during 1.5 days of culture in normal media. Shown is a typical experiment from among three with indistinguishable results.

cytes from NL4-3-infected tonsil cultures. One fraction of the cells was cultured for 3.5 days following sorting in the presence of inhibitory concentrations of AMD3100, at which time the culture media was harvested, and the cell-free p24 released by isolated naive and memory cells was measured by ELISA. As with our earlier studies, this analysis revealed that infected memory T cells released significantly greater p24 than did infected naive T cells (Figure 7A). Immediately following the sort, a second fraction of the sorted memory and naive cells was remixed with uninfected HLAC tissue derived from the same donor. Equal numbers of the sorted memory and naive cells were added back to the separate uninfected cultures in addition to AMD3100. The culture media was harvested 3.5 days later and the concentration of soluble p24 within these supernatants was quantitated. Using this "remixing assay," we found that the quantity of p24 produced by both memory and naive T cells within the lymphoid tissue microenvironment increased approximately 2-fold (range 1.57–2.15) compared with isolated cells (Figure 7A). Importantly, the relative burst sizes of memory and naive cells were unchanged within the reconstituted tissue context. Additional fractions of memory and naive T cells were similarly cultured for 1.5 days postsort in the presence or absence of uninfected lymphoid tissue. Following this incubation, the media was harvested, and the $TCID_{50}$ of the virus produced by memory or naive cells within these two culture conditions was measured. Consistent with our earlier studies, this analysis revealed an average productive capacity of 0.0055 and 0.0012 infectious units per infected memory or naive T cell, respectively (Figure 7B). Cells reinfused into HLAC released approximately 3-fold (range 2.61–3.16) more infectious virus than did isolated cells (Figure 7B). Thus, signals present within lymphoid tissues enhanced the replication of HIV-1 within infected T cells, although they did not substantially alter the relationship between the viral burst sizes of memory and naive T cells. These results further emphasize the quantitative differences between viral replication within memory and naive T cells and highlight the importance of activating or other signals in potentiating the HIV-1 viral reservoir.

Discussion

Recent studies have demonstrated that resting and naive $CD4^+$ lymphocytes are productively infected within peripheral blood and lymphoid tissues of HIV-positive individuals (Zhang et al., 1999; Ostrowski et al., 1999; Blaak et al., 2000), although these cells are highly resistant to productive infection by HIV-1 in vitro (Zack et al., 1990; Stevenson et al., 1990; Bukrinsky et al., 1991; Spina et al., 1995; Roederer et al., 1997; Chou et al., 1997). In prospective studies within lymphoid histocultures, we demonstrated that resting, naive $CD4^+$ T lymphocytes were susceptible to infection and depletion by X4 HIV-1 when present in the lymphoid microenvironment. Despite uncovering evidence of some degree of conversion of blasts into memory lymphocytes, pulse-chase and continuous DNA labeling experiments revealed an exceedingly low rate of conversion of proliferating blasts into resting, naive lymphocytes. Therefore, this small fraction of cells potentially infected during an earlier, replication-active developmental stage cannot be a significant contributor to the substantial levels of productive infection detected within the naive T cell pool. Moreover, our measurement of virus production by infected naive T cells isolated from lymphoid tissue revealed that these cells released soluble p24 in excess of the cell-associated p24 content of the cells measured at the time of collection, confirming that they had been

productively infected. Thus, nonproliferating, naive T cells within lymphoid tissue can be infected by X4 HIV-1 de novo and are permissive hosts for viral replication. However, we were unable to determine if R5 HIV-1 infection could proceed similarly in the absence of cell division. This possible behavioral difference between X4 and R5 strains may imply that naive T cells are spared during asymptomatic disease when R5 viruses predominate in vivo. Subsequent emergence of X4 viruses may then target this cell population, contributing to the loss of naive T cells and the profound immunodeficiency observed during late HIV-1 disease.

Recent work had demonstrated that productive HIV-1 infection of peripheral blood lymphocytes in culture does not require cell division per se but rather depends upon progression through a relatively early stage in the cell cycle designated as G1b (Korin and Zack, 1998). We found that nondividing $CD4^+$ lymphocytes in the lymphoid tissue context resided nearly exclusively in the G0/G1a phases that precede G1b in the absence or presence of replicating HIV-1, thus revealing that these cells were infected by HIV-1 in a G1b-independent manner. Therefore, the endogenous microenvironment within lymphoid histocultures permits HIV-1 replication in essentially all $CD4^+$ T cells regardless of phenotypic classification, proliferative status, or cell cycle progression.

