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Host and Viral Determinants of HIV-1 Influencing Cellular Tropism, Burst Size and Pathogenesis: The Role of the Lymphoid Microenvironment

by

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DISSERTATION

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Dedication

This dissertation is dedicated to my parents, Peter and Marlene, who instilled in me a life-long passion for learning, and who supported me through innumerable challenges. Without their guidance, help, and determination, this work would not have been possible. Without their love, I could not have dreamed to accomplish so much.

This thesis would not have
graduate advisor, Mark A
exceptional work ethic
process management and
worked closely to gather
strengthened our bond and

I would also like to thank M
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I am indebted to every
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All of the scientific work in this dissertation has been submitted and/or published in the following journals. Chapter 2 was published in the *Journal of Virology* and is reproduced here with permission from the American Society for Microbiology. Chapter 3 was published in *Immunity* and is reproduced here with permission from Elsevier Science. Chapter 4 has been submitted to the *Journal of Virology*. Chapter 5 has been submitted to the *Journal of Clinical Investigation*. Chapter 6 is in press at the *Journal of Experimental Medicine* and is reproduced here with permission from The Rockefeller University Press.

Host and Viral Determinants of HIV-1 Influencing Cellular Tropism, Burst Size and Pathogenesis: The Role of the Lymphoid Microenvironment

Daniel A. Eckstein

HIV-1 disease is characterized by the productive infection of macrophages and $CD4^+$ T-cells. Immunodeficiency results from the progressive loss and disregulation of T-cells. HIV-1 entry is mediated by the expression of CD4 and coreceptors. Interestingly, not all cells expressing these molecules are susceptible to HIV-1. The purpose of these studies was to identify cellular factors including coreceptor expression that impact viral infection and replication within T-cell subsets. These characteristics include maturation, activation, and proliferation. We exploited a unique lymphoid histoculture model system that permits *ex vivo* culture and infection of primary human lymphoid tissues. We found that HIV-1 infected nearly all classes of T-cells, including non-proliferating, naïve $CD4^+$ T-cells. Further studies revealed that a substantial fraction of these productively infected naïve lymphocytes were non-activated. Thus, despite considerable evidence that such cells are refractory to infection *in vitro*, we conclude that the lymphoid microenvironment supports the productive infection of these cells *de novo*. Although both naïve and memory T-cells are infected by HIV-1 within lymphoid tissues *ex vivo*, naïve cells release approximately 6-fold less virions than memory cells, implying a quantitative

difference in the infection of these cell populations. Interestingly, we also found that a subset of naïve cells were latently infected by HIV-1, implying that some naïve cells release little or no virus. We conclude that the establishment of latent provirus within these cells occurs by a novel, activation-independent pathway. A second objective of this work was to determine the role of viral gene products in mediating the infection of various cell populations. To accomplish this, we compared the behavior of viruses differing only in the presence or absence of the viral gene *vpr*. We found that Vpr contributed substantially to the productive infection of macrophages but not non-dividing T-cells. These studies also evidenced a significant role for macrophages in the generation of tissue viral burden. Thus, we infer that viral gene products have evolved that confer upon HIV-1 the ability to infect diverse and specific cellular targets. Collectively, this work highlights the complexity of HIV-1 and the importance of lymphoid tissues in the pathogenesis of HIV-1 disease *in vivo*.

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Chapter 1

Introduction

I. OVERVIEW

The hallmark of human immunodeficiency virus type 1 (HIV-1) infection is the progressive loss of $CD4^+$ T-lymphocytes. HIV-induced disease may be divided into three clinical stages: acute infection, asymptomatic infection, and acquired immunodeficiency syndrome (AIDS). During acute infection HIV-1 replicates to very high levels wherein the host's lymphoid and other tissues are seeded by the virus, and the number of circulating $CD4^+$ T-cells drops transiently (63). This period is often characterized by an acute viral syndrome, which may present clinically with flu-like symptoms. Subsequent onset of HIV-1-specific immune responses (initially cytotoxic T-cells and later B-cells) appears to limit the degree of replication, evidenced by a dramatic fall in viral titers (36, 48, 52). However, in most individuals, the immune response is inadequate to fully eradicate the virus, and replication continues at a reduced level (2, 39, 47). Nevertheless, this residual viral turnover may contribute significantly to immune pathogenesis, as the number of circulating $CD4^+$ T-cells declines slowly during asymptomatic infection (51). The level of viral replication increases dramatically and the rate of T-cell loss accelerates significantly during the final stage of disease, leading to profound immune dysfunction and the subsequent acquisition of opportunistic infections (63, 67). The viral and host determinants that impact the degree of HIV-1 replication and the rate of $CD4^+$ T-cell loss, and therefore the rate of disease progression, are not well understood, but may relate at least in part to the evolution of viral coreceptor usage.

II. HIV-1 CELLULAR ENTRY AND CORECEPTORS

In combination with CD4, the human chemokine receptors CCR5 and CXCR4 have emerged as the predominant cofactors for cellular entry of HIV-1 *in vivo* (1, 12, 20, 21, 24). CCR5 is expressed on most tissue macrophages, dendritic cells, and a subset of activated T-cells (6, 20, 60, 82). In contrast, CXCR4 is expressed on macrophages, dendritic cells and nearly all T-cells (6, 20, 23, 60, 82). It is widely reported that viruses selectively employing the CCR5 coreceptor (R5 viruses) mediate viral transmission and predominate during acute and clinically asymptomatic HIV infection, while viruses with expanded preference for CXCR4 (R5/X4 or X4 viruses) and other coreceptors often emerge in late stages of HIV-induced disease (19, 31, 43, 62, 68, 83). The evolution of X4 viruses is associated with increased viral loads, accelerated $CD4^+$ T-cell depletion, and progressive disease (19, 43, 62, 66, 74, 75). Although this phenomenon may be a key factor affecting $CD4^+$ T-cell depletion, the causal relationship between the phenotypic switch and the immunodeficiency and elevated viral titers that characterizes progressive HIV-1 disease remains speculative.

III. HUMAN LYMPHOID HISTOCULTURES AND AGGREGATE CULTURES AS MODEL SYSTEMS WITH WHICH TO INVESTIGATE HIV-1 PATHOGENESIS

To address these important questions we have utilized a unique *ex vivo* model system termed human lymphoid histoculture (HLH), which involves culturing explants from non-inflammatory human spleen and/or tonsil specimens from non-infected donors (28, 29, 32, 53, 65). This system has several important features that distinguish it from peripheral blood mononuclear cell (PBMC) culture: (1) it preserves the three-dimensional organization and diversity of mature lymphoid and myeloid cellular constituents present within lymphoid tissue in which HIV replication and pathogenesis may occur; (2) it does not depend upon exogenous stimulation or cytokines; (3) it supports progressive HIV replication over several weeks duration; and (4) it exhibits HIV-specific, reliable and quantifiable pathologic effects of infection.

During this dissertation work we have also developed an adaptation of the HLH model within our laboratory termed human lymphoid aggregate culture (HLAC) (22). Noninflammatory spleen or tonsil tissue is mechanically dispersed, and isolated cells are cultured in 96-well U-bottom plates at a density approximating that observed in lymphoid tissues. Cells reaggregate at the bottom of the wells and may then be inoculated with various strains of HIV-1. Importantly, the cellular activation state, cell diversity, and depletion and replication behavior of various HIV-1 strains within this HLAC system appear to be nearly indistinguishable from that observed in cultured explants of human lymphoid tissue (HLH). Because the cells in these cultures are physically accessible, HLAC offers experimental advantages and flexibility unachievable with other models,

including HLH, while preserving the biology of the organs from which the tissues are derived.

These models represent attractive systems in which to investigate the pathogenesis of HIV-1. Before the development of these culture techniques, most investigators relied on cultures of PBMC to study HIV. However, these cells represent just 2% of the total lymphocytes in the human immune system (14). Moreover, PBMC require mitogen activation and continuous cytokine exposure to remain viable and permissive for HIV replication. These stimuli drastically alter the phenotypes of the cells. Thus, PBMC cultures do not accurately model relevant cellular characteristics found *in vivo* and may be of limited predictive significance regarding the majority of virus-cell interactions *in vivo*. In contrast, HLH and HLAC are derived from peripheral lymphoid organs, which are the principal sites of HIV-1 replication and viral load within infected individuals. Moreover, whereas PBMC represent only a small subset of the lymphocyte pool, lymphoid organs contain the majority of lymphocytes *in vivo* (14). In addition, these organ cultures are viable and permissive for HIV replication independent of cytokine support or other signals, thereby preserving the profound cell heterogeneity found within lymphoid tissues *in vivo*. Therefore, HLH and HLAC represent unique models that recapitulate important *in vivo* features and provide a highly relevant setting in which to investigate the pathogenesis of HIV-1 *in vitro*.

IV. HIV-1 CORECEPTOR USAGE DETERMINES TARGET T-CELL DEPLETION AND CELLULAR TROPISM

Using these systems, we have shown that X4 viruses significantly infect $CD4^+$ T lymphocytes (32, 53), consistent with the widespread expression of CXCR4 on most helper T-cells. In contrast, R5 viruses infect only a small fraction of $CD4^+$ T-cells (32, 53), their entry being limited to the $CCR5^+$ subset of T lymphocytes, and mature lymphoid macrophages. Despite the limited target cell availability for R5 viruses, they nevertheless predominate during the acute and clinically asymptomatic stages of HIV infection in the majority of patients (19, 31, 43, 62, 68, 83). Moreover, studies have recently demonstrated that HIV-1 exhibits significant turnover during these phases, generating greater than $1x10^9$ virions per day (38, 80). Interestingly, despite the differences in the target T-cell availability for R5 and X4 viruses, they also replicate with similar kinetics and to similar degrees in human lymphoid tissue *ex vivo* (32). One possible explanation for this phenomenon is that the different target cells of HIV-1 may have different inherent capacities for HIV replication, a hypothesis tested in this dissertation work.

Previous investigations have shown that the variable regions of the envelope (*env*) gene contribute most significantly to the determination of viral coreceptor preference (5, 17, 37, 70, 83). In particular, within most clade B viruses, residues within the V3 loop are the dominant features influencing coreceptor usage. Indeed, in most cases incorporation of envelope sequences derived from R5-dependent viruses into X4-dependent viruses is

sufficient to transfer CCR5 usage to the formerly X4 virus (11, 53, 70, 76). We employed this principle to study matched pairs of viruses that shared a common (isogenic) genetic backbone, but differed in the V3 sequences. These recombinant viruses provided an important tool with which to investigate the importance of coreceptor preference in the tropism and pathogenesis of HIV-1 without the confounding variable of differing viral gene products. These studies allowed us to identify the cellular targets of X4 and R5 viruses and later establish the source of local R5 viral burden. We found that X4 viruses replicated within $CD4^+$ T-cells whereas R5 viruses infected a fraction of ($CCR5^+$) $CD4^+$ T-cells as well as macrophages (32). We conclude that the combined viral output of infected T-cells and macrophages accounts for the surprisingly high replication of R5 viruses within these tissues. These experiments are described in detail in Chapters 2 and 6 of this dissertation.

V. HIV-1 REPLICATES IN DIVERSE SUBSETS OF $CD4^+$ T-CELLS WITHIN HUMAN LYMPHOID TISSUES

Although the previous study demonstrated that X4 viruses replicated preferentially within $CD4^+$ T-cells, a population of cells appeared to be resistant to X4 infection and persisted in the cultures long after most other $CD4^+$ T-cells had been depleted by HIV-1, despite high levels of CXCR4 expression. These findings are consistent with studies performed with PBMC that have produced evidence that some subsets of T-cells are refractory to HIV-1 infection. Three features have emerged as

predominant factors influencing susceptibility to infection: activation, proliferation, and maturation. First, T-cells expressing the activation markers CD25, HLA-DR, and/or CD69 support the full HIV life cycle and are sensitive to the cytopathic effects of the virus (13, 58, 77). However, replication is blocked during reverse transcription and/or preceding integration within non-activated cells that do not express detectable levels of any of these markers. Second, the proliferative status of host T-cells similarly regulates viral replication, with cycling cells supporting viral replication and non-dividing cells exhibiting a pre-integration block (9, 10, 71, 73, 86). Third, maturation also appears to define cells that are susceptible to HIV-1 infection. In particular, memory cells are preferentially infected and depleted by HIV-1 *in vitro* compared to naïve cells, which are highly resistant to infection (13, 35, 59, 64, 72, 81, 85, 86).

However, substantial *in vivo* evidence for infection of naïve T-cells has recently emerged. Studies have demonstrated that naïve T-cells isolated from infected patients exhibit active gene expression (50, 89). The basis for these paradoxical observations is not immediately evident, but may relate to the inherent cross-sectional design of the *in vivo* studies. Such investigations can not determine if these cells were infected *de novo* as mature, naïve T-cells, or instead infected at an earlier, more permissive period in the cellular lifetime. More compelling is the possibility that the replication of HIV-1 is regulated differently *in vivo* and *in vitro*. That is, signals absent from PBMC culture may be essential for the productive infection of non-dividing naïve T-cells. We therefore investigated the role of lymphoid tissues in the infection of naïve T-cells. In contrast to

the findings with PBMC, we found that these cultures supported the productive infection of these cells *de novo*, though at a lower level than that seen in memory cells. These experiments are described in detail in Chapter 3 of this dissertation.

VI. HIV-1 *TRANS*-ACTIVATES BYSTANDER CD4$^+$ T-CELLS WITHIN HUMAN LYMPHOID TISSUES

Another paradox in HIV-1 biology is the surprising finding that although activated T-cells are preferentially infected both *in vitro* and *in vivo*, these cells are present at elevated frequencies in patients with progressive HIV-disease (4, 33, 42, 45, 49, 61). It is hypothesized that the chronic infection by HIV-1 stimulates the immune system, leading to enhanced expression of activation markers on formerly non-activated cells. These studies have been limited by their cross-sectional design, and this hypothesis has not been formally proven.

In the present dissertation work, having previously demonstrated that non-dividing, naïve T-cells were productively infected by X4 HIV-1, we examined the activation state of these target cells. We found that HIV-1 substantially infected and depleted both the activated and non-activated subset of naïve T-cells. Most interestingly, we also found that X4 HIV-1 activates bystander cells that escaped infection, leading to their accumulation in late infections. These studies evidence a pathway by which HIV may manipulate the host immune system so as to generate a more permissive environment for viral replication. These processes likely contribute to viral pathogenesis

by accelerating viral replication, $CD4^+$ T-cell destruction, and eventual immune collapse. These experiments are described in detail in Chapter 4 of this dissertation.

VII. GENERATION OF LATENTLY INFECTED NAÏVE $CD4^+$ T-CELLS BY HIV-1 WITHIN HUMAN LYMPHOID TISSUES

HIV-1 infection of target cells may proceed along two pathways. First, viral entry may result in productive infection, resulting in the generation of infectious, progeny virions. Second, entry may instead progress to a latent infection in which transcriptional expression of viral antigens is impossible (due to incomplete reverse transcription) or repressed. The provirus may then be maintained within these cells for varying durations. In particular, provirus arrested prior to integration is relatively unstable, and disappears in a matter of days or weeks in the absence of activating signals (10, 71, 86). In contrast, transcriptionally silent, integrated provirus is very stable, and persists nearly indefinitely (14, 16, 25, 88). Latently infected cells harboring such virus represent a clinically important reservoir for HIV and are a significant impediment to eradication of virus within infected patients receiving highly active anti-retroviral therapy (HAART).

It is thought that a transition of infected host cells from an activated to a quiescent state may be an essential factor in the generation of these cells. Consistent with this model, the majority of latently infected $CD4^+$ T-cells isolated from HIV-infected patients display a resting, memory phenotype (14-16). However, latently infected naïve $CD4^+$ T-cells, which have not undergone such a transition, have recently been identified *in vivo*

(55). Our earlier findings that lymphoid histocultures supported the productive infection of mature, naïve T-cells raised the possibility that *de novo* (activation-independent) infection may be an alternate mechanism by which latent infection is achieved in this subset. Indeed, we found that naïve T-cells within lymphoid tissues also serve as inducible reservoirs of viral persistence following *de novo* infection. Recruitment of provirus within these cells to a highly productive state may contribute to the viral loads and progressive immunodeficiency seen in HIV-1 infection *in vivo*. These experiments are described in detail in Chapter 5 of this dissertation.

VIII. HIV-1 VPR IS AN ESSENTIAL FACTOR FOR THE PRODUCTIVE INFECTION OF MACROPHAGES BUT NOT NON-DIVIDING T-CELLS BY HIV-1

HIV-1 and other lentiviruses are unique among retroviruses in that they do not depend on cellular division and the accompanying breakdown of the nuclear envelope for productive infection (40, 44). It is believed that this special property is due to the concerted and perhaps redundant activities of Matrix (MA), Integrase (IN), the DNA flap and Vpr (7-9, 18, 26, 27, 34, 46, 54, 56, 57, 79, 87). In particular, Vpr, which is incorporated into mature virions, contains two non-overlapping and unique nuclear localization signals (41, 69). Although frequently deleted during *in vitro* passaging, Vpr is believed to have numerous functions that contribute to the establishment and

pathogenesis of HIV-1 infection *in vivo* including infection of non-dividing macrophages, which presumably is linked to its nuclear localization function (3, 18, 30, 78, 84).

In the present dissertation work, we investigated the role of Vpr in the infection of various target cells in human lymphoid tissues. Having demonstrated that HIV-1 productively infected lymphoid macrophages (Chapter 2) as well as non-dividing, naïve T-cells (Chapter 3), we wished to determine the role of Vpr in mediating the infection of these diverse cell types. By selectively ablating the infection of these populations we also hoped to determine the specific contribution of these cells to the overall viral burden within lymphoid tissues. Once again, we employed matched pairs of isogenic viruses that differed only in the presence or absence of *vpr*. This strategy allowed us to isolate the effects of Vpr without confounding changes in other viral gene products. Surprisingly, we found that while Vpr augments the infection of macrophages, it does not contribute to the productive infection of non-dividing, naïve T-cells. Furthermore, Vpr-deficient R5 viruses exhibited a significant reduction in overall viral replication, emphasizing the importance of tissue macrophages as a permissive reservoir for viral replication *in vivo*. These findings suggest that other host or viral factors may be responsible for the infection of resting lymphocytes and highlight the importance of Vpr and CCR5 coreceptor specificity for HIV-1 infection of tissue macrophages. These experiments are described in detail in Chapter 6 of this dissertation.

REFERENCES

1. **Alkhatib, G., C. Combadiere, C. C. Broder, Y. Feng, P. E. Kennedy, P. M. Murphy, and E. A. Berger.** 1996. CC CKR5: a RANTES, MIP-1alpha, MIP-1beta receptor as a fusion cofactor for macrophage-tropic HIV-1. Science. **272:**1955-8.

2. **Bacchetti, P., and A. R. Moss.** 1989. Incubation period of AIDS in San Francisco. Nature. **338:**251-3.

3. **Balliet, J. W., D. L. Kolson, G. Eiger, F. M. Kim, K. A. McGann, A. Srinivasan, and R. Collman.** 1994. Distinct effects in primary macrophages and lymphocytes of the human immunodeficiency virus type 1 accessory genes vpr, vpu, and nef: mutational analysis of a primary HIV-1 isolate. Virology. **200:**623-31.

4. **Benito, J. M., J. M. Zabay, J. Gil, M. Bermejo, A. Escudero, E. Sánchez, and E. Fernández-Cruz.** 1997. Quantitative alterations of the functionally distinct subsets of CD4 and CD8 T lymphocytes in asymptomatic HIV infection: changes in the expression of CD45RO, CD45RA, CD11b, CD38, HLA-DR, and CD25 antigens. J. Acquir. Immune Defic. Syndr. Hum. Retrovirol. **14:**128-135.

5. **Bieniasz, P. D., R. A. Fridell, I. Aramori, S. S. Ferguson, M. G. Caron, and B. R. Cullen.** 1997. HIV-1-induced cell fusion is mediated by multiple regions within both the viral envelope and the CCR-5 co-receptor. EMBO J. **16:**2599-609.

6. **Bleul, C. C., L. Wu, J. A. Hoxie, T. A. Springer, and C. R. Mackay.** 1997. The HIV coreceptors CXCR4 and CCR5 are differentially expressed and regulated on human T lymphocytes [see comments]. Proc. Natl. Acad. Sci. USA. **94:**1925-30.