Despite the qualitative permissiveness of resting, naive $CD4^+$ T cells for HIV-1 replication, these studies also revealed significantly different viral burst sizes for memory and naive T cells. By several distinct measures, naive T cells released significantly fewer viruses than did memory cells. Interestingly, it was also possible to deduce that, on average, T lymphocytes release less than one $TCID_{50}$ per infected cell. This fact suggests that cell-to-cell virus transmission, a process 100- to 1000-fold more efficient than cell-free transmission, may play a crucial role in the dissemination of HIV-1 between tissue-resident lymphocytes (Sato et al., 1992; Dimitrov et al., 1993). Moreover, stimulation of these cells by exogenous IL-2 significantly augmented the number of infectious units released from both memory and naive T cells, with the effect on naive T cells being particularly dramatic. Therefore, the specific milieu of these cells quantitatively influences the support for viral replication, and although unstimulated naive cells have a significantly smaller capacity for HIV-1 replication than do memory cells, they likely contribute to total viral load, at least within the local tissue microenvironment. Furthermore, local production of growth factors may substantially enhance such production from naive cells even without the necessity of triggering cell cycle entry.

Indeed, our remixing assay demonstrated enhanced viral production when T cells were placed within the lymphoid microenvironment, although memory T cells still exhibited significantly greater burst size than did naive T cells. In addition, we found that naive $CD4^+$ T cells isolated from the lymphoid environment are resistant to productive infection by HIV-1 (D.A.E., unpublished data). These results underscore the importance of the lymphoid microenvironment to the pathogenesis of HIV-1, and we infer that secreted or cell-associated factors delivered in *trans* to naive cells within lymphoid tissues induce a state of cellular permissivity for HIV-1 infection without markedly altering the overall cellular phenotype. It has been shown that select cytokines permit infection of nonactivated and nonproliferating $CD4^+$ cells by HIV-1 or HIV-based vectors (Chun et al., 1997b; Swingler et al., 1999; Unutmaz et al., 1999). Therefore, the cytokine milieu found within these lymphoid tissues may support the full HIV-1 replication cycle within otherwise resistant populations such as resting, naive T cells, thus allowing both productive infection and depletion of these cells. It is also possible that cellular events triggered by virus-associated factors, such as auxiliary proteins, might induce a replication-permissive state in this tissue environment that is not reflected in any of the diverse experimental measures of maturation and proliferation status employed herein. Interestingly, a recent study demonstrated that expression of HIV-1 Nef within macrophages induces expression of unidentified soluble factors that stimulated resting T lymphocytes and rendered them permissive for HIV-1 infection (Swingler et al., 1999). However, our use of the strict T cell-tropic strain NL4-3 likely eliminates HIV-infected macrophages as the source of the putative signal in the present experiments, but memory T cells and other cell types within lymphoid tissues might be alternate sources.

Thus, resting, naive T cells should not be considered exclusively as silent reservoirs for latent HIV-1 (Bukrinsky et al., 1991; Spina et al., 1995; Borvak et al., 1995; Finzi et al., 1997; Chun et al., 1997a). It is likely that individual cell types have varying degrees of permissivity to HIV-1 infection depending upon their immediate environment. For example, while naive T cells that circulate through peripheral blood or other locations may be resistant to HIV-1 infection, their trafficking into lymphoid tissues—sites of active HIV-1 replication during natural infections—may convert them into permissive hosts. The ability of HIV-1 strains to infect this pool of quiescent cells may be a key factor in determining the rate of global collapse of the $CD4^+$ T cell repertoire in vivo, and the evolution of HIV-1 strains with an enhanced ability to target these cells may accelerate disease progression within infected individuals. Studies aimed at better understanding the parameters that permit viral replication within lymphoid tissues may help to limit the spread of virus within HIV-1-infected individuals.

Experimental Procedures

Preparation of Viral Stocks

NL4-3 was a gift from Malcom Martin via the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health. The molecular clone 49-5 was a gift from Bruce Chesebro. Infectious virus stocks were prepared as previously described (Atchison et al., 1996). The primary isolates 7/86 and 1/85 (Connor et al., 1997), a gift from Ruth Connor, were expanded by infection of PBMC.

Culture and Infection of Human Lymphoid Tissues Ex Vivo

Human noninflammatory tonsil tissue removed during tonsillectomy (provided by San Francisco General Hospital and Kaiser-San Francisco and San Rafael, CA) were prepared and cultured as described (Glushakova et al., 1997). Tissues were inoculated with HIV-1 at 50 $TCID_{50}$ as described previously (Glushakova et al., 1997; Penn et al., 1999).

Identification of $CCR5^+$ T Cells by Flow Cytometry

Cells dispersed from uninfected lymphoid histocultures were immunostained for the cell surface markers CCR5, CD62L, CD45RA, and

CD4. The following monoclonal antibodies (mAbs) were used from Becton Dickinson (BD, San Jose, CA): anti-CD4 (clone SK7) and anti-CD62L. The following mAbs from BD Pharmingen (San Jose, CA) were also used: anti-CD45RA and anti-CCR5. The cells were then labeled with the Alexa Fluor 488 Signal-Amplification Kit (Molecular Probes, Eugene, OR) per manufacturer instructions.

Assessment of CD4$^+$ T Cell Infection and Depletion

For infection, cells were immunostained for cell surface markers CD4, CD62L or CD45RO (BD Pharmingen), and CD45RA. Cells were then fixed in 1% PFA and subsequently permeabilized and immunostained for intracellular CD4 and p24. To identify infected cells, anti-p24 from Coulter (Miami, FL) was also used. Titration/mixing studies demonstrated that the intracellular p24 staining assay was sensitive to a frequency of infected cells below 0.6%. AMD3100 was a gift from Dominique Schols and Erik De Clerq.

Depletion was assessed as described previously (Glushakova et al., 1997; Penn et al., 1999; Schramm et al., 2000). Depletion was also assessed by absolute cell numbers, determined with Fluorospheres (Coulter Immunotech, Miami, FL), and calculated per manufacturer instructions.

Flow Cytometric Analysis of Cellular Proliferation and Cell Cycle Status

For PI staining, cells were stained with an anti-CD4 mAb and then fixed with 2% PFA. Cells were resuspended in a solution of RNase A (1 μg/ml, Sigma, St. Louis, MO) and PI (10 μg /ml, Molecular Probes, Eugene, OR) and analyzed by flow cytometry. For BrdU labeling, lymphoid histocultures were cultured in media supplemented with BrdU (50 μM, Sigma, St. Louis, MO). Cells were dispersed and fixed and permeabilized in a solution of 1% PFA and 0.01% Tween 20. Cells were then treated with DNase (10 mg/ml, Sigma, St. Louis, MO) in PBS (with Ca^{2+} and Mg^{2+}) and then immunostained with a combination of mAbs recognizing CD4, CD45RA, CD45RO, and BrdU (BD Pharmingen). Titration/mixing studies demonstrated that the BrdU assay was sensitive to a labeling frequency below 0.5%. Four-color cell cycle analysis was performed as described previously (Schmid et al., 2000).

HIV Replication and Burst Size in Human Lymphoid Aggregate Cultures

Lymphoid tissue was mechanically dispersed, and the isolated cells were transferred to 96-well U-bottom plates at a concentration of 1×10^7 cells per ml, 200 μl per well. Tissues were inoculated with HIV-1 NL4-3 at approximately 100 $TCID_{50}$/well. Following 5 day infections, cell aggregates were resuspended and stained with mAbs recognizing CD62L and CD45RA (Coulter). Naive and memory cells were sorted and replated. Reanalysis of sorted populations demonstrated purities in excess of 95%.

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References

Adachi, A., Koenig, S., Gendelman, H.E., Daugherty, D., Gattoni-Celli, S., Fauci, A.S., and Martin, M.A. (1987). Productive, persistent infection of human colorectal cell lines with human immunodeficiency virus. J. Virol. *61*, 209–213.