7. **Bouyac-Bertoia, M., J. D. Dvorin, R. A. Fouchier, Y. Jenkins, B. E. Meyer, L. I. Wu, M. Emerman, and M. H. Malim.** 2001. HIV-1 infection requires a functional integrase NLS. Mol. Cell. **7:**1025-35.

8. **Bukrinsky, M. I., S. Haggerty, M. P. Dempsey, N. Sharova, A. Adzhubel, L. Spitz, P. Lewis, D. Goldfarb, M. Emerman, and M. Stevenson.** 1993. A nuclear localization signal within HIV-1 matrix protein that governs infection of non-dividing cells. Nature. **365:**666-9.

9. **Bukrinsky, M. I., N. Sharova, M. P. Dempsey, T. L. Stanwick, A. G. Bukrinskaya, S. Haggerty, and M. Stevenson.** 1992. Active nuclear import of human immunodeficiency virus type 1 preintegration complexes. Proc. Natl. Acad. Sci. USA. **89:**6580-6584.

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

11. **Chesebro, B., K. Wehrly, J. Nishio, and S. Perryman.** 1996. Mapping of independent V3 envelope determinants of human immunodeficiency virus type 1 macrophage tropism and syncytium formation in lymphocytes. J. Virol. **70:**9055-9.

12. **Choe, H., M. Farzan, Y. Sun, N. Sullivan, B. Rollins, P. D. Ponath, L. Wu, C. R. Mackay, G. LaRosa, W. Newman, N. Gerard, C. Gerard, and J. Sodroski.** 1996. The beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. Cell. **85:**1135-48.

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

14. **Chun, T. W., L. Carruth, D. Finzi, X. Shen, J. A. DiGiuseppe, H. Taylor, M. Hermankova, K. Chadwick, J. Margolick, T. C. Quinn, Y. H. Kuo, R. Brookmeyer, M. A. Zeiger, P. Barditch-Crovo, and R. F. Siliciano.** 1997. Quantification of latent tissue reservoirs and total body viral load in HIV-1 infection. Nature. **387:**183-188.

15. **Chun, T. W., D. Engel, M. M. Berrey, T. Shea, L. Corey, and A. S. Fauci.** 1998. Early establishment of a pool of latently infected, resting CD4(+) T cells during primary HIV-1 infection. Proc. Natl. Acad. Sci. USA. **95:**8869-8873.

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

17. **Cocchi, F., A. L. DeVico, A. Garzino-Demo, A. Cara, R. C. Gallo, and P. Lusso.** 1996. The V3 domain of the HIV-1 gp120 envelope glycoprotein is critical for chemokine-mediated blockade of infection [see comments]. Nat. Med. **2:**1244-7.

18. **Connor, R. I., B. K. Chen, S. Choe, and N. R. Landau.** 1995. Vpr is required for efficient replication of human immunodeficiency virus type-1 in mononuclear phagocytes. Virology. **206:**935-44.

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

20. **Deng, H., R. Liu, W. Ellmeier, S. Choe, D. Unutmaz, M. Burkhart, P. Di Marzio, S. Marmon, R. E. Sutton, C. M. Hill, C. B. Davis, S. C. Peiper, T. J. Schall, D. R. Littman, and N. R. Landau.** 1996. Identification of a major co-receptor for primary isolates of HIV-1 [see comments]. Nature. **381:**661-6.

21. **Dragic, T., V. Litwin, G. P. Allaway, S. R. Martin, Y. Huang, K. A. Nagashima, C. Cayanan, P. J. Maddon, R. A. Koup, J. P. Moore, and W. A. Paxton.** 1996. HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5 [see comments]. Nature. **381:**667-73.

22. **Eckstein, D. A., M. L. Penn, Y. D. Korin, D. D. Scripture-Adams, J. A. Zack, J. F. Kreisberg, M. Roederer, M. P. Sherman, P. S. Chin, and M. A. Goldsmith.** 2001. HIV-1 actively replicates in naive CD4+ T-cells residing within human lymphoid tissue. Immunity. **15:**

23. **Endres, M. J., P. R. Clapham, M. Marsh, M. Ahuja, J. D. Turner, A. McKnight, J. F. Thomas, B. Stoebenau-Haggarty, S. Choe, P. J. Vance, T. N. Wells,**

C. A. Power, S. S. Sutterwala, R. W. Doms, N. R. Landau, and J. A. Hoxie. 1996. CD4-independent infection by HIV-2 is mediated by fusin/CXCR4. Cell. **87:**745-56.

24. **Feng, Y., C. C. Broder, P. E. Kennedy, and E. A. Berger.** 1996. HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor [see comments]. Science. **272:**872-7.

25. **Finzi, D., J. Blankson, J. D. Siliciano, J. B. Margolick, K. Chadwick, T. Pierson, K. Smith, J. Lisziewicz, F. Lori, C. Flexner, T. C. Quinn, R. E. Chaisson, E. Rosenberg, B. Walker, S. Gange, J. Gallant, and R. F. Siliciano.** 1999. Latent infection of CD4+ T cells provides a mechanism for lifelong persistence of HIV-1, even in patients on effective combination therapy. Nat. Med. **5:**512-7.

26. **Gallay, P., T. Hope, D. Chin, and D. Trono.** 1997. HIV-1 infection of nondividing cells through the recognition of integrase by the importin/karyopherin pathway. Proc. Natl. Acad. Sci. USA. **94:**9825-30.

27. **Gallay, P., V. Stitt, C. Mundy, M. Oettinger, and D. Trono.** 1996. Role of the karyopherin pathway in human immunodeficiency virus type 1 nuclear import. J. Virol. **70:**1027-32.

28. **Glushakova, S., B. Baibakov, L. B. Margolis, and J. Zimmerberg.** 1995. Infection of human tonsil histocultures: a model for HIV pathogenesis. Nat. Med. **1:**1320-2.

29. **Glushakova, S., B. Baibakov, J. Zimmerberg, and L. B. Margolis.** 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.

30. **Goh, W. C., M. E. Rogel, C. M. Kinsey, S. F. Michael, P. N. Fultz, M. A. Nowak, B. H. Hahn, and M. Emerman.** 1998. HIV-1 Vpr increases viral expression by manipulation of the cell cycle: a mechanism for selection of Vpr in vivo. Nat. Med. **4:**65-71.

31. **Goudsmit, J.** 1995. The role of viral diversity in HIV pathogenesis. J. Acquir. Immune Defic. Syndr. Hum. Retrovirol. **10 Suppl 1:**S15-9.

32. **Grivel, J. C., M. L. Penn, D. A. Eckstein, B. Schramm, R. F. Speck, N. W. Abbey, B. Herndier, L. Margolis, and M. A. Goldsmith.** 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.

33. **Gröttrup-Wolfers, E., R. Strzelecki, T. Grünewald, W. Schüler-Maue, and B. Ruf.** 1997. Enhanced expression of activation antigens HLA-DR and CD69 on gamma delta T cells but impaired interleukin-2-induced upregulation in HIV-1 infection [letter]. AIDS. **11:**838-9.

34. **Heinzinger, N. K., M. I. Bukinsky, S. A. Haggerty, A. M. Ragland, V. Kewalramani, M. A. Lee, H. E. Gendelman, L. Ratner, M. Stevenson, and M. Emerman.** 1994. The Vpr protein of human immunodeficiency virus type 1 influences

nuclear localization of viral nucleic acids in nondividing host cells. Proc. Natl. Acad. Sci. USA. **91:**7311-5.

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

36. **Henrard, D. R., E. Daar, H. Farzadegan, S. J. Clark, J. Phillips, G. M. Shaw, and M. P. Busch.** 1995. Virologic and immunologic characterization of symptomatic and asymptomatic primary HIV-1 infection. J. Acquir. Immune. Defic. Syndr. Hum. Retroviro. **9:**305-10.

37. **Hill, C. M., H. Deng, D. Unutmaz, V. N. Kewalramani, L. Bastiani, M. K. Gorny, S. Zolla-Pazner, and D. R. Littman.** 1997. Envelope glycoproteins from human immunodeficiency virus types 1 and 2 and simian immunodeficiency virus can use human CCR5 as a coreceptor for viral entry and make direct CD4-dependent interactions with this chemokine receptor. J. Virol. **71:**6296-304.

38. **Ho, D. D., A. U. Neumann, A. S. Perelson, W. Chen, J. M. Leonard, and M. Markowitz.** 1995. Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. Nature. **373:**123-6.

39. **Horsburgh, C. R., Jr., C. Y. Ou, J. Jason, S. D. Holmberg, I. M. Longini, Jr., C. Schable, K. H. Mayer, A. R. Lifson, G. Schochetman, J. W. Ward, and et al.** 1989. Duration of human immunodeficiency virus infection before detection of antibody. Lancet. **2:**637-40.

40. **Humphries, E. H., and H. M. Temin.** 1974. Requirement for cell division for initiation of transcription of Rous sarcoma virus RNA. J. Virol. **14:**531-46.

41. **Jenkins, Y., M. McEntee, K. Weis, and W. C. Greene.** 1998. Characterization of HIV-1 vpr nuclear import: analysis of signals and pathways. J. Cell Biol. **143:**875-85.

42. **Kestens, L., G. Vanham, C. Vereecken, M. Vandenbruaene, G. Vercauteren, R. L. Colebunders, and P. L. Gigase.** 1994. Selective increase of activation antigens HLA-DR and CD38 on CD4+ CD45RO+ T lymphocytes during HIV-1 infection. Clin. Exp. Immunol. **95:**436-41.

43. **Koot, M., I. P. Keet, A. H. Vos, R. E. de Goede, M. T. Roos, R. A. Coutinho, F. Miedema, P. T. Schellekens, and M. Tersmette.** 1993. Prognostic value of HIV-1 syncytium-inducing phenotype for rate of CD4+ cell depletion and progression to AIDS [see comments]. Ann. Intern. Med. **118:**681-8.

44. **Lewis, P. F., and M. Emerman.** 1994. Passage through mitosis is required for oncoretroviruses but not for the human immunodeficiency virus. J. Virol. **68:**510-6.

45. **Mahalingam, M., M. Peakman, E. T. Davies, A. Pozniak, T. J. McManus, and D. Vergani.** 1993. T cell activation and disease severity in HIV infection. Clin. Exp. Immunol. **93:**337-43.

46. **Miller, M. D., C. M. Farnet, and F. D. Bushman.** 1997. Human immunodeficiency virus type 1 preintegration complexes: studies of organization and composition. J. Virol. **71:**5382-90.

47. **Muñoz, A., M. C. Wang, S. Bass, J. M. Taylor, L. A. Kingsley, J. S. Chmiel, and B. F. Polk.** 1989. Acquired immunodeficiency syndrome (AIDS)-free time after human immunodeficiency virus type 1 (HIV-1) seroconversion in homosexual men. Multicenter AIDS Cohort Study Group. Am. J. Epidemiol. **130:**530-9.

48. **Niu, M. T., D. S. Stein, and S. M. Schnittman.** 1993. Primary human immunodeficiency virus type 1 infection: review of pathogenesis and early treatment intervention in humans and animal retrovirus infections. J. Infect. Dis. **168:**1490-501.

49. **Orendi, J. M., A. C. Bloem, J. C. Borleffs, F. J. Wijnholds, N. M. de Vos, H. S. Nottet, M. R. Visser, H. Snippe, J. Verhoef, and C. A. Boucher.** 1998. Activation and cell cycle antigens in CD4+ and CD8+ T cells correlate with plasma human immunodeficiency virus (HIV-1) RNA level in HIV-1 infection. J. Infect. Dis. **178:**1279-87.

50. **Ostrowski, M. A., T. W. Chun, S. J. Justement, I. Motola, M. A. Spinelli, J. Adelsberger, L. A. Ehler, S. B. Mizell, C. W. Hallahan, and A. S. Fauci.** 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.

51. **Pantaleo, G., C. Graziosi, J. F. Demarest, L. Butini, M. Montroni, C. H. Fox, J. M. Orenstein, D. P. Kotler, and A. S. Fauci.** 1993. HIV infection is active and progressive in lymphoid tissue during the clinically latent stage of disease [see comments]. Nature. **362:**355-8.

52. **Pantaleo, G., C. Graziosi, and A. S. Fauci.** 1997. Virologic and immunologic events in primary HIV infection. Springer Semin. Immunopathol. **18:**257-66.

53. **Penn, M. L., J. C. Grivel, B. Schramm, M. A. Goldsmith, and L. Margolis.** 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.

54. **Petit, C., O. Schwartz, and F. Mammano.** 2000. The karyophilic properties of human immunodeficiency virus type 1 integrase are not required for nuclear import of proviral DNA. J. Virol. **74:**7119-26.

55. **Pierson, T., T. L. Hoffman, J. Blankson, D. Finzi, K. Chadwick, J. B. Margolick, C. Buck, J. D. Siliciano, R. W. Doms, and R. F. Siliciano.** 2000. Characterization of chemokine receptor utilization of viruses in the latent reservoir for human immunodeficiency virus type 1. J.Virol. **74:**7824-33.

56. **Popov, S., M. Rexach, L. Ratner, G. Blobel, and M. Bukrinsky.** 1998. Viral protein R regulates docking of the HIV-1 preintegration complex to the nuclear pore complex. J. Biol. Chem. **273:**13347-52.

57. **Popov, S., M. Rexach, G. Zybarth, N. Reiling, M. A. Lee, L. Ratner, C. M. Lane, M. S. Moore, G. Blobel, and M. Bukrinsky.** 1998. Viral protein R regulates nuclear import of the HIV-1 pre-integration complex. EMBO J. **17:**909-17.

58. **Reddy, M. M., and M. H. Grieco.** 1991. Quantitative changes in T helper inducer (CD4+ CD45RA-), T suppressor inducer (CD4+ CD45RA+), T suppressor

(CD8+ CD11b+), and T cytotoxic (CD8+ CD11b-) subsets in human immunodeficiency virus infection. J. Clin. Lab. Anal. **5:**96-100.

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

60. **Rottman, J. B., K. P. Ganley, K. Williams, L. Wu, C. R. Mackay, and D. J. Ringler.** 1997. Cellular localization of the chemokine receptor CCR5. Correlation to cellular targets of HIV-1 infection. Am. J. Pathol. **151:**1341-51.

61. **Salazar-Gonzalez, J. F., O. Martinez-Maza, P. Nishanian, N. Aziz, L. P. Shen, S. Grosser, J. Taylor, R. Detels, and J. L. Fahey.** 1998. Increased immune activation precedes the inflection point of CD4 T cells and the increased serum virus load in human immunodeficiency virus infection. J. Infect. Dis. **178:**423-30.

62. **Scarlatti, G., E. Tresoldi, A. Björndal, R. Fredriksson, C. Colognesi, H. K. Deng, M. S. Malnati, A. Plebani, A. G. Siccardi, D. R. Littman, E. M. Fenyö, and P. Lusso.** 1997. In vivo evolution of HIV-1 co-receptor usage and sensitivity to chemokine-mediated suppression. Nat. Med. **3:**1259-65.

63. **Schacker, T. W., J. P. Hughes, T. Shea, R. W. Coombs, and L. Corey.** 1998. Biological and virologic characteristics of primary HIV infection [see comments]. Ann. Intern. Med. **128:**613-20.

64. **Schnittman, S. M., H. C. Lane, J. Greenhouse, J. S. Justement, M. Baseler, and A. S. Fauci.** 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.

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

66. **Schuitemaker, H., M. Koot, N. A. Kootstra, M. W. Dercksen, R. E. de Goede, R. P. van Steenwijk, J. M. Lange, J. K. Schattenkerk, F. Miedema, and M. Tersmette.** 1992. Biological phenotype of human immunodeficiency virus type 1 clones at different stages of infection: progression of disease is associated with a shift from monocytotropic to T-cell-tropic virus population. J. Virol. **66:**1354-60.

67. **Selik, R. M., H. W. Jaffe, S. L. Solomon, and J. W. Curran.** 1986. CDC's definition of AIDS. New England J. of Med. **315:**761.

68. **Shankarappa, R., J. B. Margolick, S. J. Gange, A. G. Rodrigo, D. Upchurch, H. Farzadegan, P. Gupta, C. R. Rinaldo, G. H. Learn, X. He, X. L. Huang, and J. I. Mullins.** 1999. Consistent viral evolutionary changes associated with the progression of human immunodeficiency virus type 1 infection. J. Virol. **73:**10489-502.

69. **Sherman, M. P., C. M. de Noronha, M. I. Heusch, S. Greene, and W. C. Greene.** 2001. Nucleocytoplasmic shuttling by human immunodeficiency virus type 1 Vpr. J. Virol. **75:**1522-32.

70. **Speck, R. F., K. Wehrly, E. J. Platt, R. E. Atchison, I. F. Charo, D. Kabat, B. Chesebro, and M. A. Goldsmith.** 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.

71. **Spina, C. A., J. C. Guatelli, and D. D. Richman.** 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.

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

73. **Tang, S., B. Patterson, and J. A. Levy.** 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.

74. **Tersmette, M., R. A. Gruters, F. de Wolf, R. E. de Goede, J. M. Lange, P. T. Schellekens, J. Goudsmit, H. G. Huisman, and F. Miedema.** 1989. Evidence for a role of virulent human immunodeficiency virus (HIV) variants in the pathogenesis of acquired immunodeficiency syndrome: studies on sequential HIV isolates. J. Virol. **63:**2118-25.

75. **Tersmette, M., J. M. Lange, R. E. de Goede, F. de Wolf, J. K. Eeftink-Schattenkerk, P. T. Schellekens, R. A. Coutinho, J. G. Huisman, J. Goudsmit, and F. Miedema.** 1989. Association between biological properties of human immunodeficiency virus variants and risk for AIDS and AIDS mortality. Lancet. **1:**983-5.

76. **Toohey, K., K. Wehrly, J. Nishio, S. Perryman, and B. Chesebro.** 1995. Human immunodeficiency virus envelope V1 and V2 regions influence replication efficiency in macrophages by affecting virus spread. Virology. **213:**70-9.

77. **Veazey, R. S., I. C. Tham, K. G. Mansfield, M. DeMaria, A. E. Forand, D. E. Shvetz, L. V. Chalifoux, P. K. Sehgal, and A. A. Lackner.** 2000. Identifying the target cell in primary simian immunodeficiency virus (SIV) infection: highly activated memory CD4(+) T cells are rapidly eliminated in early SIV infection in vivo. J. Virol. **74:**57-64.

78. **Vodicka, M. A., D. M. Koepp, P. A. Silver, and M. Emerman.** 1998. HIV-1 Vpr interacts with the nuclear transport pathway to promote macrophage infection. Genes Dev. **12:**175-85.

79. **von Schwedler, U., R. S. Kornbluth, and D. Trono.** 1994. The nuclear localization signal of the matrix protein of human immunodeficiency virus type 1 allows the establishment of infection in macrophages and quiescent T lymphocytes. Proc. Natl. Acad. Sci. USA. **91:**6992-6.

80. **Wei, X., S. K. Ghosh, M. E. Taylor, V. A. Johnson, E. A. Emini, P. Deutsch, J. D. Lifson, S. Bonhoeffer, M. A. Nowak, B. H. Hahn, and et al.** 1995. Viral dynamics in human immunodeficiency virus type 1 infection. Nature. **373:**117-22.

81. **Woods, T. C., B. D. Roberts, S. T. Butera, and T. M. Folks.** 1997. Loss of inducible virus in CD45RA naive cells after human immunodeficiency virus-1 entry accounts for preferential viral replication in CD45RO memory cells. Blood. **89:**1635-1641.

82. **Wu, L., W. A. Paxton, N. Kassam, N. Ruffing, J. B. Rottman, N. Sullivan, H. Choe, J. Sodroski, W. Newman, R. A. Koup, and C. R. Mackay.** 1997. CCR5 levels and expression pattern correlate with infectability by macrophage-tropic HIV-1, in vitro. J. Exp. Med. **185:**1681-91.

83. **Xiao, L., D. L. Rudolph, S. M. Owen, T. J. Spira, and R. B. Lal.** 1998. Adaptation to promiscuous usage of CC and CXC-chemokine coreceptors in vivo correlates with HIV-1 disease progression. AIDS. **12:**F137-43.

84. **Yedavalli, V. R., C. Chappey, and N. Ahmad.** 1998. Maintenance of an intact human immunodeficiency virus type 1 vpr gene following mother-to-infant transmission. J. Virol. **72:**6937-43.

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

86. **Zack, J. A., A. M. Haislip, P. Krogstad, and I. S. Chen.** 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.