Atchison, R.E., Gosling, J., Monteclaro, F.S., Franci, C., Digilio, L., Charo, I.F., and Goldsmith, M.A. (1996). Multiple extracellular elements of CCR5 and HIV-1 entry: dissociation from response to chemokines. Science *274*, 1924–1926.

Blaak, H., van't Wout, A.B., Brouwer, M., Hooibrink, B., Hovenkamp, E., and Schuitemaker, H. (2000). In vivo HIV-1 infection of CD45RA(+)CD4(+) T cells is established primarily by syncytium-inducing variants and correlates with the rate of CD4(+) T cell decline. Proc. Natl. Acad. Sci. USA *97,* 1269–1274.

Borvak, J., Chou, C.S., Bell, K., Van Dyke, G., Zola, H., Ramilo, O., and Vitetta, E.S. (1995). Expression of CD25 defines peripheral blood mononuclear cells with productive versus latent HIV infection. J. Immunol. *155*, 3196–3204.

Bukrinsky, M.I., Stanwick, T.L., Dempsey, M.P., and Stevenson, M. (1991). Quiescent T lymphocytes as an inducible virus reservoir in HIV-1 infection. Science *254*, 423–427.

Chesebro, B., Wehrly, K., Nishio, J., and Perryman, S. (1992). Macrophage-tropic human immunodeficiency virus isolates from different patients exhibit unusual V3 envelope sequence homogeneity in comparison with T-cell-tropic isolates: definition of critical amino acids involved in cell tropism. J. Virol. *66*, 6547–6554.

Chou, C.S., Ramilo, O., and Vitetta, E.S. (1997). Highly purified CD25−resting T cells cannot be infected de novo with HIV-1. Proc. Natl. Acad. Sci. USA *94*, 1361–1365.

Chun, T.W., Carruth, L., Finzi, D., Shen, X., DiGiuseppe, J.A., Taylor, H., Hermankova, M., Chadwick, K., Margolick, J., Quinn, T.C., et al. (1997a). Quantification of latent tissue reservoirs and total body viral load in HIV-1 infection. Nature *387*, 183–188.

Chun, T.W., Stuyver, L., Mizell, S.B., Ehler, L.A., Mican, J.A., Baseler, M., Lloyd, A.L., Nowak, M.A., and Fauci, A.S. (1997b). Presence of an inducible HIV-1 latent reservoir during highly active antiretroviral therapy. Proc. Natl. Acad. Sci. USA *94*, 13193–13197.

Connor, R.I., Sheridan, K.E., Ceradini, D., Choe, S., and Landau, N.R. (1997). Change in coreceptor use coreceptor use correlates with disease progression in HIV-1-infected individuals. J. Exp. Med. *185*, 621–628.

Darzynkiewicz, Z., Sharpless, T., Staiano-Coico, L., and Melamed, M.R. (1980). Subcompartments of the G1 phase of cell cycle detected by flow cytometry. Proc. Natl. Acad. Sci. USA *77*, 6696–6699.

Dimitrov, D.S., Willey, R.L., Sato, H., Chang, L.J., Blumenthal, R., and Martin, M.A. (1993). Quantitation of human immunodeficiency virus type 1 infection kinetics. J. Virol. *67*, 2182–2190.

Donzella, G.A., Schols, D., Lin, S.W., Esté, J.A., Nagashima, K.A., Maddon, P.J., Allaway, G.P., Sakmar, T.P., Henson, G., De Clercq, E., et al. (1998). AMD3100, a small molecule inhibitor of HIV-1 entry via the CXCR4 co-receptor. Nat. Med. *4*, 72–77.

Finzi, D., Hermankova, M., Pierson, T., Carruth, L.M., Buck, C., Chaisson, R.E., Quinn, T.C., Chadwick, K., Margolick, J., Brookmeyer, R., et al. (1997). Identification of a reservoir for HIV-1 in patients on highly active antiretroviral therapy. Science *278*, 1295–1300.

Frankel, A.D., and Young, J.A. (1998). HIV-1: fifteen proteins and an RNA. Annu. Rev. Biochem. *67*, 1–25.

Gilbert, K.M., Ernst, D.N., Hobbs, M.V., and Weigle, W.O. (1992). Effects of tolerance induction on early cell cycle progression by Th1 clones. Cell. Immunol. *141*, 362–372.

Glushakova, S., Baibakov, B., Zimmerberg, J., and Margolis, L.B. (1997). Experimental HIV infection of human lymphoid tissue: correlation of CD4+ T cell depletion and virus syncytium-inducing/non-

syncytium-inducing phenotype in histocultures inoculated with laboratory strains and patient isolates of HIV type 1. AIDS Res. Hum. Retroviruses *13*, 461–471.

Grivel, J.C., Penn, M.L., Eckstein, D.A., Schramm, B., Speck, R.F., Abbey, N.W., Herndier, B., Margolis, L., and Goldsmith, M.A. (2000). Human immunodeficiency virus type 1 coreceptor preferences determine target T-cell depletion and cellular tropism in human lymphoid tissue. J. Virol. *74*, 5347–5351.

Hamann, D., Baars, P.A., Hooibrink, B., and van Lier, R.W. (1996). Heterogeneity of the human CD4+ T-cell population: two distinct CD4+ T-cell subsets characterized by coexpression of CD45RA and CD45RO isoforms. Blood *88*, 3513–3551.

Helbert, M.R., Walter, J., L'Age, J., and Beverley, P.C. (1997). HIV infection of CD45RA+ and CD45RO+ CD4+ T cells. Clin. Exp. Immunol. *107*, 300–305.

Korin, Y.D., and Zack, J.A. (1998). Progression to the G1b phase of the cell cycle is required for completion of human immunodeficiency virus type 1 reverse transcription in T cells. J. Virol. *72*, 3161–3168.

LaSalle, J.M., and Hafler, D.A. (1991). The coexpression of CD45RA and CD45RO isoforms on T cells during the S/G2/M stages of cell cycle. Cell. Immunol. *138*, 197–206.

Lee, W.T., and Pelletier, W.J. (1998). Visualizing memory phenotype development after in vitro stimulation of CD4(+) T cells. Cell. Immunol. *188*, 1–11.

Luftig, R., Ikuta, K., Bu, M., and Calkins, P. (1990). Terminal stages of retroviral morphogenesis. In: Retrovial Proteases: Maturation and Morphogenesis, L.H. Pearl, ed. (London: Macmillan), pp. 141–148.

Ostrowski, M.A., Chun, T.W., Justement, S.J., Motola, I., Spinelli, M.A., Adelsberger, J., Ehler, L.A., Mizell, S.B., Hallahan, C.W., and Fauci, A.S. (1999). Both memory and CD45RA+/CD62L+ naive CD4(+) T cells are infected in human immunodeficiency virus type 1-infected individuals. J. Virol. *73*, 6430–6435.

Penn, M.L., Grivel, J.C., Schramm, B., Goldsmith, M.A., and Margolis, L. (1999). CXCR4 utilization is sufficient to trigger CD4+ T cell depletion in HIV-1-infected human lymphoid tissue. Proc. Natl. Acad. Sci. USA *96*, 663–668.

Perelson, A.S., Essunger, P., Cao, Y., Vesanen, M., Hurley, A., Saksela, K., Markowitz, M., and Ho, D.D. (1997). Decay characteristics of HIV-1-infected compartments during combination therapy. Nature *387*, 188–191.

Piatak, M., Jr., Saag, M.S., Yang, L.C., Clark, S.J., Kappes, J.C., Luk, K.C., Hahn, B.H., Shaw, G.M., and Lifson, J.D. (1993). High levels of HIV-1 in plasma during all stages of infection determined by competitive PCR. Science *259*, 1749–1754.

Roederer, M., Raju, P.A., Mitra, D.K., and Herzenberg, L.A. (1997). HIV does not replicate in naive CD4 T cells stimulated with CD3/CD28. J. Clin. Invest. *99*, 1555–1564.

Sato, H., Orenstein, J., Dimitrov, D., and Martin, M. (1992). Cell-to-cell spread of HIV-1 occurs within minutes and may not involve the participation of virus particles. Virology *186*, 712–724.

Schmid, I., Cole, S.W., Korin, Y.D., Zack, J.A., and Giorgi, J.V. (2000). Detection of cell cycle subcompartments by flow cytometric estimation of DNA-RNA content in combination with dual-color immunofluorescence. Cytometry *39*, 108–116.