87. **Zennou, V., C. Petit, D. Guetard, U. Nerhbass, L. Montagnier, and P. Charneau.** 2000. HIV-1 genome nuclear import is mediated by a central DNA flap. Cell. **101:**173-85.

88. **Zhang, L., B. Ramratnam, K. Tenner-Racz, Y. He, M. Vesanen, S. Lewin, A. Talal, P. Racz, A. S. Perelson, B. T. Korber, M. Markowitz, and D. D. Ho.** 1999.

Quantifying residual HIV-1 replication in patients receiving combination antiretroviral therapy. N. Engl. J. Med. **340:**1605-13.

89. **Zhang, Z., T. Schuler, M. Zupancic, S. Wietgrefe, K. A. Staskus, K. A. Reimann, T. A. Reinhart, M. Rogan, W. Cavert, C. J. Miller, R. S. Veazey, D. Notermans, S. Little, S. A. Danner, D. D. Richman, D. Havlir, J. Wong, H. L. Jordan, T. W. Schacker, P. Racz, K. Tenner-Racz, N. L. Letvin, S. Wolinsky, and A. T. Haase.** 1999. Sexual transmission and propagation of SIV and HIV in resting and activated CD4+ T cells. Science. **286:**1353-1357.

Chapter 2

Human Immunodeficiency Virus Type 1 Coreceptor Preferences Determine Target T-cell Depletion and Cellular Tropism in Human Lymphoid Tissue

Prologue

Our laboratory had previously demonstrated that viruses employing CCR5 deplete T-cells only mildly, whereas X4 viruses deplete T-cells massively. Moreover, R5 viruses replicate to a level comparable to that of X4 viruses despite that T-cells displaying CCR5 represent only a small fraction of all T-cells. The studies in this chapter sought to determine the role of coreceptor usage in target cell cytopathicity and cell tropism. We found that R5 viruses resulted in the marked depletion principally of T-cells expressing detectable levels of CCR5. We also found that R5 viruses productively infect both a subset of T-cells as well as tissue macrophages, and we infer that the combined output of these viral reservoirs accounts for the surprisingly high replication of R5 viruses. We conclude that cell tropism and cytopathicity is regulated at the level of *env* expression.

The experiments presented in this chapter were principally conducted by M. Penn, who shared first-authorship with J.C. Grivel. B. Schramm and R. Speck made intellectual contributions to the work. N. Abbey and B. Herndier performed the immunohistochemical studies presented in Figure 4A and B. I developed the flow-cytometric assay to monitor productive infection of tissue macrophages and generated the data presented in Figure 4C. L. Margolis and M. Goldsmith jointly supervised this work. This chapter was published in the *Journal of Virology* and is reproduced here with permission from the American Society for Microbiology.

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NOTES

Human Immunodeficiency Virus Type 1 Coreceptor Preferences Determine Target T-Cell Depletion and Cellular Tropism in Human Lymphoid Tissue

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The present study sought to determine how usage of coreceptors by human immunodeficiency virus type 1 dictates cell tropism and depletion of CD4$^+$ T cells in human lymphoid tissues cultured ex vivo. We found that coreceptor preferences control the marked, preferential depletion of coreceptor-expressing CD4$^+$ lymphocytes. In addition, there was a strong, but not absolute, preference shown by CXCR4-using strains for lymphocytes and by CCR5-using strains for macrophages.

The hallmark of human immunodeficiency virus type 1 (HIV-1) disease is the progressive depletion of CD4$^+$ lymphocytes. Different strains of HIV vary with respect to their target cell range and cytopathic potential. The molecular basis for differential cell tropism and virulence remained obscure until the discovery of select chemokine receptors that act as essential cofactors for cellular entry by HIV-1 (1). We previously reported that HIV-1 envelope glycoprotein (gp120) determinants controlling a preference for CXCR4 resulted in marked depletion of CD4$^+$ T cells in human lymphoid histocultures, while those specifying a preference for CCR5 resulted in only mild depletion of such cells. These results suggested that either X4 viruses are intrinsically more cytopathic than R5 viruses or viruses with different coreceptor specificities target quantitatively distinct CD4$^+$ T-cell pools. Our earlier study established that R5 HIV-1 variants exclusively deplete CCR5-expressing CD4$^+$ lymphocytes, while X4 HIV-1 variants preferentially deplete CXCR4-expressing cells (5). However, the diverse HIV-1 isolates used in this work differed from each other by many parameters other than coreceptor usage that could influence cytopathicity.

The present study sought to establish a specific causative relationship among coreceptor usage, tropism, and CD4$^+$ T-cell depletion in mature lymphoid tissue. Human tonsil histocultures were inoculated with pairs of recombinant strains of HIV-1 that differ exclusively in small regions of gp120 that control coreceptor preference. Three pairs of viruses based on an isogenic (NL4-3) viral backbone were studied: (i) NL4-3 (X4) and 49-5 (R5), virus chimeras that differ only in the gp120 V3 loop region that specifies strict reciprocal tropism for CXCR4 and CCR5, respectively (9, 12, 13); (ii) 134 (X4) and 126 (R5), site-directed mutants that differ in a single V3 amino acid residue that likewise dictates preference for CXCR4 or CCR5, respectively (3, 12); and 123 (X4) and USV3 (R5), chimeras that contain V3 loop segments derived from primary X4 and R5 viral isolates (references 3 and 12 and unpublished data).

T-cell depletion and viral replication were measured 12 to 15 days following inoculation as described previously (4). Consistent with our earlier report (9), NL4-3 (X4) severely depleted these cultures of CD4$^+$ T cells, while the paired virus 49-5 (R5) depleted these cells only mildly (Fig. 1A). Recombinant strain 134 (X4) also severely depleted these cells, while its paired strain, 126 (R5), which differs by a single amino acid within the V3 loop region, mildly depleted these cells (Fig. 1A). Viruses 123 and USV3, which encode V3 loop segments derived from primary viral isolates, likewise depleted CD4$^+$ T cells according to coreceptor usage (Fig. 1A). These results confirm and extend previous observations by demonstrating that sequences within the V3 loop that control coreceptor preference dictate severe or mild CD4$^+$ lymphocyte depletion. We have shown previously (9) and verified here for each virus pair (see Fig. 3) (data not shown) that the differential depletion effects occur despite comparable viral replication kinetics.

To test the hypothesis that target cell availability influences the magnitude of cellular depletion by each viral strain, we used flow cytometry to determine the relative prevalence of potential target cells as defined by CXCR4 and CCR5 expression. CXCR4 was expressed on the overwhelming majority of CD4$^+$ T cells in resting tissues (mean, 88.5% ± 1.6%, n = 25), whereas CCR5 expression was restricted to a much smaller subset of these cells (mean, 10.4% ± 0.8%, n = 25) (reference 5 and data not shown). We determined whether these expression profiles are linked to preferential depletion by inoculating

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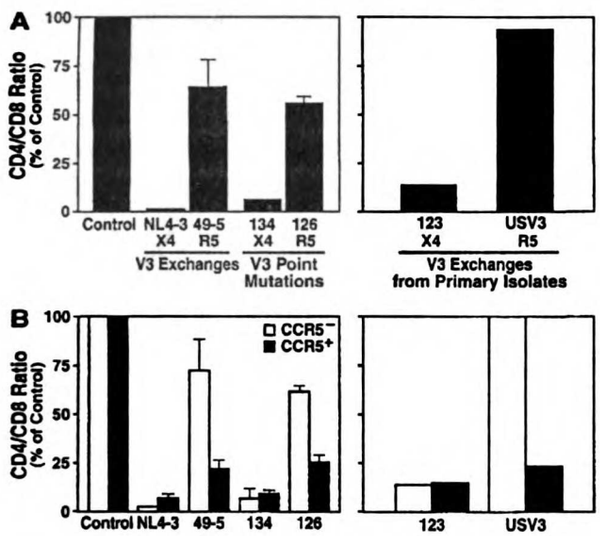

FIG. 1. CD4⁺ T-cell depletion in human tonsil histocultures infected ex vivo by matched HIV-1 strains. (A) Left: CD4⁺ T-cell depletion as indicated by mean relative CD4/CD8 ratio on day 15 after infection by recombinant viruses NL4-3, 49-5, 134, and 126. For each data point, cells were pooled from 6 to 10 tissue blocks (mean plus standard error of the mean, $n = 3$) and analyzed by flow cytometry. Right: CD4 depletion on day 13 by viruses 123 and USV3, which were tested in a separate experiment. Presented are data from typical experiments with tissues from two to five donors. (B) CD4⁺ T-cell subset depletion data as indicated by the relative CD4/CD8 ratio for the CCR5⁻ or CCR5⁺ T-cell subsets in the tissue samples presented in panel A.

histocultures with viruses that vary only in coreceptor phenotype. Since CXCR4 is widely expressed on both CCR5⁺ and CCR5⁻ cells, our hypothesis predicts that X4 viruses would deplete cells in both subsets, whereas R5 viruses would deplete preferentially within the CCR5⁺ subset. We therefore focused our analysis on quantitation of the CCR5⁺ and CCR5⁻ subsets of CD4⁺ lymphocytes following infection. Indeed, all three of the X4 viruses (NL4-3, 134, and 123) massively and comparably depleted both CCR5⁻ and CCR5⁺ cells, while the R5 viruses (49-5, 126, and USV3) caused depletion preferentially within the CCR5⁺ subset and comparatively modest depletion within the CCR5⁻ subset (Fig. 1B). We speculate, but cannot prove, that the partial depletion of CCR5⁻ cells by R5 viruses is explained by subthreshold levels of CCR5 expression on some cells, since previous work established that certain CD4⁺ lymphocytes that do not express CCR5 at levels detectable by flow cytometric methods were infectable by R5 viruses (11). Nonetheless, in all of our experiments, depletion within the CCR5⁺ subset by R5 viruses exceeded that in the CCR5⁻ subset by twofold or more. These results together demonstrate that envelope-determined coreceptor preferences direct the selective depletion of cognate coreceptor-expressing CD4⁺ lymphocytes in human lymphoid histocultures.

Despite a marked difference in the frequencies of potential cell targets for X4 and R5 viruses and the selective depletion of targets by each virus type, the replication kinetics of these viruses based on virus production were quite similar. Although the absolute levels of virus replication varied among individual tissue donors, there were no consistent differences in the peak levels of viral replication or the kinetics of virus accumulation in the culture medium upon inoculation of any particular tissue specimen by pairs of X4 and R5 strains. To elucidate further the relationship between virus-induced CD4⁺ T-cell depletion and virus replication, we performed concurrent kinetic measurements of these parameters in histocultures infected by NL4-3 or 49-5. In a typical experiment, NL4-3 progressively depressed the overall CD4/CD8 ratio with early effects evident by day 3 and progressive effects on days 6 and 12 (Fig. 2A, left). In contrast, the effect of 49-5 on CD4/CD8 ratio was very modest at all time points (Fig. 2A, right).

Stratification by CCR5 expression revealed a more complex pattern. In NL4-3-infected cultures both CCR5⁻ (Fig. 2B, left) and CCR5⁺ (Fig. 2C, right) lymphocytes were lost markedly and progressively over the 12-day period with kinetics that paralleled the overall depletion of CD4⁺ T cells (Fig. 1A). In contrast, 49-5 had minimal effects on the CCR5⁻ population (Fig. 2B, right) but severely and progressively depleted CCR5⁺ cells (Fig. 2C, right). In each of these cases, the kinetics of depletion of CD4⁺ T cells overall corresponded to the kinetics of depletion within the CCR5⁻ population, which reflects the small contribution of the minor CCR5⁺ pool. These kinetic analyses provide further evidence that R5 viruses deplete T cells in a coreceptor-dependent process leading to preferential loss of CCR5⁺ cells. It should be noted that, in some experiments with 49-5, partial loss of CCR5⁺ cells was detected by

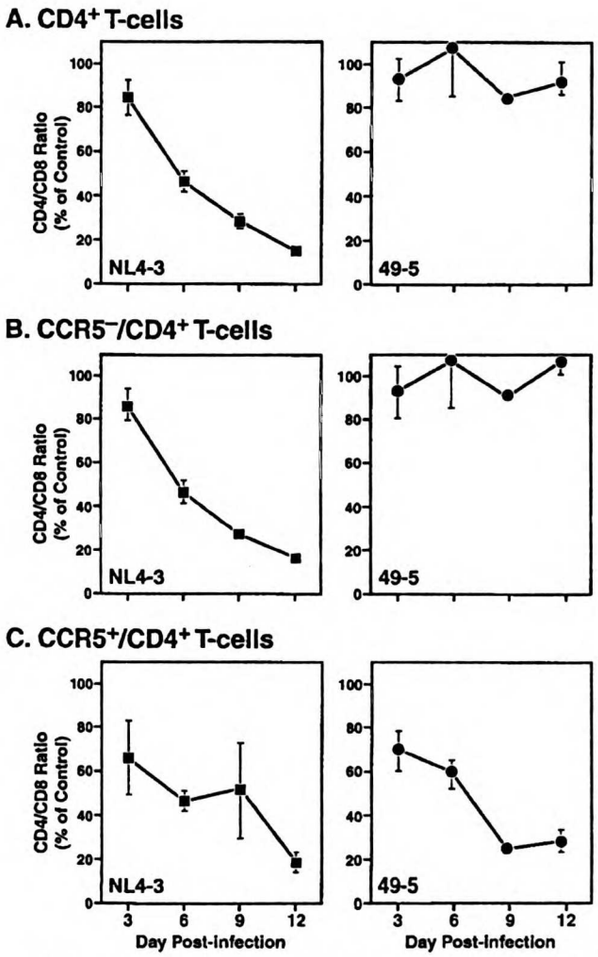

FIG. 2. Kinetic analysis of overall and subset-specific T-cell depletion in human tonsil histocultures in NL4-3- and 49-5-infected tonsillar tissue (left and right panels, respectively). Mean values (± standard errors of the means) from a representative experiment ($n = 3$) are shown.

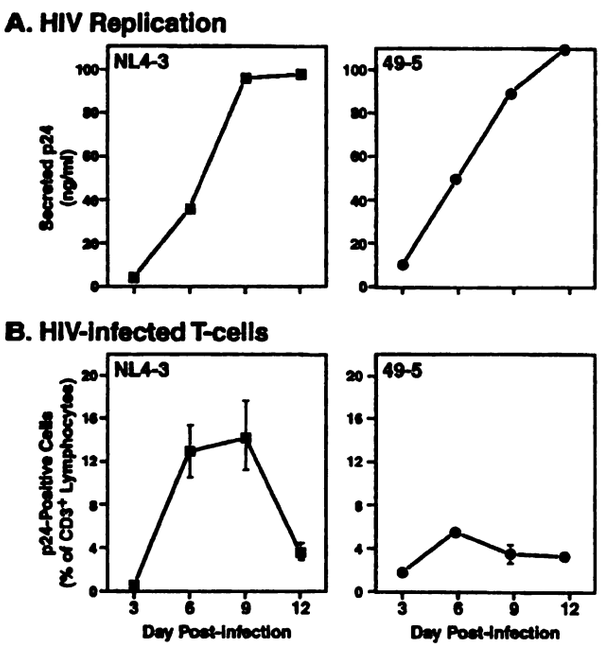

FIG. 3. Kinetic analysis of HIV-1 replication and lymphocyte infection in human tonsil histocultures. (A) HIV-1 p24 concentration in the culture medium of NL4-3- and 49-5-infected histocultures (Fig. 2) and sampled at various time points. (B) Frequencies of productively infected lymphocytes in these cultures as demonstrated by flow cytometry of cells isolated from infected tissues and co-immunostained with MAbs recognizing CD3 and p24.

day 3 without further loss on subsequent days (data not shown). This donor-specific effect suggests that under some conditions CCR5 expression is necessary but not sufficient for $CD4^+$ T-cell depletion by R5 viruses and that cellular properties other than coreceptor expression may also influence susceptibility to depletion.

In view of the coreceptor-dependent depletion of major and minor T-cell subsets by X4 and R5 strains, respectively, the relationship among cellular coreceptor expression, productive viral infection, and cell killing is not immediately evident. In particular, R5 and X4 viruses differentially depleted $CD4^+$ T cells yet exhibited similar replication profiles. Also, we previously found that the frequencies of productively infected lymphocytes were not consistently different in X4 and R5 virus-infected cultures as assessed at day 12 postinfection (9). To clarify this apparent discrepancy, we concurrently examined viral output and infected T-cell frequencies in infected cultures. As described earlier, in a typical experiment NL4-3- and 49-5-infected tonsil histocultures produced nearly identical amounts of virus with very similar kinetics as assessed by the p24 content of culture supernatants (Fig. 3A). To measure the frequency of infected cells in these cultures, cells were harvested at various time points, coimmunostained with monoclonal antibodies (MAbs) to CD3 and p24, and analyzed by flow cytometry. By this measurement, at days 6 to 9 postinfection, NL4-3 productively infected a relatively large proportion of the T cells, reaching approximately 15% of total $CD3^+$ lymphocytes and declining by the end of the experiment (Fig. 3B). In contrast, 49-5 infected fewer T cells, reaching approximately 5% of $CD3^+$ cells (Fig. 3B).

The difference in T-cell infection efficiency exhibited by these two viruses corresponds to the more aggressive depletion effect of X4 viruses compared to that of R5 viruses, but it also represents a paradox in the context of their very similar viral output profiles (Fig. 3A). One hypothesis to account for this paradox is that macrophages constitute an additional source of virus output that is not reflected in the analysis of infected T cells. We therefore used immunohistochemistry to visualize the full spectrum of infected cells in these histocultures. Immunostaining of formalin-fixed tonsil sections for the macrophage-specific antigen CD68 (6) revealed large numbers of macrophages distributed throughout the tissue (reference 4 and data not shown). Immunostaining for p24 revealed striking differences between the X4 and R5 virus-infected cultures. NL4-3-infected tissue demonstrated a predominance of p24-positive cells that appeared to be lymphocytes based on their small size and high nucleus/cytoplasm ratio (Fig. 4A, left, and 4B). In contrast, in 49-5-infected tissue many of the p24-positive cells appeared to be macrophages, based on their large size and abundant cytoplasm (Fig. 4A, right). Interestingly, some of these macrophage-like cells were also p24-positive in the NL4-3-infected cultures, and these were distinguishable as moderately and intensely stained cells. Likewise, a small number of intensely staining p24-positive lymphocytes was observed in the 49-5-infected samples. To compare target cell frequencies quantitatively, p24-positive lymphocytes and macrophage-like cells in the two cultures were counted by visual inspection (Fig. 4B), which confirmed the strong but not absolute preference of an X4 strain for lymphocytes and of an R5 strain for macrophage-like cells. Thus, as analyzed by immunohistochemistry, productive infection of both lymphocytes and macrophage-like cells appears to contribute to the total output of virus in HIV-1-infected histocultures.

We also developed a flow cytometric approach to identify and quantify macrophage-like cell types in HIV-infected lymphoid tissues. Macrophages were identified by immunostaining cells dispersed from histocultures for markers that distinguish T cells (CD3) from macrophages (CD14 and CD68). Cytospin and transmission electron microscopic analysis of cells sorted by positive staining for these markers validated this separation technique (data not shown). To analyze infection in this population, cells from infected tonsil histocultures were also immunostained for viral p24. These experiments demonstrated that the R5 virus infected a significantly higher proportion of macrophages in these tissues than did the matched X4 virus (Fig. 4C); similar results were obtained using two other distinct pairs of isogenic X4 and R5 viruses (data not shown). These results confirm that macrophages serve as significant cellular hosts for productive infection by R5 viruses, presumably contributing to the total viral output. The relative contribution macrophages make to replication of X4 viruses in these lymphoid tissues appears to be smaller (Fig. 4). Additionally, other cells not identified in this analysis (e.g., dendritic cells) may also contribute to total viral output in tonsil histocultures.