Schnittman, S.M., Lane, H.C., Greenhouse, J., Justement, J.S., Baseler, M., and Fauci, A.S. (1990). Preferential infection of CD4+ memory T cells by human immunodeficiency virus type 1: evidence for a role in the selective T-cell functional defects observed in infected individuals. Proc. Natl. Acad. Sci. USA *87*, 6058–6062.

Schols, D., Struyf, S., Van Damme, J., Esté, J.A., Henson, G., and De Clercq, E. (1997). Inhibition of T-tropic HIV strains by selective antagonization of the chemokine receptor CXCR4. J. Exp. Med. *186*, 1383–1388.

Schols, D., Esté, J.A., Cabrera, C., and De Clercq, E. (1998). T-cell-line-tropic human immunodeficiency virus type 1 that is made resistant to stromal cell-derived factor 1alpha contains mutations in the envelope gp120 but does not show a switch in coreceptor use. J. Virol. *72*, 4032–4037.

Schramm, B., Penn, M.L., Speck, R.F., Chan, S.Y., De Clercq, E., Schols, D., Connor, R.I., and Goldsmith, M.A. (2000). Viral entry through CXCR4 is a pathogenic factor and therapeutic target in human immunodeficiency virus type 1 disease. J. Virol. *74*, 184–192.

Speck, R.F., Wehrly, K., Platt, E.J., Atchison, R.E., Charo, I.F., Kabat, D., Chesebro, B., and Goldsmith, M.A. (1997). Selective employment of chemokine receptors as human immunodeficiency virus type 1 coreceptors determined by individual amino acids within the envelope V3 loop. J. Virol. *71*, 7136–7139.

Spina, C.A., Guatelli, J.C., and Richman, D.D. (1995). Establishment of a stable, inducible form of human immunodeficiency virus type 1 DNA in quiescent CD4 lymphocytes in vitro. J. Virol. *69*, 2977–2988.

Spina, C.A., Prince, H.E., and Richman, D.D. (1997). Preferential replication of HIV-1 in the CD45RO memory cell subset of primary CD4 lymphocytes in vitro. J. Clin. Invest. *99*, 1774–1785.

Stevenson, M., Stanwick, T.L., Dempsey, M.P., and Lamonica, C.A. (1990). HIV-1 replication is controlled at the level of T cell activation and proviral integration. EMBO J. *9*, 1551–1560.

Swingler, S., Mann, A., Jacqué, J., Brichacek, B., Sasseville, V.G., Williams, K., Lackner, A.A., Janoff, E.N., Wang, R., Fisher, D., and Stevenson, M. (1999). HIV-1 Nef mediates lymphocyte chemotaxis and activation by infected macrophages. Nat. Med. *5*, 997–1003.

Tang, S., Patterson, B., and Levy, J.A. (1995). Highly purified quiescent human peripheral blood CD4+ T cells are infectible by human immunodeficiency virus but do not release virus after activation. J. Virol. *69*, 5659–5665.

Unutmaz, D., KewalRamani, V.N., Marmon, S., and Littman, D.R. (1999). Cytokine signals are sufficient for HIV-1 infection of resting human T lymphocytes. J. Exp. Med. *189,* 1735–1746.

Zack, J.A., Arrigo, S.J., Weitsman, S.R., Go, A.S., Haislip, A., and Chen, I.S. (1990). HIV-1 entry into quiescent primary lymphocytes: molecular analysis reveals a labile, latent viral structure. Cell *61*, 213–222.

Zack, J.A., Haislip, A.M., Krogstad, P., and Chen, I.S. (1992). Incompletely reverse-transcribed human immunodeficiency virus type 1 genomes in quiescent cells can function as intermediates in the retroviral life cycle. J. Virol. *66*, 1717–1725.

Zhang, Z., Schuler, T., Zupancic, M., Wietgrefe, S., Staskus, K.A., Reimann, K.A., Reinhart, T.A., Rogan, M., Cavert, W., Miller, C.J., et al. (1999). Sexual transmission and propagation of SIV and HIV in resting and activated CD4+ T cells. Science *286*, 1353–1357.