In the present studies of matched recombinant viruses, we confirmed that X4 HIV-1 strains are more pathogenic toward the overall $CD4^+$ T-cell population than are isogenic R5 strains. We also verified that CXCR4 is expressed very widely among $CD4^+$ T cells in these lymphoid cultures, while CCR5 is expressed on fewer cells. Correspondingly, all X4 strains depleted $CD4^+$ T cells broadly in these experiments, while their matched R5 counterparts potently and preferentially depleted $CD4^+$ T cells within the smaller $CCR5^+$ pool. Additionally, the overall frequency of productively infected lymphocytes in X4 HIV-1-infected tissues is higher than in R5 HIV-1-infected tissues, suggesting that the measured frequencies of productively infected lymphocytes are proportional and related to the cumulative attrition of infected cells. These exper-

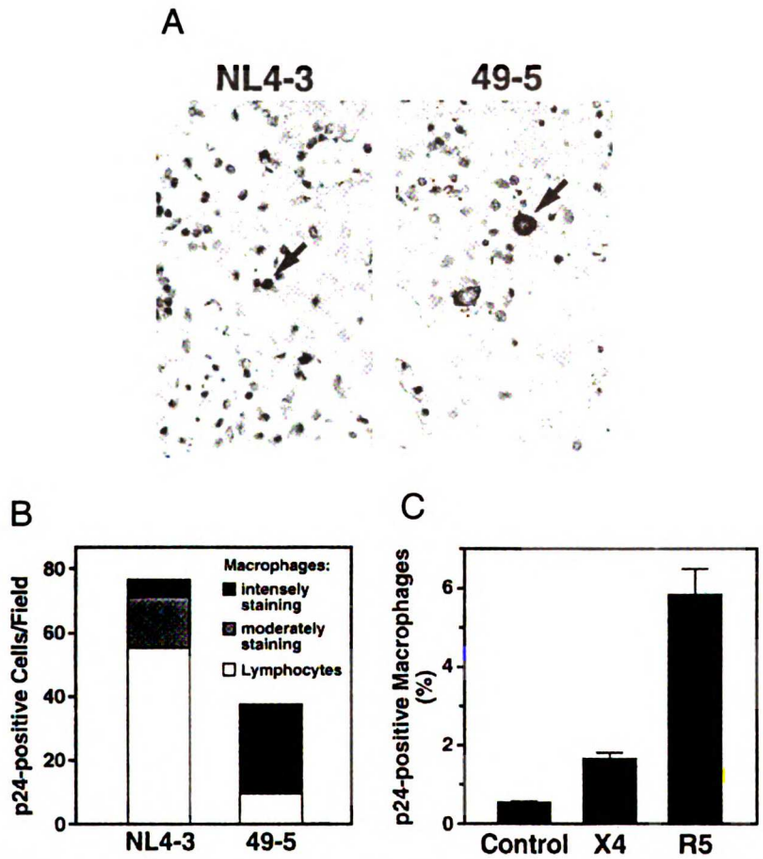

FIG. 4. Immunohistochemical and flow cytometric detection of intracellular HIV-1 p24 in infected tonsil histocultures. (A) Representative examples of p24-positive lymphocytes and macrophages in tonsil tissue infected ex vivo with NL4-3 and 49-5. Arrows indicate a typical lymphocyte (left) or macrophage (right). (B) Quantitation by counting p24-positive cells in these NL4-3- and 49-5-infected tissue sections that have comparable cross-sectional areas. (C) Frequencies of productively infected macrophages following a matched inoculation by a pair of isogenic viruses, NL4-3 (X4) and NL107 (R5). On day 9, cultures were coimmunostained with MAbs recognizing CD14 (phycoerythrin conjugated), CD68 (phycoerythrin conjugated), and p24 (fluorescein-isothiocyanate conjugated). NL107 is isogenic with NL4-3 but harbors the V1-V3 loop gp120 segments from BaL in the backbone of NL4-3 (13). Results are means of triplicate samples (plus standard errors of the means).

iments strongly support the hypothesis that X4 viruses infect lymphocytes at high frequencies and exhibit high overall virulence because CXCR4-expressing targets are abundant, while R5 viruses are also pathogenic for cell targets but exhibit lower overall virulence because CCR5-expressing cells are much less abundant. Furthermore, they prove that sequences in gp120 controlling coreceptor specificity alone are sufficient to determine which subset of $CD4^+$ T cells is depleted by each virus.

These principles should be relevant for other lymphoid organs as well (4, 9). Moreover, no consistent differences were observed between pathologic tonsil specimens and nonpathologic spleen specimens in the expression of various common markers of activation (data not shown), suggesting that a particular inflammatory state is not required for these viral properties to be manifest. In addition, CXCR4 and CCR5 expression patterns were comparable in these tissues (data not shown). However, there may be differences in relative activation status and/or coreceptor expression in other lymphoid tissues not examined here, such as gut-associated lymphoid tissue or thymus tissue. It is reasonable to expect that in these tissue contexts as well, the level of pathogenicity demonstrated by X4 and R5 viruses would be governed by the relative expression levels of CXCR4 and CCR5. For example, the level of CCR5 is significantly higher in gut-associated lymphoid tissue than in peripheral blood (7), and this tissue in rhesus monkeys was found to be highly susceptible to the pathogenic effects of an R5 simian immunodeficiency virus strain (14). In contrast, CCR5 expression in the human thymus is very low, and R5 viruses replicate poorly in this tissue and cause minimal cytopathic effects (2).

Despite differences in the pattern of selective CD4 depletion, X4 and R5 strains produced comparable amounts of virus with similar kinetics, in contrast to other reports based on xenotransplant model systems (2, 8, 10). The presence of significant numbers of tissue macrophages and dendritic cells is one feature that distinguishes the histoculture model (4). Indeed, we detected large, p24-positive macrophage-like cells in both R5- and X4-infected tissues by immunohistochemistry and flow cytometry. It is possible that this p24 staining represented cells that had endocytosed infected lymphocytes rather than those directly infected by HIV-1. By this interpretation, one would expect a higher proportion of p24-positive macrophage-like cells in X4-infected cultures, given the higher level of infection of T cells by these strains. However, a larger proportion of macrophage-like cells were found to be p24-positive in R5-infected cultures than in X4-infected cultures, making it likely that the majority of large, p24-positive cells represent direct and productive infection. Thus, macrophages

may contribute to viral output for both virus types but make a greater contribution for R5 strains. Therefore, the combined viral output from infected T cells, macrophages, and possibly other related cell types not identified in these analyses could account for the overall similarities in replication kinetics. Unfortunately, current technology has not yet permitted a robust and direct determination of the actual sources of virus production in these histocultures. Nonetheless, one interesting speculation is that the relative contribution of cell types other than $CD4^+$ T cells (e.g., macrophages) to viral load may increase over time in conjunction with progressive loss of specific T cells that are susceptible to the cytopathic effects of HIV-1.

These data provide compelling reasons to continue anti-HIV-1 therapeutic efforts aimed at developing antagonists to both CCR5 and CXCR4. Because macrophages can be infected detectably by both X4 and R5 strains, such antagonists may be effective not only in preventing CD4 depletion but also in limiting viral replication in alternative non-T-cell reservoirs.

J.-C.G. and M.L.P. contributed equally to this work.

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REFERENCES

1. **Berger, E. A., P. M. Murphy, and J. M. Farber.** 1999. Chemokine receptors as HIV-1 coreceptors: roles in viral entry, tropism, and disease. Annu. Rev. Immunol. **17**:657–700.
2. **Berkowitz, R. D., S. Alexander, C. Bare, V. Linquist-Stepps, M. Bogan, M. E. Moreno, L. Gibson, E. D. Wieder, J. Kosek, C. A. Stoddart, and J. M. McCune.** 1998. CCR5- and CXCR4-utilizing strains of human immunodeficiency virus type 1 exhibit differential tropism and pathogenesis in vivo. J. Virol. **72**:10108–10117.
3. **Chesebro, B., K. Wehrly, J. Nishio, and S. Perryman.** 1996. Mapping of independent V3 envelope determinants of human immunodeficiency virus type 1 macrophage tropism and syncytium formation in lymphocytes. J. Virol. **70**:9055–9059.
4. **Glushakova, S., B. Baibakov, J. Zimmerberg, and L. B. Margolis.** 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. Retrovir. **13**:461–471.
5. **Grivel, J.-C., and L. B. Margolis.** 1999. CCR5- and CXCR4-tropic HIV-1 are equally cytopathic for their T-cell targets in human lymphoid tissue. Nat. Med. **5**:344–346.
6. **Herndier, B. G., A. Werner, P. Arnstein, N. W. Abbey, F. Demartis, R. L. Cohen, M. A. Shuman, and J. A. Levy.** 1994. Characterization of a human Kaposi's sarcoma cell line that induces angiogenic tumors in animals. AIDS **8**:575–581.
7. **Lapenta, C., M. Boirivant, M. Marini, S. M. Santini, M. Logozzi, M. Viora, F. Belardelli, and S. Fais.** 1999. Human intestinal lamina propria lymphocytes are naturally permissive to HIV-1 infection. Eur. J. Immunol. **29**:1202–1208.
8. **Mosier, D. E.** 1996. Human immunodeficiency virus infection of human cells transplanted to severe combined immunodeficient mice. Adv. Immunol. **63**:79–125.
9. **Penn, M. L., J. C. Grivel, B. Schramm, M. A. Goldsmith, and L. Margolis.** 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.
10. **Picchio, G. R., R. J. Gulizia, K. Wehrly, B. Chesebro, and D. E. Mosier.** 1998. The cell tropism of human immunodeficiency virus type 1 determines the kinetics of plasma viremia in SCID mice reconstituted with human peripheral blood leukocytes. J. Virol. **72**:2002–2009.
11. **Riley, J. L., B. L. Levine, N. Craighead, T. Francomano, D. Kim, R. G. Carroll, and C. H. June.** 1998. Naïve and memory CD4 T cells differ in their susceptibilities to human immunodeficiency virus type 1 infection following CD28 costimulation: implications for transmission and pathogenesis. J. Virol. **72**:8273–8280.
12. **Speck, R. F., K. Wehrly, E. J. Platt, R. E. Atchison, I. F. Charo, D. Kabat, B. Chesebro, and M. A. Goldsmith.** 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.
13. **Toohey, K., K. Wehrly, J. Nishio, S. Perryman, and B. Chesebro.** 1995. Human immunodeficiency virus envelope V1 and V2 regions influence replication efficiency in macrophages by affecting virus spread. Virology **213**: 70–79.
14. **Veazey, R. S., M. DeMaria, L. V. Chalifoux, D. E. Shvetz, D. R. Pauley, H. L. Knight, M. Rosenzweig, R. P. Johnson, R. C. Desrosiers, and A. A. Lackner.** 1998. Gastrointestinal tract as a major site of CD4+ T cell depletion and viral replication in SIV infection. Science **280**:427–431.

Chapter 3

HIV-1 Actively Replicates in Naïve CD4$^+$ T-cells Residing within Human Lymphoid Tissue

Prologue

A paradoxical finding in HIV-1 biology is the identification of productively infected naïve $CD4^+$ T-cells *in vivo* despite that these cells are refractory to infection *in vitro*. Thus, the mechanism by which these cells are generated within infected individuals is not known. Moreover, the importance of these cells to the pathogenesis of HIV and immune collapse is also unknown. We therefore investigated the role of lymphoid tissue in mediating the infection of these cells. In a comprehensive analysis based on detailed examination of cellular characteristics, lymphoid trafficking, and cellular proliferation, we found that all conventional subsets of T-cells are permissive for infection when present within this microenvironment. In particular, non-dividing, naïve cells support *de novo* infection by X4 HIV. These cells are also sensitive to the cytopathic effects of the virus and are extensively depleted, which may prevent the generation of novel immune responses *in vivo*. Therefore, these cells should not be considered mere reservoirs for latent virus, but rather are permissive targets of HIV-1. Replication within these cells augments local viral burden, and their destruction invariably contributes to the collapse of the immune system within infected individuals.

These studies were performed in close collaboration with co-first author, M. Penn. He contributed data to Figures 1, 3, and 4. In addition, he also contributed invaluable intellectual insight and wrote a draft of the manuscript at a much earlier time when the work was formative. Y. Korin, D. Scripture-Adams, and J. Zack generated the data

presented in Figure 5. J. Kreisberg and M. Roederer made important intellectual contributions. M. Sherman provided important technical assistance and advice, and P. Chin provided valuable experimental assistance with this work. I designed and performed the studies presented in Figures 1A, 2, 3C, 4B, 6, and 7, and wrote a new manuscript (presented here) that incorporated the earlier work. M. Goldsmith supervised this work. This chapter was published in *Immunity* and is reproduced here with permission from Elsevier Science.

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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

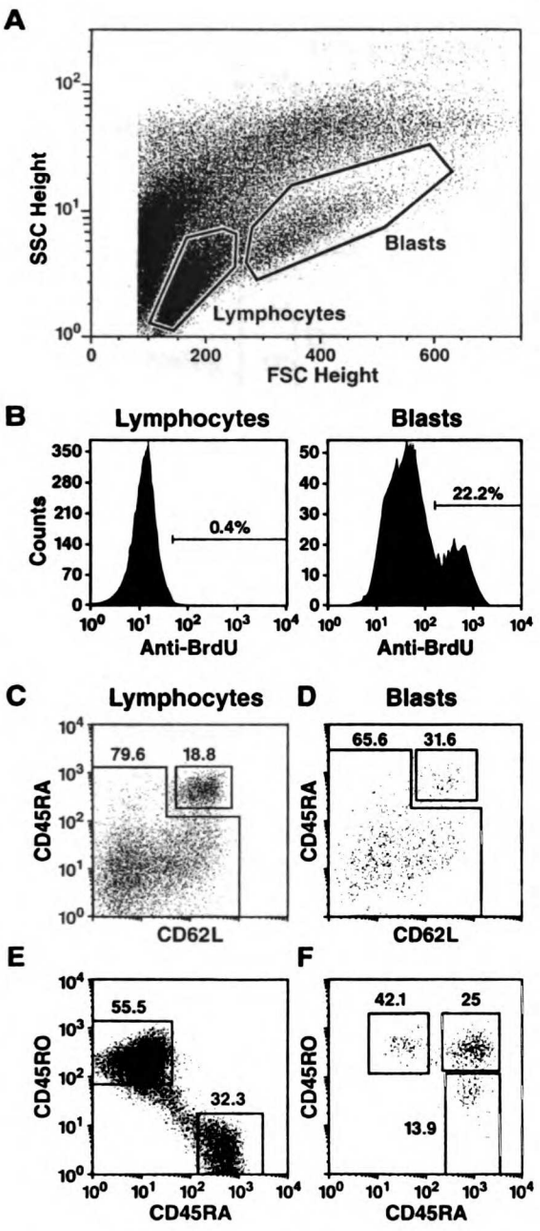

Figure 1. Flow Cytometric Analysis of Lymphocytes and Blasts within Human Lymphoid Histocultures Immediately following Tissue Procurement

(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).

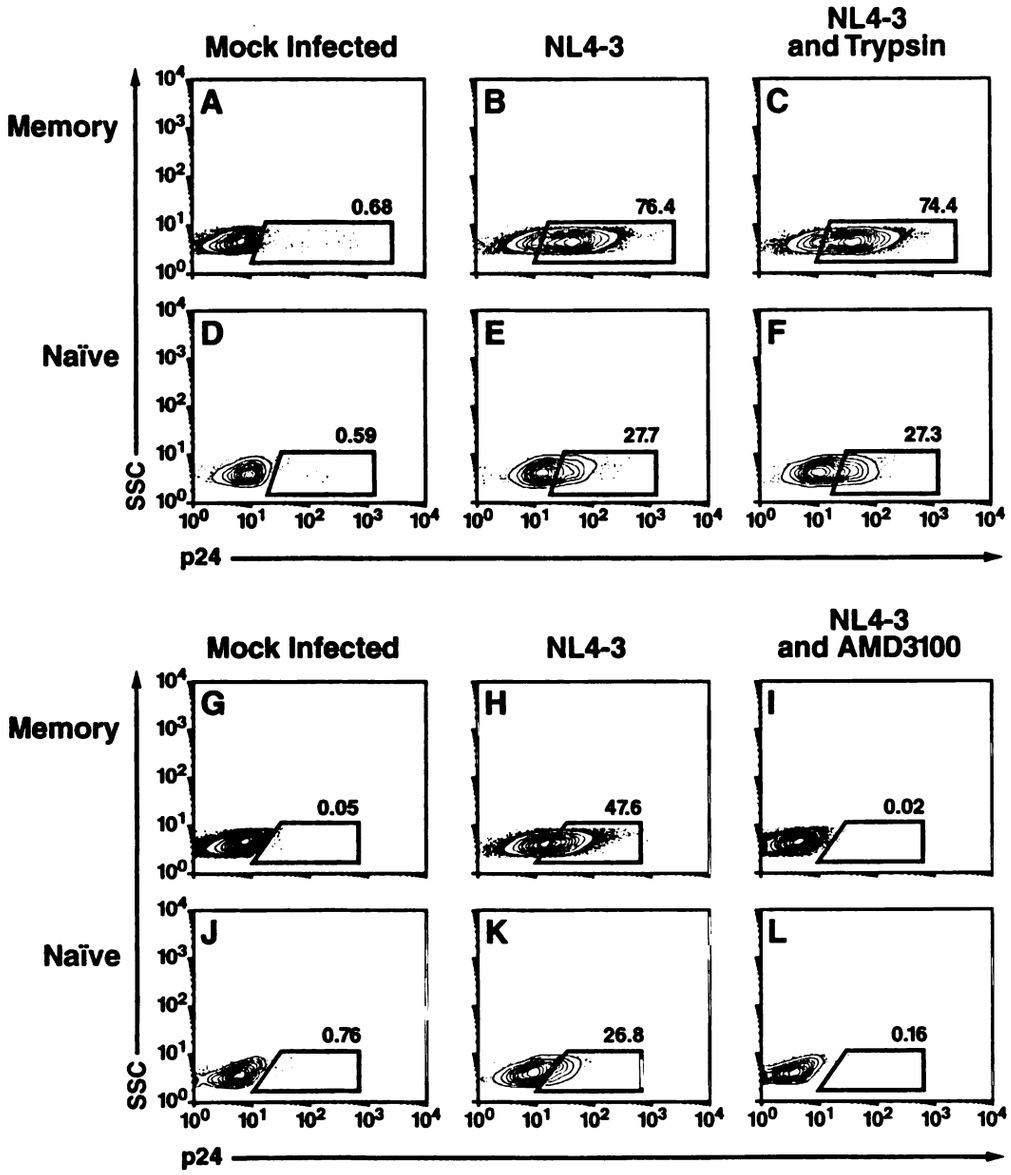

Figure 2. HIV-1 Infection of Naive and Memory CD4⁺ T Cells

(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

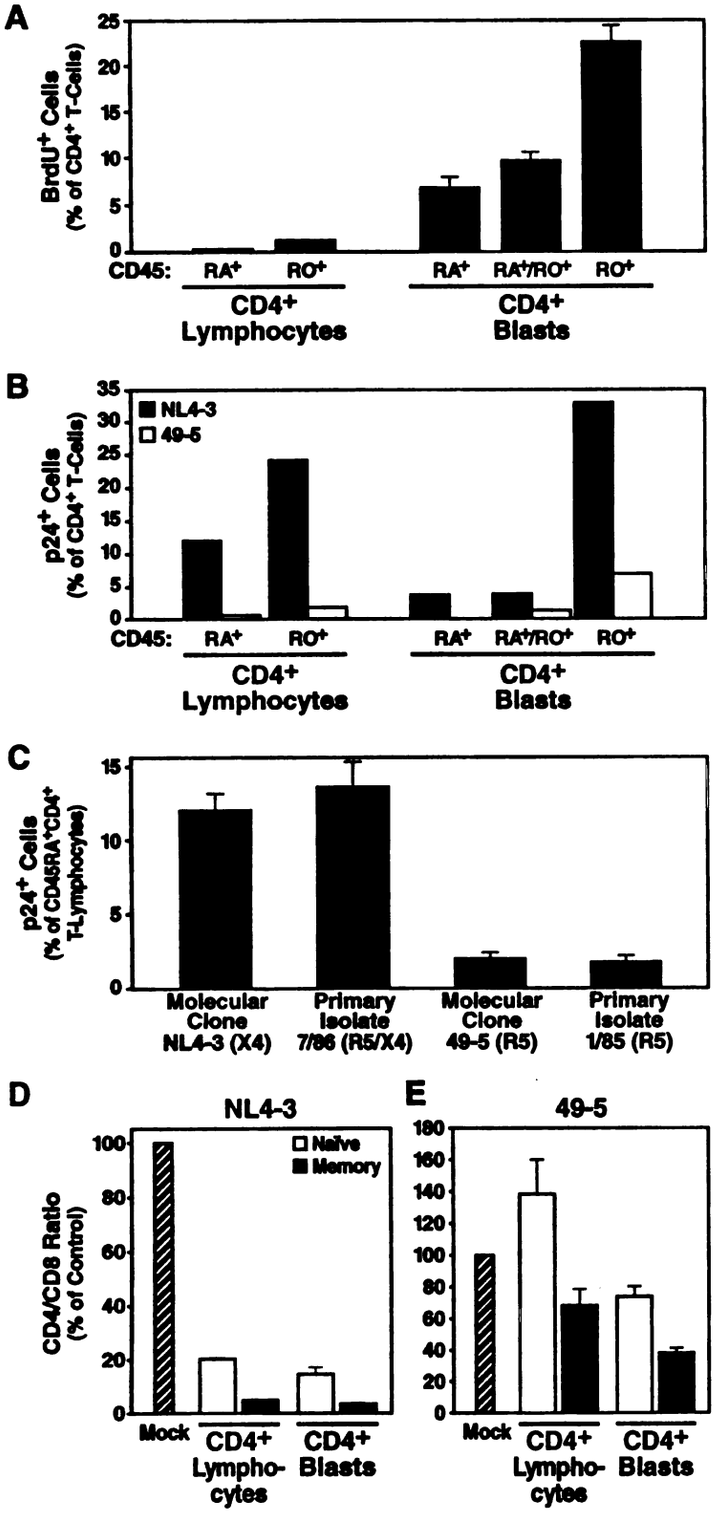

Figure 3. Cellular Proliferation and HIV-1 Infection and Depletion of Naive and Memory CD4+ T Cell Subsets

(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

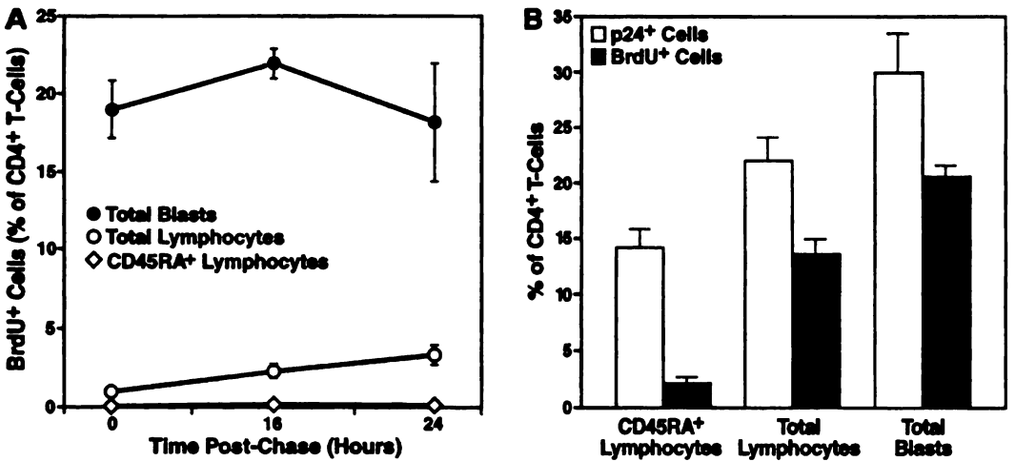

Figure 4. Kinetic Analysis of BrdU Incorporation in Tonsil Histoculture Lymphocytes and Blasts

(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

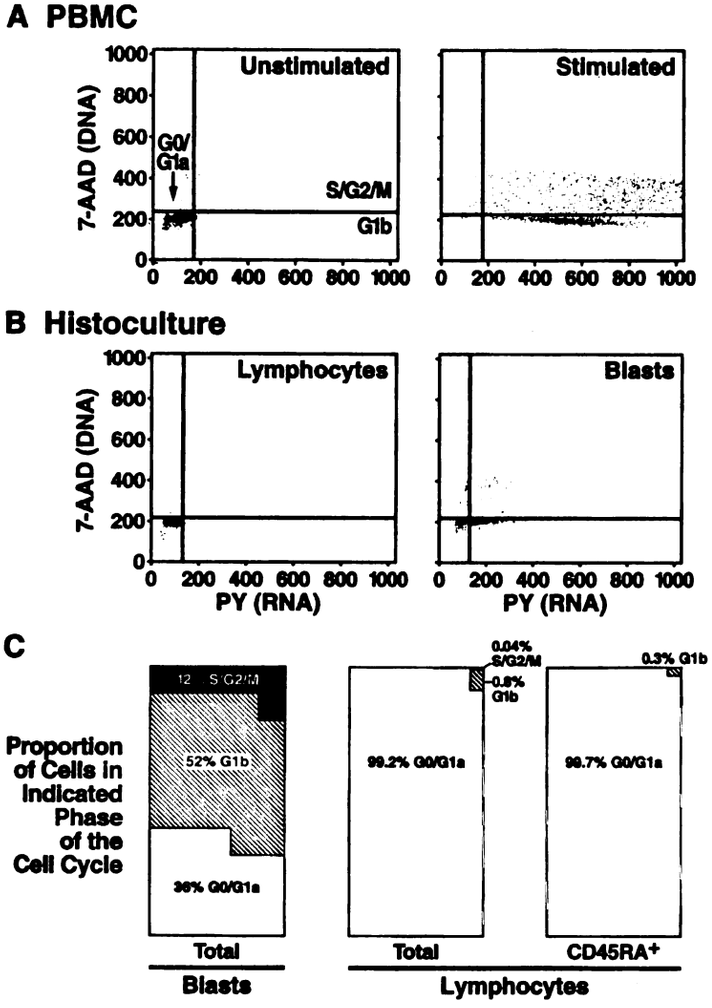

Figure 5. Cell Cycle Analysis of CD4⁺ Lymphocytes and Blasts in PBMC and HIV-1-Infected Tonsil Histocultures

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

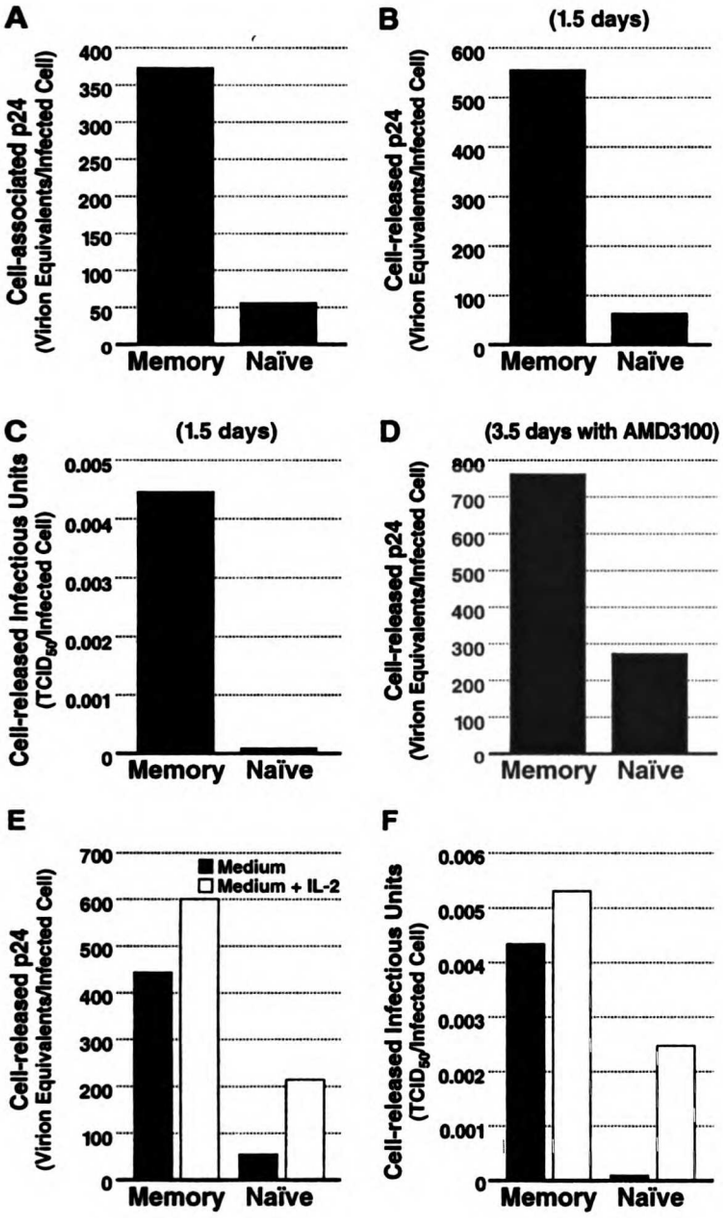

Figure 6. Burst Size of NL4-3-Infected Memory and Naive T Cells Isolated from Tonsil Cultures

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-

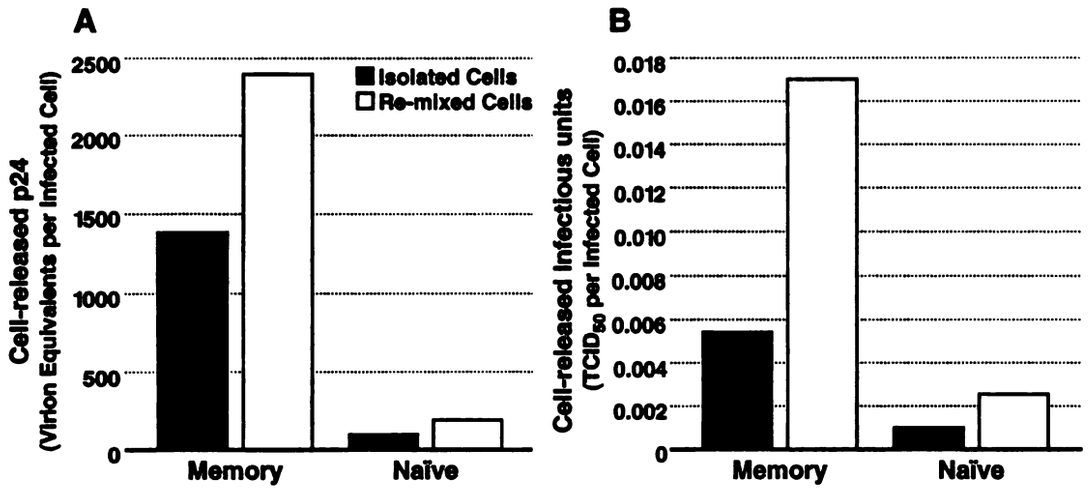

Figure 7. Burst Size of NL4-3-Infected Memory and Naive T Cells Reincorporated into Tonsil Cultures

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.

Hellert, 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: Retroviral 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.

Chapter 4

Induction of Cellular Activation and Proliferation Among Bystander $CD4^+$ T-cells within Human Lymphoid Tissue by HIV-1

Prologue

We had previously demonstrated that non-dividing, naïve $CD4^+$ T-cells within lymphoid tissues supported the productive infection by X4 HIV-1 (Chapter 3). Studies of samples from infected patients revealed that non-activated T-cells also supported productive infection despite resistance of these cells *in vitro*. Surprisingly, studies of infected patients have also demonstrated an increase in the proportion of activated T-cells during late-stage HIV-disease. The studies herein sought to determine the activation state, as evidenced by surface expression of activation markers, of the HIV-1 target cells within human lymphoid tissues. We found that the majority of naïve T-cells in lymphoid tissues cultured *ex vivo* were non-activated, but nonetheless, supported active viral replication. However, HIV-1 preferentially infected the activated subset, consistent with *in vivo* findings. Interestingly, we observed an "overrepresentation" of activated cells in lymphoid tissue explants following depletion by X4 HIV-1 due to an ability of the virus to effectively induce proliferation and activation of bystander T-cells. We speculate that such *trans*-activation may contribute to or augment the chronic immune activation observed in patients with long-standing HIV infection, and may underlie the *in vivo* phenomena. Collectively, these findings implicate non-activated cells in the replication of HIV and underscore the role of the lymphoid microenviroment in the pathogenesis of HIV-disease.

I designed, conducted and/or supervised the work presented in this manuscript. A. Jekle provided valuable experimental advice and also contributed important data for this work. M. Penn provided intellectual advice, and P. Chin contributed experimental support. M. Goldsmith supervised this work. This chapter has been submitted to the *Journal of Virology*.

Induction of Cellular Activation and Proliferation Among Bystander CD4$^+$ T-Cells within Human Lymphoid Tissues by HIV-1

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ABSTRACT

We have previously demonstrated that naïve $CD4^+$ T-cells within human lymphoid tissues *ex vivo* support productive infection by human immunodeficiency virus type 1 (HIV-1). In the present studies, CXCR4-dependent (X4) strains of HIV-1 substantially infected and depleted the non-activated fraction of naïve $CD4^+$ lymphocytes within these tissues, although HIV-1 nonetheless replicated preferentially within the activated T-cell subset. Despite this preferential replication within activated T-cells, non-activated T-cells appeared to be more severely depleted by X4 strains than were activated T-cells. To investigate this paradoxical finding we labeled bystander $CD4^+$ T-cells with the fluorochrome 5-carboxyfluorescein diacetate, succinimidyl ester (CFSE). Labeled cells were combined with HIV-1-inoculated cultures in the presence of inhibitory concentrations of AZT to prevent spread of HIV-1 to the bystander cells. We found that infection of the cultures with X4 viruses induced activation of these bystander cells, a process characterized by enhanced proliferation of activated T-cells as well as increased expression of activation markers on formerly non-activated cells. Through these effects on bystander cells, X4 strains of HIV-1 may thus locally increase the activation state of the lymphoid tissue. This process generates a more permissive environment for active HIV-1 replication and may therefore contribute to the increase in viremia observed in HIV-1-infected patients following the emergence of X4 variants.

INTRODUCTION

The hallmark of human immunodeficiency virus type 1 (HIV-1) disease is the progressive loss and eventual collapse of the $CD4^+$ T-cell compartment thereby contributing significantly to immunodeficiency. *In vitro* studies have demonstrated that HIV-1 tropism is principally determined by cellular coreceptor expression, whereas productive infection is influenced by many additional factors. For example, we have previously demonstrated that within lymphoid tissues CXCR4-dependent (X4) HIV-1 strains, including both molecular clones and primary isolates, preferentially replicate in memory $CD4^+$ T-cells compared to naïve $CD4^+$ T-cells despite high levels of CXCR4 expression on both populations (Eckstein *et al.*, submitted for publication). In addition, others have shown that the maturation and activation state of the infected T-cells also contributes to the efficiency of productive infection and thus the pathogenesis of HIV-1 disease. In particular, the expression of CD45RO, CD25, HLA-DR, or CD69 appears to define cells that are highly permissive for HIV-1 infection both *in vitro* and *in vivo* (6, 9, 15, 24, 26, 29, 31, 36, 41, 42). Nevertheless, studies of infected individuals have revealed productive infection of non-activated, naïve $CD4^+$ T-lymphocytes *in vivo*, albeit at frequencies below that of activated cells (45). However, the possibility exists that these cells were not infected by HIV-1 as mature, non-activated T-cells, but rather acquired virus at an earlier, more permissive phase of the cellular lifetime.

Paradoxically, although these activation markers define cell populations that are particularly susceptible to HIV-1 infection, and presumably virus-mediated cytopathic

effects, the proportion of activated $CD4^+$ T-cells among total circulating $CD4^+$ T-cells in HIV-1 infected individuals increases in association with disease progression (5, 14, 17, 19, 22, 23, 30). The basis for these contradictory findings is not well established, but may relate to chronic immune stimulation during long-term HIV-1 infection leading to enhanced activation marker expression on formerly non-activated T-cells (3, 18). However, a full understanding of these latter studies has been limited by the inherent cross-sectional nature of obtaining patient samples.

We have investigated these complex host-virus interactions in prospective studies by culturing mature human lymphoid tissues and inoculating them with HIV-1 *ex vivo*. Because these tissues are permissive for HIV-1 infection independent of exogenous stimulation (13), they preserve the cytokine milieu and lymphoid cell diversity found *in vivo* (Eckstein *et al.*, submitted for publication). Our previous studies revealed that within the endogenous lymphoid microenvironment, HIV-1 productively infects non-dividing, naïve $CD4^+$ T-cells *de novo* (Eckstein *et al.*, submitted for publication). We now demonstrate productive infection and depletion of the non-activated fraction of this naïve $CD4^+$ T-cell subset. Unexpectedly, we also found that X4 HIV-1 activates bystander cells, thereby manipulating the host immune system so as to generate a more permissive environment for viral replication. These processes likely contribute to viral pathogenesis by accelerating viral replication, $CD4^+$ T-cell destruction, and eventual immune collapse.

MATERIALS AND METHODS

Preparation of viral stocks. NL4-3 was a gift from Malcom Martin via the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health. The molecular clone 49-5 was a gift from Bruce Chesebro. Infectious virus stocks were prepared by transfecting 293T cells with proviral DNA as previously described (2). The primary isolates 7/86 and 1/85 (11), a gift from Ruth Connor, were expanded by infection of heterologous peripheral blood mononuclear cells (PBMC). The p24 Gag concentration of viral stocks was assessed by enzyme linked immunosorbent assay (NEN Life Sciences, Boston, MA).

Culture and infection of human lymphoid tissues *ex vivo*. Human noninflammatory tonsil tissue removed during tonsillectomy (provided by Mills Peninsula Hospital, Kaiser-San Francisco, South San Francisco and San Rafael, CA) was prepared for lymphoid aggregate culture as previously described (Eckstein *et al.*, submitted for publication). Briefly, tonsil tissue was mechanically dispersed and the isolated cells were transferred to 96-well U-bottom plates at a concentration of $1x10^7$ cells per ml, 200µl per well. Cells were allowed to aggregate at the bottom of the well and were not disturbed for the remainder of the culture period. Tissues were inoculated within 24 hours of preparation with HIV-1 at approximately 100 $TCID_{50}$/well, as determined by terminal dilution of the virus stocks in quadruplicate on heterologous phytohemagglutinin-activated PBMC as described previously (13, 25).

Identification of activated $CD4^+$ T-cell populations by flow cytometry. Six days post-inoculation (at peak infection), dispersed cells from infected and uninfected lymphoid cultures were immunostained for cell surface markers CD4, CD45RA and CD62L in combination with CD25, HLA-DR, and/or CD69. 50,000 $CD4^+$ lymphocytes were counted and the data were analyzed with FlowJo software (Treestar). To identify activated, non-activated, memory, and naïve subsets, the following monoclonal antibodies (mAbs) were used from Becton Dickinson (BD): anti-CD4 (clone SK7, allophycocyanin-conjugated, 1:20 dilution), anti-CD25 (phycoerythrin-conjugated, 1:20 dilution), anti-HLA-DR (phycoerythrin-conjugated, 1:40 dilution), anti-CD69 (phycoerythrin-conjugated, 1:20 dilution), and anti-CD62L (fluoroscein isothiocyanate-conjugated, 1:20 dilution). The following mAb from BD Pharmingen (San Jose, CA) was also used: anti-CD45RA (cychrome-conjugated, 1:20 dilution).

Assessment of $CD4^+$ T-cell infection by flow cytometry. At the indicated time points post-inoculation (at peak infection, approximately one-week post-inoculation), dispersed cells from infected and uninfected lymphoid cultures were immunostained for cell surface markers CD4 and CD45RA in combination with CD25, HLA-DR, and/or CD69. Cells were then fixed in 1% paraformaldehyde (PFA) for 10 minutes on ice and subsequently permeabilized with 0.1% Triton X-100 and immunostained for intracellular CD4 and p24. 50,000 $CD4^+$ lymphocytes were counted and the data were analyzed with FlowJo. To identify activated, non-activated, memory, and naïve subsets the following mAbs were used from BD: anti-CD4, anti-CD25, anti-HLA-DR, and anti-CD69. The

following mAb from BD Pharmingen was also used: anti-CD45RA. To identify infected cells, anti-p24 (fluoroscein isothiocyanate-conjugated, 1:100 dilution) from Coulter (Miami, FL) was also used. Results are reported as the mean with standard error of the mean (SEM).

Assessment of $CD4^+$ T-cell depletion by flow cytometry. At the indicated time points post-inoculation, dispersed cells from infected and uninfected lymphoid histocultures were immunostained for cell surface markers CD3, CD4, and CD8 as described previously (25, 33). 10,000 $CD3^+$ lymphocytes were counted and the data were analyzed with FlowJo. $CD4^+$ T-cell depletion was calculated as the ratio of $CD4^+$ to $CD8^+$ T-cells in infected relative to uninfected tissues as described previously (13). To determine $CD4^+$ depletion in more complex subsets we also enumerated the frequency of activated, non-activated, memory and naïve T-cells among total $CD4^+$ T-cells in uninfected as well as infected tissues. To identify activated, non-activated, naïve and memory subsets the following mAbs were used from BD: anti-CD3 (clone SK7, allophycocyanin-conjugated, 1:80 dilution) anti-CD4 (clone SK3, fluoroscein isothiocyanate-conjugated, 1:20 dilution), anti-CD8 (clone SK1, phycoerythrin-conjugated, 1:20 dilution), anti-CD25, anti-HLA-DR, and anti-CD69. The following mAb from BD Pharmingen was also used: anti-CD45RA. $CD4^+$ T-cell subset frequencies in infected tissues were compared to those obtained for uninfected control tissues. Results are reported as the mean with standard error of the mean (SEM).

Flow cytometric analysis of cellular proliferation and activation marker upregulation. Six days post-inoculation, uninfected lymphoid cultures were labeled with carboxyfluorescein diacetate, succinimidyl ester (CFDA, SE; CFSE) as per manufacturer instructions (1 μM, Molecular Probes, OR). $5x10^5$ labeled cells were mixed with $2x10^6$ infected cells from the same donor. Mixed cultures were incubated for 3-days in standard histoculture media supplemented with AZT (Sigma, St. Louis, MO). Following this incubation, cells were harvested, washed, and immunostained with a combination of mAbs recognizing CD4, CD45RA, and CD25, HLA-DR, or CD69 in 3% fetal bovine serum in PBS for 30 minutes on ice. 100,000 lymphocytes were collected and analyzed by FlowJo.

RESULTS

Identification of activated and non-activated memory and naïve $CD4^+$ T-cells within human lymphoid tissue. We previously used flow cytometry to identify memory and naïve $CD4^+$ T-cells within histocultures derived from human tonsils (Eckstein *et al.*, submitted for publication). In the present study we employed multicolor flow cytometry further to identify activated and non-activated subsets of both memory and naïve T-cells within these same tissues. Following 5 days of culture, we immunostained tonsil explants for memory surface markers as well as the prototypic activation markers CD25, HLA-DR, or CD69 (Fig. 1A–C). Among memory $CD4^+$ T-cells we found that expression of each of these markers was limited to a minority of the population. Interestingly, we also found detectable levels of all three markers on naïve $CD4^+$ T-cells as well, but the frequency of naïve cells expressing CD25, HLA-DR, or CD69 was typically below the frequency of memory cells expressing the same marker. Expression of activation markers on a measurable fraction of naïve T-cells contrasts sharply with the negligible proliferative activity of this T-cell population and their failure to progress beyond the G0 and G1a phases of the cell cycle (Eckstein *et al.*, submitted for publication). Thus, expression of surface activation markers does not immediately correlate with other traditional measures of T-cell activation.

To determine if the activation status of T-cells was influenced by productive infection by HIV-1, cultures were inoculated with the X4 HIV-1 molecular clone NL4-3 (1, 25, 32, 34), the R5 molecular clone 49-5 (8, 25), the dual-tropic (X4R5) primary

isolate 7/86 or the mono-tropic R5 primary isolate 1/85 (11). Following 5 days of culture, and preceding peak T-cell infection and significant T-cell depletion by HIV-1, we immunostained the explants for activation marker expression (Fig. 1A–C). We found that expression of these markers was unchanged by X4 or R5 HIV-1 infection within this acute time-frame, as memory and naïve $CD4^+$ T-cells isolated from cultures infected with HIV-1 exhibited the same frequency of CD25, HLA-DR, and CD69 expression as was found in mock-infected cultures (Fig. 1A–C).

We also stained infected tonsil cultures with the combination of antibodies recognizing CD25, HLA-DR, and CD69 as well as CD4 and the maturation markers CD45RA and CD62L. By using activation marker antibodies conjugated to a single fluorochrome and combining the detection into the same channel, two distinct cell subsets were identified: one subset that did not express any of these markers examined, a population we refer to as "non-activated," and a second subset that expressed one or more of the markers, a population we refer to as "activated." This strategy revealed non-activated subsets within both the memory and naïve $CD4^+$ T-cell populations (Fig. 1D). Interestingly, the frequency of cells scored as activated in this analysis was very similar to the frequency of cells expressing a single activation marker alone (Fig. 1A–C), implying that expression of a given marker likely corresponds to expression of others. Furthermore, infection with any of the four HIV-1 strains caused no significant change in the proportion of activated cells expressing one or more such markers.

Non-activated, naïve T-cells are permissive for HIV-1 infection. To determine the relative susceptibility of various T-cell subpopulations for HIV-1 infection, we again inoculated tonsil cultures with the HIV-1 strains NL4-3, 49-5, 7/86, or 1/85. At a time point previously determined to represent a peak of infection (8 days post-infection), cultures were harvested and immunostained to detect intracellular p24, a marker of productive HIV-1 infection, along with CD25, HLA-DR, or CD69 as independent markers of cellular activation. Both activated and non-activated subsets of memory and naïve $CD4^+$ T-cells were infected at significant frequencies by the X4 strains NL4-3 and 7/86 (Fig. 2A–C). However, within the memory or naïve T-cell populations, both viruses demonstrated a preference for the activated subsets. Preferential infection of activated memory and activated naïve T-cells was likewise observed for the R5 viruses, 49-5 and 1/85 (Fig. 2A–C). Infection of naïve T-cells overall by these R5 strains was much less frequent than that by the X4 strains, and in particular was negligible in the non-activated subset of naïve $CD4^+$ T-cells.

To establish definitively whether a subset of T-cells refractory to productive HIV-1 infection could be identified, we again inoculated cultures with NL4-3, 7/86, 49-5, or 1/85, and following 7–9 days of incubation immunostained the cultures with antibodies to intracellular p24 and the cocktail of antibodies to CD25, HLA-DR, and CD69. We observed productive infection of all subsets by the two X4 viruses, but preferential infection of activated cells was evident (Fig. 2D). The R5 viruses infected these subsets at significantly lower frequencies, but also demonstrated a strong preference for the

activated subset. As before, infection of the naïve T-cell subsets by these R5 viruses was minimal. Therefore, X4 strains of HIV-1 are capable of productively infecting naïve and memory $CD4^+$ T-cells that do not express detectable quantities of any of these prototypic activation markers.

Collectively, these results demonstrate that both HIV-1 molecular clones and primary isolates are capable of infecting activated and non-activated $CD4^+$ T-cells within human lymphoid tissue, although they demonstrate a preference for activated cells. Furthermore, time course studies examining HIV-1 infection of these same cell subsets at early, peak, and late infection revealed no significant deviation in this preferential infection of activated cells (data not shown). At all time points examined, activated T-cells were infected at a higher frequency than were non-activated cells, although this trend was less pronounced at later time points when significant T-cell depletion by HIV-1 had occurred. Therefore, the preferential infection of activated cells reported above is not a consequence of delayed kinetics of infection of non-activated cells, but rather a feature inherent to HIV-1.

Differential infection of non-activated T-cells by X4 HIV-1 does not correlate with sensitivity to HIV-1-induced cytopathic effects. To determine whether there was a relationship between sensitivity to infection and susceptibility to depletion, we inoculated lymphoid tissues with NL4-3, 49-5, 7/86, or 1/85, and on day 13 post-infection immunostained the cell cultures for expression of CD3, CD4, CD8, memory and activation markers. The samples were then analyzed for depletion of various T-cell

subpopulations by flow cytometry. As expected, the R5 strains, 49-5 and 1/85, depleted $CD4^+$ T-cells only minimally, the cytopathic effects of the viruses being largely restricted to the memory T-cell subsets, consistent with the preferential infection of these cell types (Fig. 3A–D). In contrast, we observed severe depletion of all $CD4^+$ T-cells by both X4 viruses, NL4-3 and 7/86, although depletion of memory cells was more extensive than was depletion of naïve cells (Fig. 3A–D). Surprisingly, preferential depletion of activated cells was not observed, despite preferential infection of these cell types (Fig. 2A–D). That is, among either memory or naïve T-cells, activated cells—identified by expression of CD25, HLA-DR, and/or CD69—were depleted detectably less severely than were non-activated cells. Thus, although the absolute number of virtually all $CD4^+$ T-cells declined in the cultures inoculated with X4 viruses, the relative abundance of activated cells compared to non-activated cells increased measurably during the culture period. Time-course analysis of $CD4^+$ T-cell subset depletion revealed that the abundance of activated cells increased progressively following peak infection, before reaching a stable, higher level toward the end of the culture period (data not shown). Therefore, the apparent preferential depletion of non-activated cells by X4 strains of HIV-1 is not an artifact due to delayed depletion of the activated subset.

X4 HIV-1 increases expression of activation markers on non-activated bystander T-cells. One hypothesis to explain these counterintuitive findings is that factors released into the tissue microenvironment during X4 infections may indirectly increase the activation state of residual (bystander) T-cells that escaped depletion. Such

effects directed against bystander T-cells may be manifest as increased expression of activation markers on formerly non-activated bystander $CD4^+$ T-cells. This process would tend to increase the relative abundance of activated cells in these lymphoid tissues, despite ongoing depletion associated with infection.

To test this hypothesis, we developed an assay to monitor the activation state of bystander cells labeled with the inert and stable fluorescent dye, CFSE. Labeled lymphoid tissue cells were mixed with unlabeled mock-, NL4-3-, or 49-5-pre-infected tissues at a ratio of 1:4 in the presence of inhibitory concentrations of AZT to prevent viral spread to CFSE-tagged (bystander) cells. Intracellular p24 staining of CFSE-labeled cells treated with AZT following 3 additional days of coculture (day 9 post-inoculation), revealed no detectable infection of the bystander population (data not shown). The remaining samples were harvested and stained for CD4, and memory and activation markers. Importantly, cell division of CFSE-labeled cells is accompanied by a reduction in the CFSE fluorescence intensity due to a cytosolic dilution effect ($CFSE^{low}$). To avoid the confounding effects of proliferation, we measured the expression of CD25, HLA-DR, or CD69 on $CFSE^+$ bystander T-cells that had not divided during the coculture by gating on the $CFSE^{high}$ fraction and subsequently gating on the memory or naïve $CD4^+$ T-cell subsets (schematized in Fig. 4A, B, E, and F).

Within this non-dividing population, expression of CD25, HLA-DR, and CD69 did not increase significantly in 49-5-infected tissues compared to non-infected tissues (Fig. 5A–C). In contrast, expression of these markers on both memory and naïve

bystander $CD4^+$ T-cells increased significantly in cultures inoculated with NL4-3 (Fig. 5A–C). Therefore, replication by an X4 virus promotes an interconversion of cell types that increases the size of the activated T-cell subset while simultaneously decreasing the size of the non-activated subset. These dynamics contribute to the apparent sparing of activated cells in the depletion studies reported above.

X4 HIV-1 induces proliferation of bystander naïve $CD4^+$ T-cells. We have shown above that X4 infections activate bystander cells thereby increasing the relative abundance of activated memory and naïve T-cells. Since highly activated cells generally have a higher rate of proliferation than non-activated cells, we also investigated the related hypothesis that activation of bystander cells by X4 infections likewise induces the selective proliferation of activated bystander cells, thereby further contributing to the increased representation of activated cells within these tissues. As stated earlier, cellular division of $CFSE^+$ cells is accompanied by a reduction in the fluorescent intensity of CFSE within the daughter cells. Therefore, to monitor the proliferation of various cell subsets, we measured the frequency of $CFSE^{low}$ $CD4^+$ T-cells among activated ($CD25^+$, $HLA\text{-}DR^+$, or $CD69^+$) $CFSE^+$ bystander memory and naïve T-cells in mock- and HIV-1-infected cultures (schematized in Fig. 4A–D).

As previously reported (Eckstein *et al.*, submitted for publication), we confirmed that within mock-inoculated tissues memory T-cells exhibited a higher rate of proliferation than naïve cells (Fig. 6A–C). Additionally, within each subset, activated T-cells exhibited a higher rate of cell division compared to non-activated cells (Fig. 6A–C).

An identical pattern was also seen in tissues inoculated with 49-5 (Fig. 6A–C). However, in tissues inoculated with NL4-3, the proliferative activity of activated T-cells was significantly higher than in mock- or 49-5-infected cultures (Fig. 6A–C). Among memory cells, the percentage of both activated and non-activated cells that had divided during this incubation typically doubled in the presence of X4 HIV-1, thereby preserving the ratio of activated memory cells relative to non-activated memory cells. Thus, this affect of bystander activation may not account for the surprising abundance of activated memory cells following X4 infections. However, among naïve $CD4^+$ T-cells, the proliferative activity of non-activated cells was unchanged or even declined in the presence of HIV-1, whereas a dramatic (3-fold) increase in the proliferation of activated cells was observed. These findings imply that X4 HIV-1 induces selective expansion of activated naïve cells. Therefore, rather than preferential depletion of the non-activated naïve T-cell subset by HIV-1 accounting for the relative abundance of the activated naïve subset following X4 infection, we infer that there is a relative expansion of the activated naïve T-cell subset within X4 HIV-1-infected cultures due to induction of proliferation. Thus, the enhanced rate of division of these cells contributes to the apparent preservation of activated cells relative to non-activated $CD4^+$ T-cells in tissues incubated with X4 HIV-1. Collectively, these results suggest that factors released in conjunction with infection and/or killing of target cells by X4 strains activates bystander lymphocytes, resulting in the selective expansion of the activated T-cell fraction through enhanced activation marker expression and increased proliferation.

DISCUSSION

Although non-activated and/or naïve $CD4^+$ T-lymphocytes have been reported to be resistant to infection *in vitro*, studies have recently demonstrated that within HIV-1-infected individuals these cell subsets support active virus replication *in vivo* (7, 9, 27, 35, 37, 43, 44). We have recently found that naïve $CD4^+$ T-cells cultured within human lymphoid tissue *ex vivo* are indeed permissive for productive infection by HIV-1 (Eckstein *et al.*, submitted for publication). Here we extend these observations by demonstrating that the lymphoid microenvironment supports the infection and HIV-mediated depletion of both activated and non-activated $CD4^+$ T-cells, including naïve $CD4^+$ T-cells lacking cell surface expression of CD25, HLA-DR, or CD69. Previous work has identified cytokines that promote HIV-1 infection of non-activated and quiescent $CD4^+$ T-cells (10, 38, 40). Therefore, while non-activated cells in the peripheral circulation are relatively refractory to HIV-1 infection, upon entering lymphoid organs and subsequent exposure to the locally high concentrations of cytokines and other permissivity factors therein, such cells become permissive for productive infection even in the absence of proliferation or overt signs of activation. Thus, by preserving the endogenous cellular heterogeneity found within lymphatic organs *in vivo*, the lymphoid culture techniques employed herein provide a biologically relevant and informative model in which to investigate HIV-1 pathogenesis.

Within this system the activation markers CD25, HLA-DR, and CD69 identify populations of cells with varying degrees of permissiveness for viral infection. Like the

maturation markers that define memory and naïve cells, these markers—and perhaps others—define the relative susceptibilities of $CD4^+$ T-cells to HIV-1 infection. With them we may identify distinct $CD4^+$ T-cell populations that constitute a spectrum of HIV-1 permissivity. The effects of these markers appears to be additive, such that activated memory cells exhibit the highest rates of infection, non-activated memory cells and activated naïve cells support lower levels of infection, and non-activated naïve cells are the least permissive. Importantly, these studies did not reveal any $CD4^+$ T-cell subset—defined by activation, maturation, or proliferation—that does not support X4 HIV-1 replication within the lymphoid microenvironment. Interestingly, we have obtained preliminary evidence that naïve $CD4^+$ T-cells are less permissive when isolated from the lymphoid tissue by flow-based cell sorting (Eckstein *et al.*, manuscript in preparation). We infer that signals derived from other cell types within the lymphoid organ act upon non-activated, naïve $CD4^+$ T-cells *in trans*, and thereby render them permissive for HIV-1 infection. These results further highlight the importance of the cytokine milieu (7, 9, 27, 35, 37, 43, 44) and antigen presenting cell (APC)-derived signals (12, 20, 39) found within lymphoid tissues to the replication and pathogenesis of HIV-1.

Although these studies demonstrated preferential infection of activated T-cells within lymphoid tissues, they also revealed a surprising overabundance of these cells in late stages of infection. This pattern recapitulates the behavior of HIV-1 in infected individuals, although the kinetics of the *ex vivo* and *in vivo* situations differ substantially.

At least two mechanisms may reconcile these counterintuitive findings. First, the present results demonstrate that X4 HIV-1 infection results in activation of bystander cells within lymphoid tissues. Interestingly, this effect is seen only in late infections. At earlier time points preceding significant HIV-1-induced cytopathicity the expression of CD25, HLA-DR, and CD69 in cultures inoculated with either NL4-3 or 49-5 closely parallels the expression observed in uninfected tissues. Later, increased marker expression is observed in NL4-3-inoculated cultures but not in mock- or 49-5-inoculated cultures. We infer that the massive cell death occurring within X4-infected tissues enhances the expression of pro-inflammatory cytokines and other immune-mediators (such as IL-1, IL-2, IL-6, and TNF-α) as is observed within the blood of HIV-1-infected patients with late-stage disease (4, 16, 21, 28). Thus, within X4-inoculated cultures these signals induce activation marker expression on bystander cells. In contrast, R5 viruses, which result in far less overall cytopathicity than do X4 viruses, likewise do not induce significantly greater expression of these putative signals compared to mock-infected cultures. Activation maker expression in the context of an R5 infection therefore remains stable.

Second, activated T-cells exhibit a significantly higher rate of proliferation than do non-activated cells. Ordinarily, this high rate of cell division is coupled with a similarly high rate of apoptosis (data not shown). By this mechanism, the size of the T-cell population within the lymphoid cultures remains at equilibrium, and the ratio of activated to non-activated T-cells is relatively constant. Presumably by releasing pro-inflammatory mediators, cytokines, and other signaling molecules, HIV-1 disrupts these

normal homeostatic processes. We found that the few activated cells that escaped previous rounds of HIV-1-mediated infection and depletion significantly increase their rate of division, presumably in response to these signals. The small remaining T-cell population that later emerges is thus a reflection of two populations: the subset representing non-activated cells that survived the cytopathic effects of HIV-1, and the expanded subset of activated T-cells.

Both R5 and X4 viruses significantly infect and deplete the activated T-cells, but they exhibit profound differences in the infection and depletion of non-activated cells. It is this difference in the capacity of R5 and X4 viruses to replicate within non-activated T-cells that apparently accounts for the different patterns of depletion observed. Specifically, infection (and depletion) by R5 viruses is restricted principally to activated T-cells, and the large number of non-activated cells are thus spared. The associated cell loss lowers the abundance of activated T-cells relative to non-activated T-cells. The basal level of proliferation of the activated cells is insufficient to fully counter the depletion of this subset, and we therefore detect preferential depletion of activated cells by R5 viruses. In contrast, in X4-infected cultures there is profound depletion of both activated and non-activated cells. Unlike the situation with R5 viruses, this severe depletion by X4 strains is accompanied by bystander activation. By this mechanism, non-activated cells begin to express activation markers, and the proliferative activity of activated cells increases substantially. These processes expand the size of the activated cell population while simultaneously reducing the size of the non-activated subset. This results in the increased

relative abundance of activated cells during late infection and may account, at least in part, for the apparent "resistance" of this population to HIV-1-mediated depletion by X4 viruses *ex vivo* and *in vivo*.

It thus appears that X4 HIV-1 manipulates its host to increase the pool of permissive target cells. X4 infections result in increased proliferation of $CD4^+$ T-cells and enhanced activation of bystander cells. We and others have demonstrated that such cells (proliferating and activated) are the most permissive for HIV-1 replication. X4 infection therefore establishes a local microenvironment more suitable for further HIV-1 replication and spread. This phenomenon likely contributes to the dissemination of virus within and between lymphoid organs. Although most cells present within these tissues are normally non-activated, seeding by a few productively infected cells or activation by secondary infections may nucleate high levels of HIV-1 replication locally. One possible mechanism to explain the bystander activation by X4 viruses exclusively is envelope-mediated immune stimulation. However, studies with a CXCR4-specific antagonist did not provide evidence of an envelope-driven effect on bystander cells (D.A.E. and M.A.G., unpublished observations). Moreover, a similar increase in the proliferation and activation of $CD4^-$ bystander lymphocytes was also observed, implying independence from strictly receptor-mediated processes. We reason that the bystander effects of X4 HIV-1 described herein are not due to selective coreceptor engagement by envelope, but rather to immune mediators released in conjunction with widespread X4-mediated cytopathicity.

Interestingly, the modest cytopathicity of R5 viruses limits their ability to activate the immune system, which may restrict the ability of such viruses to spread and thus defines the lower viral burdens observed in patients harboring exclusively R5 viruses. Subsequent emergence of X4 viruses may re-seed the tissues, activate the resident cells, increase target cell availability, and boost viremia. This complex interplay between HIV-1 and the host immune system within lymphoid tissues *ex vivo* may in part underlie the complex viral dynamics and evolutionary patterns observed *in vivo*.

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REFERENCES

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

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

3. **Aukrust, P., N. B. Liabakk, F. Müller, T. Espevik, and S. S. Frøland.** 1995. Activation of tumor necrosis factor--alpha system in HIV-1 infection: association with markers of immune activation. Infection. **23:**9-15.

4. **Bélec, L., D. Meillet, A. Hernvann, G. Grésenguet, and R. Gherardi.** 1994. Differential elevation of circulating interleukin-1 beta, tumor necrosis factor alpha, and interleukin-6 in AIDS-associated cachectic states. Clin. Diagn. Lab. Immunol. **1:**117-20.

5. **Benito, J. M., J. M. Zabay, J. Gil, M. Bermejo, A. Escudero, E. Sánchez, and E. Fernández-Cruz.** 1997. Quantitative alterations of the functionally distinct subsets of CD4 and CD8 T lymphocytes in asymptomatic HIV infection: changes in the expression of CD45RO, CD45RA, CD11b, CD38, HLA-DR, and CD25 antigens. J. Acquir. Immune Defic. Syndr. Hum. Retrovirol. **14:**128-135.

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

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

8. **Chesebro, B., K. Wehrly, J. Nishio, and S. Perryman.** 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.

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

10. **Chun, T. W., D. Engel, S. B. Mizell, L. A. Ehler, and A. S. Fauci.** 1998. Induction of HIV-1 replication in latently infected CD4+ T cells using a combination of cytokines. J. Exp. Med. **188:**83-91.

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

12. **Frank, I., L. Kacani, H. Stoiber, H. Stössel, M. Spruth, F. Steindl, N. Romani, and M. P. Dierich.** 1999. Human immunodeficiency virus type 1 derived from cocultures of immature dendritic cells with autologous T cells carries T-cell-specific molecules on its surface and is highly infectious. J. Virol. **73:**3449-3454.

13. **Glushakova, S., B. Baibakov, J. Zimmerberg, and L. B. Margolis.** 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.

14. **Gröttrup-Wolfers, E., R. Strzelecki, T. Grünewald, W. Schüler-Maue, and B. Ruf.** 1997. Enhanced expression of activation antigens HLA-DR and CD69 on gamma delta T cells but impaired interleukin-2-induced upregulation in HIV-1 infection. AIDS. **11:**838-9.

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

16. **Hober, D., A. Haque, P. Wattre, G. Beaucaire, Y. Mouton, and A. Capron.** 1989. Production of tumour necrosis factor-alpha (TNF-alpha) and interleukin-1 (IL-1) in patients with AIDS. Enhanced level of TNF-alpha is related to a higher cytotoxic activity. Clin. Exp. Immunol. **78:**329-33.

17. **Kestens, L., G. Vanham, C. Vereecken, M. Vandenbruaene, G. Vercauteren, R. L. Colebunders, and P. L. Gigase.** 1994. Selective increase of activation antigens HLA-DR and CD38 on CD4+ CD45RO+ T lymphocytes during HIV-1 infection. Clin. Exp. Immunol. **95:**436-41.

18. **Lederman, M. M., L. A. Kalish, D. Asmuth, E. Fiebig, M. Mileno, and M. P. Busch.** 2000. 'Modeling' relationships among HIV-1 replication, immune activation and CD4+ T-cell losses using adjusted correlative analyses. AIDS. **14:**951-8.

19. **Mahalingam, M., M. Peakman, E. T. Davies, A. Pozniak, T. J. McManus, and D. Vergani.** 1993. T cell activation and disease severity in HIV infection. Clin. Exp. Immunol. **93:**337-43.

20. **Moran, P. A., M. L. Diegel, J. C. Sias, J. A. Ledbetter, and J. M. Zarling.** 1993. Regulation of HIV production by blood mononuclear cells from HIV-infected donors: I. Lack of correlation between HIV-1 production and T cell activation. AIDS Res. Hum. Retroviruses. **9:**455-464.

21. **Navikas, V., J. Link, C. Persson, T. Olsson, B. Höjeberg, A. Ljungdahl, H. Link, and B. Wahren.** 1995. Increased mRNA expression of IL-6, IL-10, TNF-alpha, and perforin in blood mononuclear cells in human HIV infection. J. Acquir. Immune. Defic. Syndr. Hum. Retrovirol. **9:**484-9.

22. **Orendi, J. M., A. C. Bloem, J. C. Borleffs, F. J. Wijnholds, N. M. de Vos, H. S. Nottet, M. R. Visser, H. Snippe, J. Verhoef, and C. A. Boucher.** 1998.

Activation and cell cycle antigens in CD4+ and CD8+ T cells correlate with plasma human immunodeficiency virus (HIV-1) RNA level in HIV-1 infection. J. Infect. Dis. **178:**1279-87.

23. **Ostrowski, M. A., S. J. Justement, A. Catanzaro, C. A. Hallahan, L. A. Ehler, S. B. Mizell, P. N. Kumar, J. A. Mican, T. W. Chun, and A. S. Fauci.** 1998. Expression of chemokine receptors CXCR4 and CCR5 in HIV-1-infected and uninfected individuals. J. Immunol. **161:**3195-201.

24. **Pedroza-Martins, L., K. B. Gurney, B. E. Torbett, and C. H. Uittenbogaart.** 1998. Differential tropism and replication kinetics of human immunodeficiency virus type 1 isolates in thymocytes: coreceptor expression allows viral entry, but productive infection of distinct subsets is determined at the postentry level. J. Virol. **72:**9441-52.

25. **Penn, M. L., J. C. Grivel, B. Schramm, M. A. Goldsmith, and L. Margolis.** 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.

26. **Ramilo, O., K. D. Bell, J. W. Uhr, and E. S. Vitetta.** 1993. Role of CD25+ and CD25-T cells in acute HIV infection in vitro. J. Immunol. **150:**5202-5208.

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

28. **Roux-Lombard, P., C. Modoux, A. Cruchaud, and J. M. Dayer.** 1989. Purified blood monocytes from HIV 1-infected patients produce high levels of TNF alpha and IL-1. Clin. Immunol. Immunopathol. **50:**374-84.

29. **Saifuddin, M., G. T. Spear, C. Chang, and K. A. Roebuck.** 2000. Expression of MHC class II in T cells is associated with increased HIV-1 expression. Clin. Exp. Immunol. **121:**324-31.

30. **Salazar-Gonzalez, J. F., O. Martinez-Maza, P. Nishanian, N. Aziz, L. P. Shen, S. Grosser, J. Taylor, R. Detels, and J. L. Fahey.** 1998. Increased immune activation precedes the inflection point of CD4 T cells and the increased serum virus load in human immunodeficiency virus infection. J. Infect. Dis. **178:**423-30.

31. **Schnittman, S. M., H. C. Lane, J. Greenhouse, J. S. Justement, M. Baseler, and A. S. Fauci.** 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.

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

33. **Schramm, B., M. L. Penn, R. F. Speck, S. Y. Chan, E. De Clercq, D. Schols, R. I. Connor, and M. A. Goldsmith.** 2000. Viral entry through CXCR4 is a

pathogenic factor and therapeutic target in human immunodeficiency virus type 1 disease. J. Virol. **74:**184-192.

34. **Speck, R. F., K. Wehrly, E. J. Platt, R. E. Atchison, I. F. Charo, D. Kabat, B. Chesebro, and M. A. Goldsmith.** 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.

35. **Spina, C. A., J. C. Guatelli, and D. D. Richman.** 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.

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

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

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

39. **Tsunetsugu-Yokota, Y., K. Akagawa, H. Kimoto, K. Suzuki, M. Iwasaki, S. Yasuda, G. Häusser, C. Hultgren, A. Meyerhans, and T. Takemori.** 1995.

Monocyte-derived cultured dendritic cells are susceptible to human immunodeficiency virus infection and transmit virus to resting T cells in the process of nominal antigen presentation. J. Virol. **69:**4544-4547.

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

41. **Veazey, R. S., I. C. Tham, K. G. Mansfield, M. DeMaria, A. E. Forand, D. E. Shvetz, L. V. Chalifoux, P. K. Sehgal, and A. A. Lackner.** 2000. Identifying the target cell in primary simian immunodeficiency virus (SIV) infection: highly activated memory CD4(+) T cells are rapidly eliminated in early SIV infection in vivo. J. Virol. **74:**57-64.

42. **Woods, T. C., B. D. Roberts, S. T. Butera, and T. M. Folks.** 1997. Loss of inducible virus in CD45RA naive cells after human immunodeficiency virus-1 entry accounts for preferential viral replication in CD45RO memory cells. Blood. **89:**1635-1641.

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

44. **Zack, J. A., A. M. Haislip, P. Krogstad, and I. S. Chen.** 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.

45. **Zhang, Z., T. Schuler, M. Zupancic, S. Wietgrefe, K. A. Staskus, K. A. Reimann, T. A. Reinhart, M. Rogan, W. Cavert, C. J. Miller, R. S. Veazey, D. Notermans, S. Little, S. A. Danner, D. D. Richman, D. Havlir, J. Wong, H. L. Jordan, T. W. Schacker, P. Racz, K. Tenner-Racz, N. L. Letvin, S. Wolinsky, and A. T. Haase.** 1999. Sexual transmission and propagation of SIV and HIV in resting and activated CD4+ T cells. Science. **286:**1353-1357.

FIGURE LEGENDS

FIG. 1. Flow cytometric analysis of activation marker expression on $CD4^+$ lymphocytes within human lymphoid cultures either mock infected or productively infected by HIV-1. Tonsil specimens were inoculated with NL4-3, 49-5, 7/86, or 1/85 and incubated for 6 days. Tissues were then harvested and immunostained with anti-CD4, anti-CD45RA and anti-CD62L mAbs as well as anti-CD25 (A), anti-HLA-DR (B), anti-CD69 (C) or a combination of anti-CD25, anti-HLA-DR, and anti-CD69 (D). Shown are mean frequencies with SEM (n=3) of activated memory (open bars) and naïve (closed bars) $CD4^+$ T-cells from a representative experiment from among experiments performed with 3–6 different donor tissues.

FIG. 2. HIV-1 infection of activated and non-activated $CD4^+$ T-cells. Cultures were inoculated with the molecular clones NL4-3 or 49-5 or the primary isolates 7/86 or 1/85. Productively infected $CD25^-$ (open bars) and $CD25^+$ (closed bars) naïve and memory $CD4^+$ T-cell subsets (A), $HLA\text{-}DR^-$ (open bars) and $HLA\text{-}DR^+$ (closed bars) naïve and memory $CD4^+$ T-cell subsets (B), $CD69^-$ (open bars) and $CD69^+$ (closed bars) naïve and memory $CD4^+$ T-cell subsets (C), and non-activated (open bars) and activated (closed bars) naïve and memory $CD4^+$ T-cell subsets (D). Shown are mean values with SEM (n=3) of a representative experiment from among experiments performed with 3-6 different donor tissues.

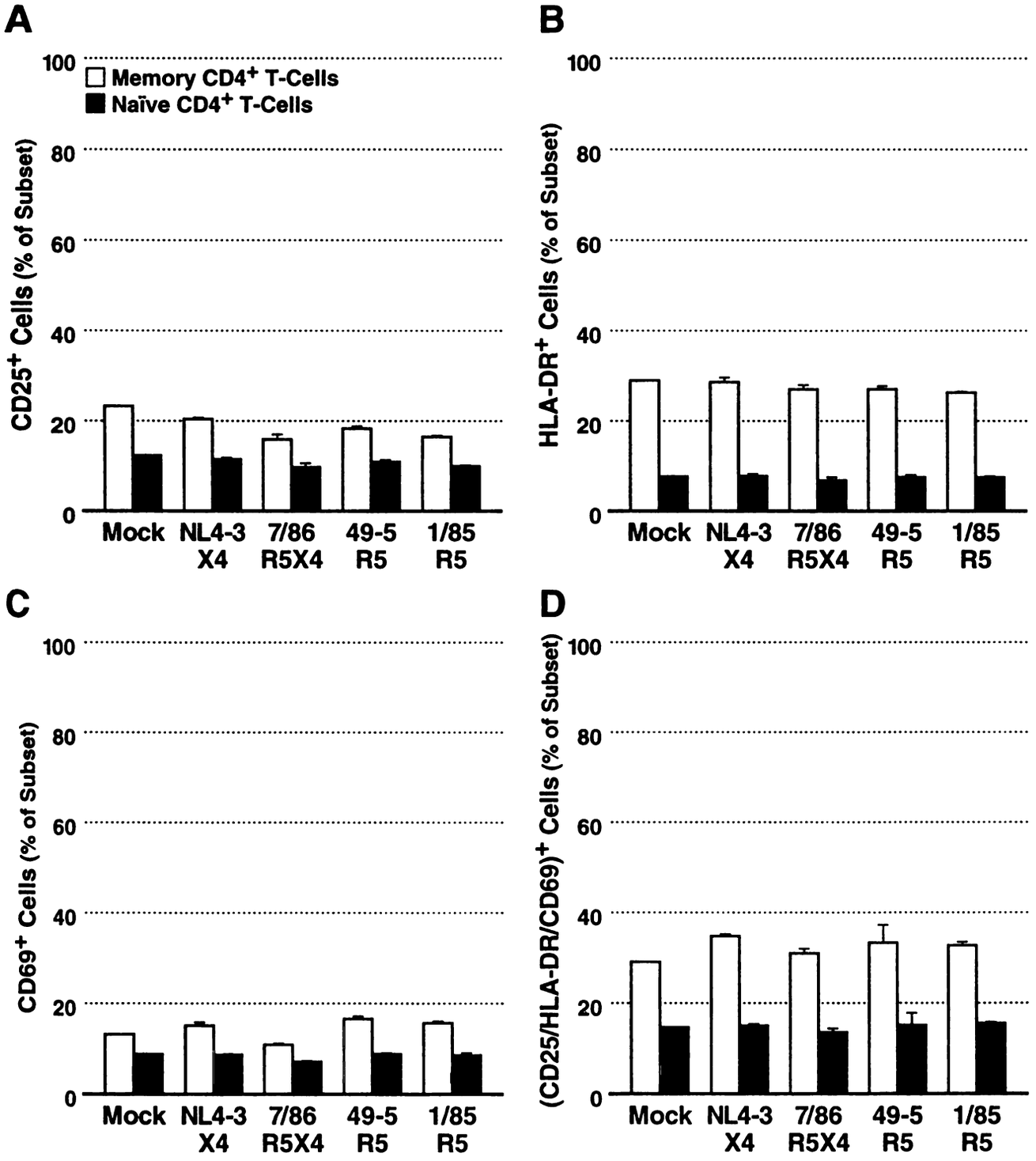
FIG. 3. Depletion of activated and non-activated $CD4^+$ T-cells. Tonsil specimens were inoculated with NL4-3, 49-5, 7/86, or 1/85, incubated for 12 days, and immunostained with anti-CD4, anti-CD3, and anti-CD8 mAbs or anti-CD4, anti-CD45RA and anti-CD62L mAbs as well as anti-CD25 (A), anti-HLA-DR (B), anti-CD69 (C) or a combination of anti-CD25, anti-HLA-DR, and anti-CD69 (D). Shown are mean frequencies with SEM (n=3) of activated (open bars) and non-activated (closed bars) memory and naïve $CD4^+$ T-cells from a representative experiment from among experiments performed with 3–6 different donor tissues.

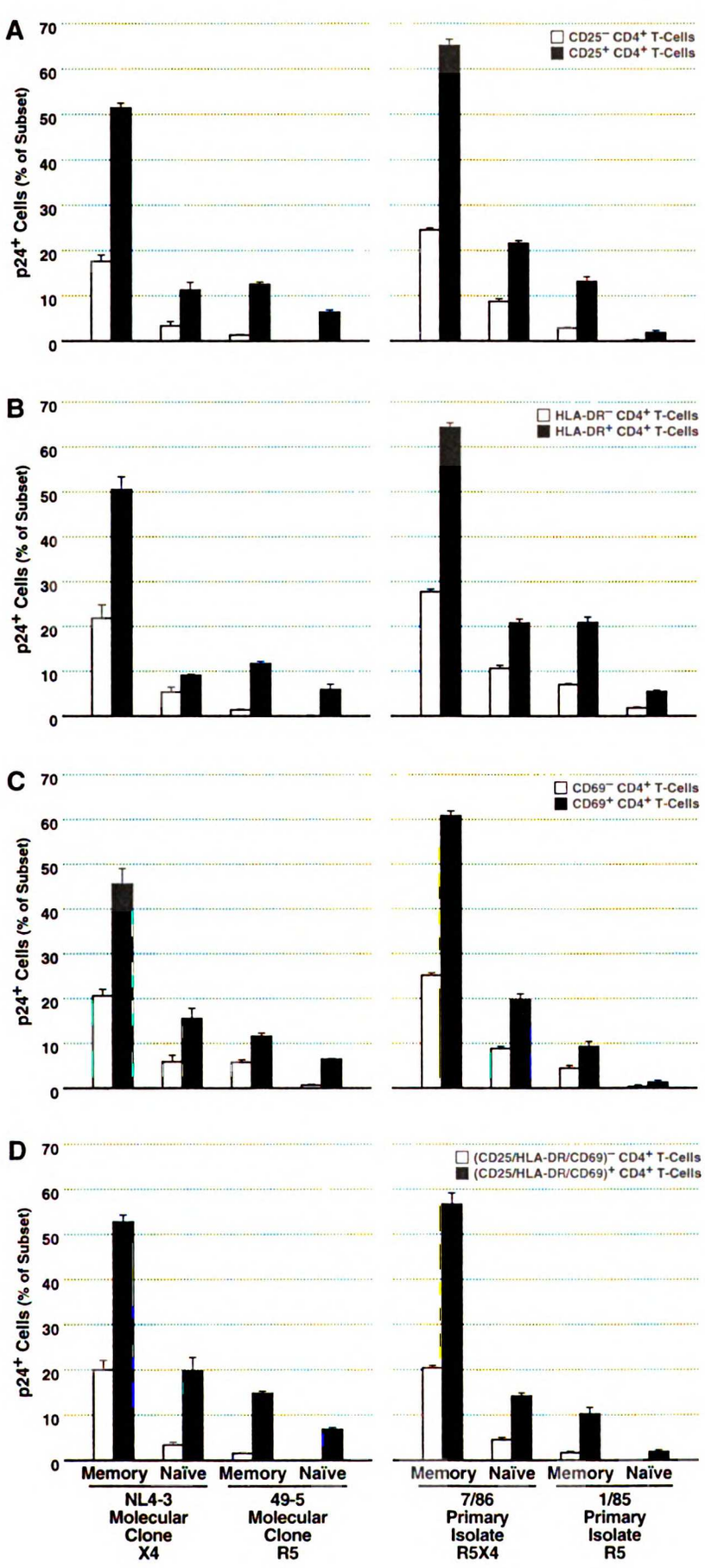
FIG. 4. Quantitation of proliferation and activation of CFSE-labeled bystander $CD4^+$ T-cells within human lymphoid tissue *ex vivo*. (A) Lymphocytes were identified within lymphoid cultures by forward- and side-scatter characteristics. (B) $CD4^+$ CFSE-labeled lymphocytes were subdivided into total bystanders ($CFSE^+$) or non-proliferating bystanders ($CFSE^{high}$). (C) $CFSE^+$ memory and naïve cells were further divided into activated or non-activated subsets. (D) The fraction of proliferating cells ($CFSE^{low}$) within these subsets was quantified. (E) $CFSE^{high}$ cells were subdivided into memory and naïve fractions, and (F) the frequency of activated cells among these naïve and memory populations was quantified.

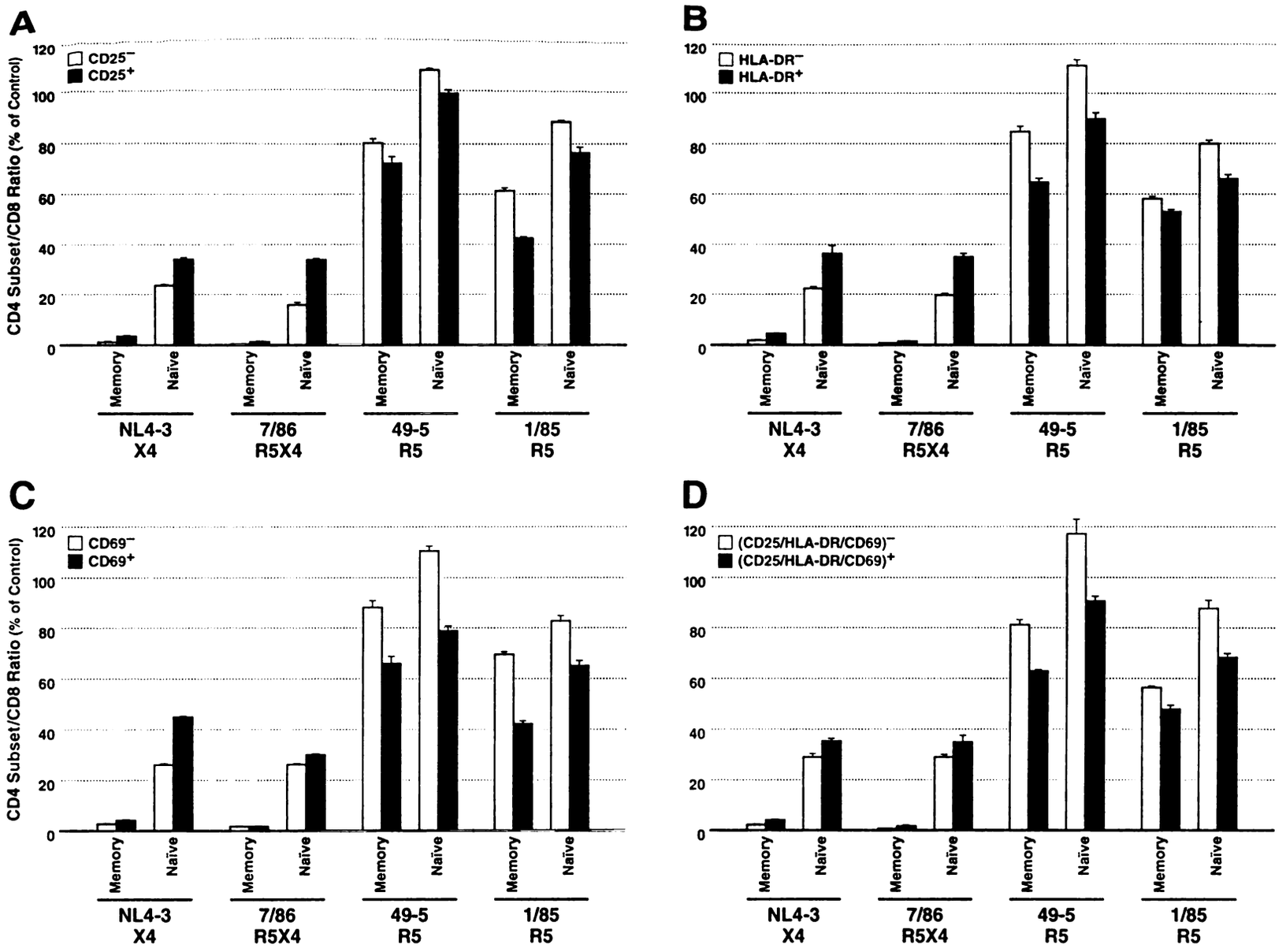
FIG. 5. Enhanced expression of activation markers on bystander $CD4^+$ T-cells. Tonsil specimens were inoculated with NL4-3 or 49-5 and incubated for 6 days.

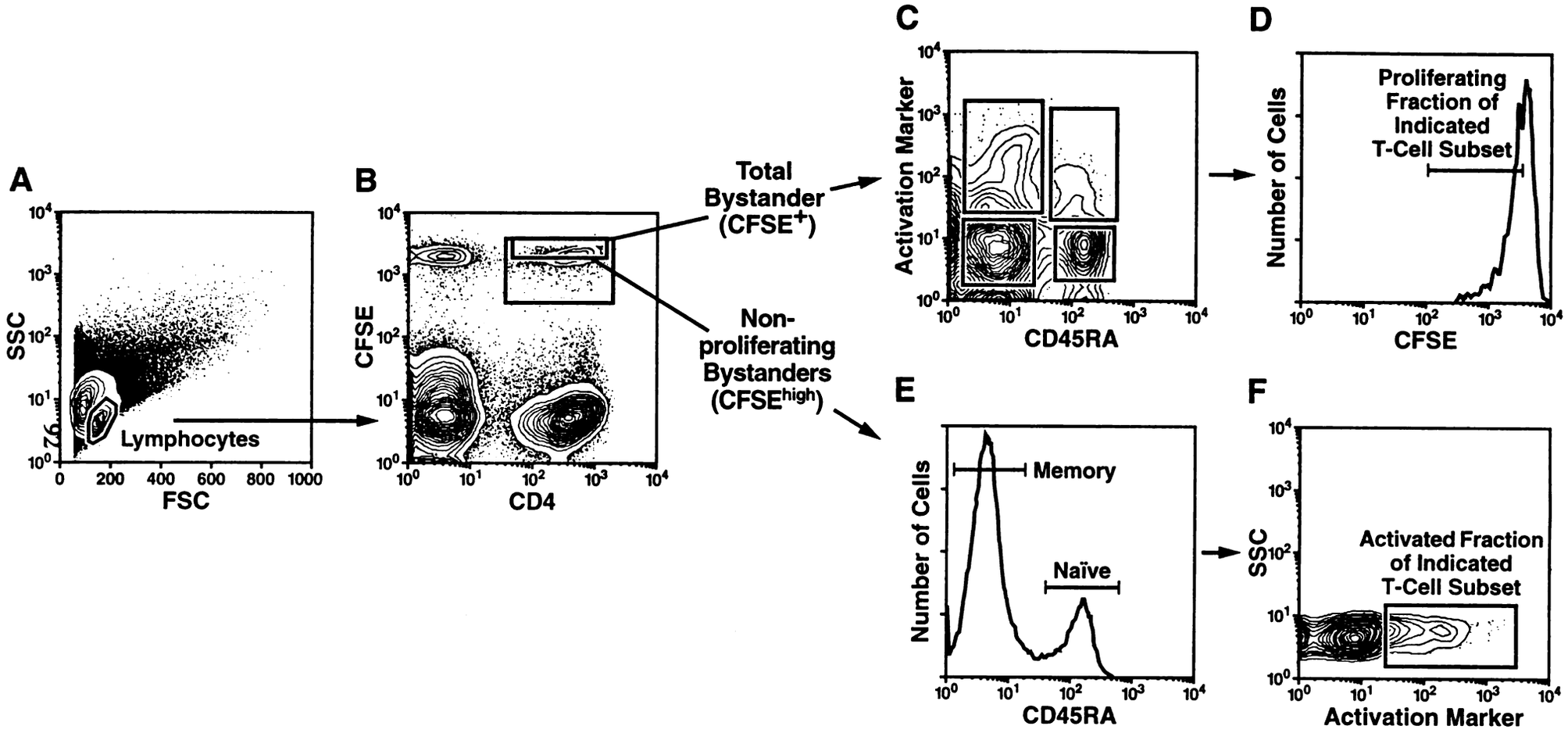
Uninfected donor-matched tonsil tissue was then labeled with CFSE, reaggregated with infected samples, and cultured for an additional 3 days in the presence of AZT. Cells were then immunostained with anti-CD4 and anti-CD45RA mAbs and anti-CD25 (A), anti-HLA-DR (B), or anti-CD69 (C). Shown are mean values with SEM (n=3) of a representative experiment from among experiments performed with 8 different donor tissues.

FIG. 6. Proliferation of activated and non-activated $CD4^+$ T-cells. Tonsil specimens were inoculated with NL4-3 or 49-5 and incubated for 6 days. Uninfected donor-matched tonsil tissue was then labeled with CFSE, reaggregated with infected samples, and cultured for an additional 3 days in the presence of AZT. Cells were then immunostained with anti-CD4 and anti-CD45RA mAbs and anti-CD25 (A), anti-HLA-DR (B), or anti-CD69 (C). Shown are mean values with SEM (n=3) of a representative experiment from among experiments performed with 8 different donor tissues.


A
B
C
D
Memory CD4+ T-Cells
Naïve CD4+ T-Cells
CD25+ Cells (% of Subset)
HLA-DR+ Cells (% of Subset)
CD69+ Cells (% of Subset)
(CD25/HLA-DR/CD69)+ Cells (% of Subset)
100
80
60
40
20
0
Mock
NL4-3 X4
7/86 R5X4
49-5 R5
1/85 R5


A
B
C
D
p24+ Cells (% of Subset)
0
10
20
30
40
50
60
70
CD25− CD4+ T-Cells
CD25+ CD4+ T-Cells
HLA-DR− CD4+ T-Cells
HLA-DR+ CD4+ T-Cells
CD69− CD4+ T-Cells
CD69+ CD4+ T-Cells
(CD25/HLA-DR/CD69)− CD4+ T-Cells
(CD25/HLA-DR/CD69)+ CD4+ T-Cells
Memory
Naïve
NL4-3 Molecular Clone X4
49-5 Molecular Clone R5
7/86 Primary Isolate R5X4
1/85 Primary Isolate R5


A
CD4 Subset/CD8 Ratio (% of Control)
CD25⁻
CD25⁺
B
HLA-DR⁻
HLA-DR⁺
C
CD69⁻
CD69⁺
D
(CD25/HLA-DR/CD69)⁻
(CD25/HLA-DR/CD69)⁺
0
20
40
60
80
100
120
Memory
Naïve
NL4-3
X4
7/86
R5X4
49-5
R5
1/85
R5



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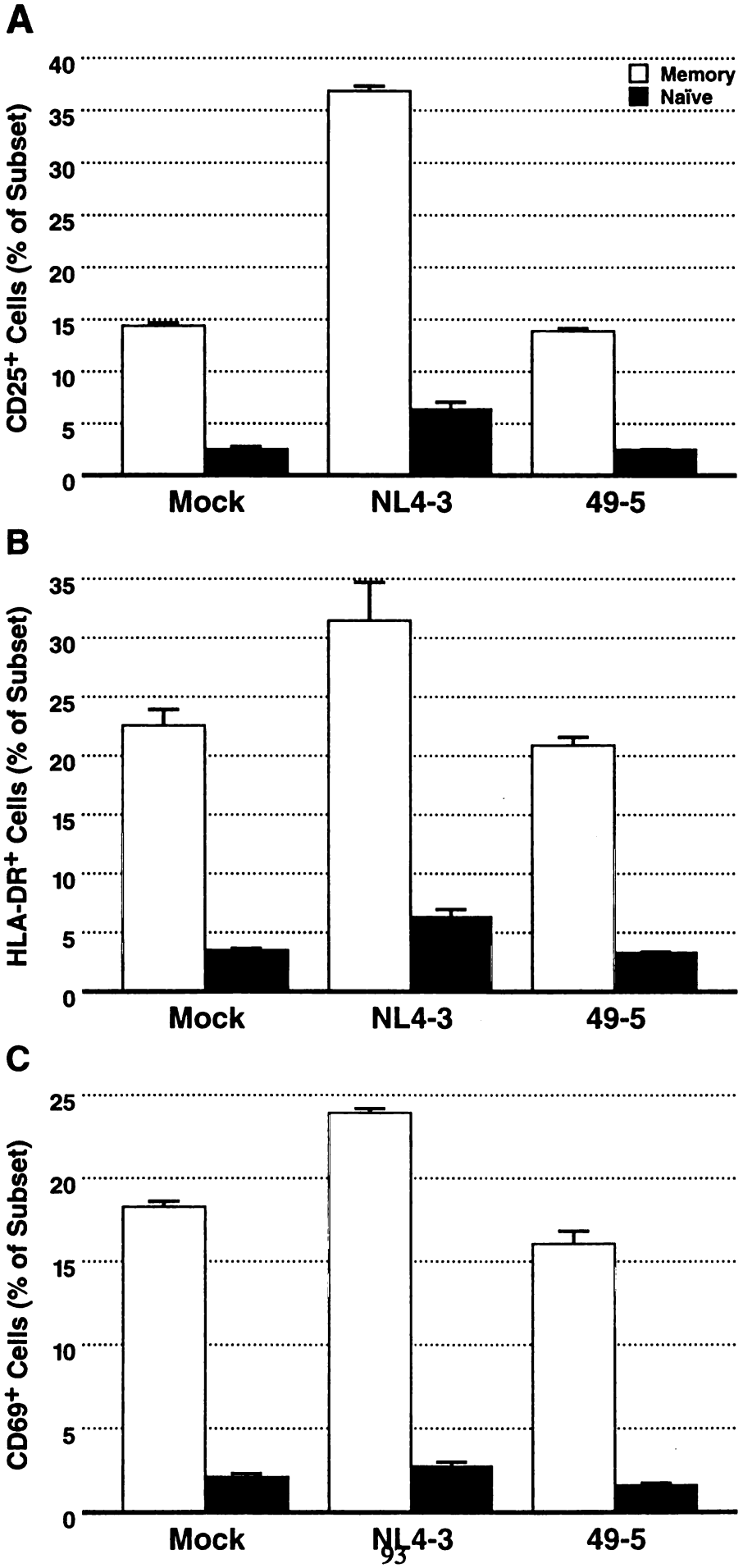
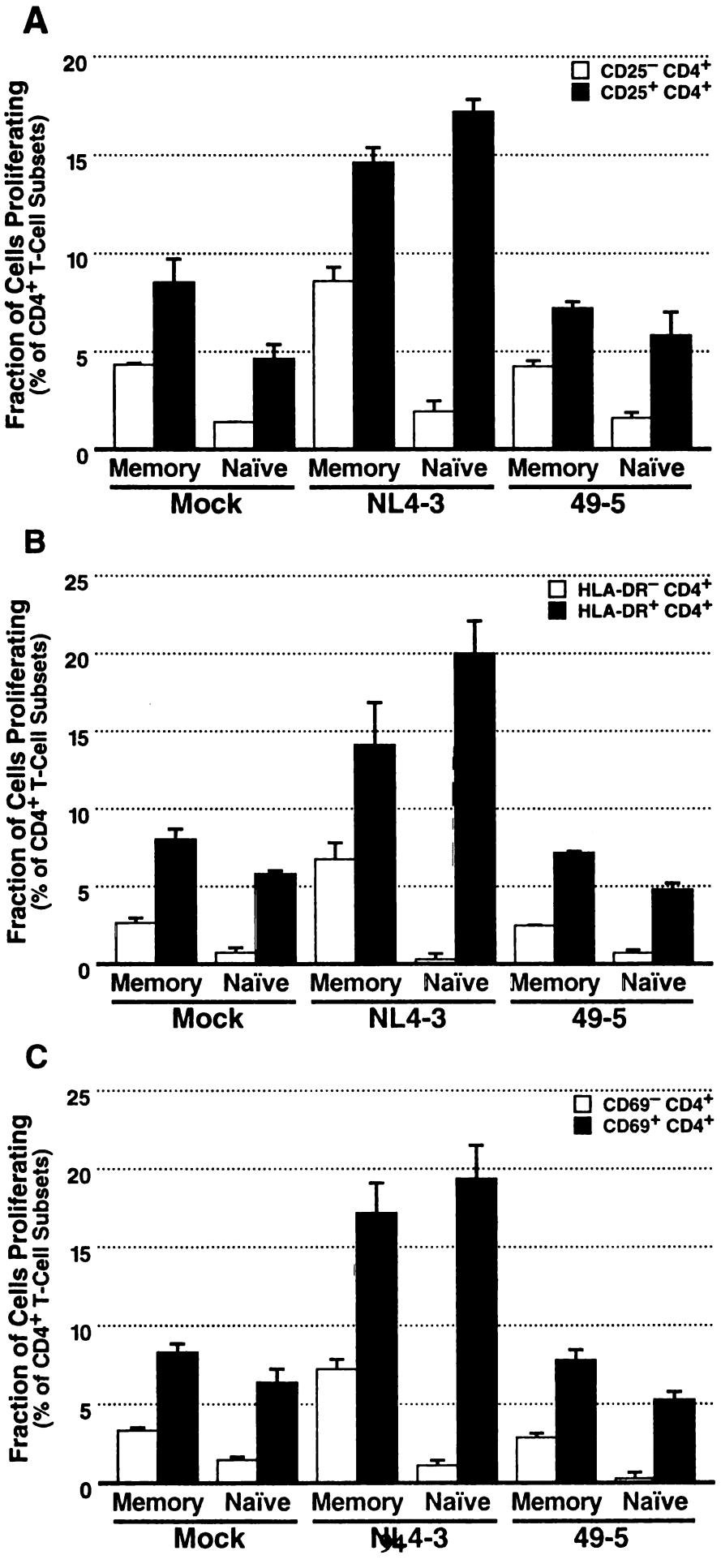

A
Memory
Naïve
CD25+ Cells (% of Subset)
40
35
30
25
20
15
10
5
0
Mock
NL4-3
49-5
B
HLA-DR+ Cells (% of Subset)
35
30
25
20
15
10
5
0
Mock
NL4-3
49-5
C
CD69+ Cells (% of Subset)
25
20
15
10
5
0
Mock
NL4-3
49-5


A
Fraction of Cells Proliferating (% of CD4+ T-Cell Subsets)
20
15
10
5
0
CD25− CD4+
CD25+ CD4+
Memory Naïve Memory Naïve Memory Naïve
Mock NL4-3 49-5
B
Fraction of Cells Proliferating (% of CD4+ T-Cell Subsets)
25
20
15
10
5
0
HLA-DR− CD4+
HLA-DR+ CD4+
Memory Naïve Memory Naïve Memory Naïve
Mock NL4-3 49-5
C
Fraction of Cells Proliferating (% of CD4+ T-Cell Subsets)
25
20
15
10
5
0
CD69− CD4+
CD69+ CD4+
Memory Naïve Memory Naïve Memory Naïve
Mock NL4-3 49-5

Chapter 5

De Novo HIV-1 Infection of Naïve CD4$^+$ T-cells Leading to an Inducible Viral Reservoir within Lymphoid Tissues

Prologue

One of the most significant obstacles to eradication of HIV-1 within infected patients receiving HAART is the presence of a stable, inducible viral reservoir within latently infected cells. Estimates suggest that elimination of this reservoir may take the lifetime of the infected individual. It is therefore of critical importance to understand the generation and regulation of this reservoir. Previous studies have demonstrated that latency may be induced upon inactivation of productively infected cells. However, recent studies have isolated latently infected naïve T-cells from infected individuals. As these cells may not have undergone a similar transition to a non-activated state, the mechanism by which these cells are generated is not known. We therefore investigated the possibility that *de novo* infection of mature T-cells within human lymphoid tissue may result in latent infection. We found that naïve T-cells infected *de novo* within the lymphoid microenvironment can support a form of virologic latency. These findings implicate a novel pathway by which latently infected T-cells may be generated in the absence of cellular transitions from activated to non-activated states, and may contribute to the formation of these cells *in vivo*.

I designed, conducted and/or supervised the work presented in this manuscript. B. Schweighardt and A. Jekle provided valuable experimental assistance and performed the studies shown in Figure 3B. M. Goldsmith supervised this work. This chapter has been submitted to the *Journal of Clinical Investigation*.

De Novo HIV-1 Infection of Naïve CD4$^+$ T-cells Leading to an Inducible Viral Reservoir within Lymphoid Tissues

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ABSTRACT

We have previously demonstrated that non-proliferating, naïve $CD4^+$ T-cells within human lymphoid tissues support productive infection by HIV-1. In the present studies we found that HIV-1 replication within these tissues *ex vivo* was enhanced by treatment with the cytokines IL-2, IL-6, and TNF-α despite experimental conditions that prevented viral spread. The elevation in virus output was derived only partially from a modest cytokine-induced increase in the "burst-size" of infected T-cells. We also discovered a marked cytokine-induced increase in the proportion of memory and naïve T-cells that were producing new virions, which appears to be a major contributor to the overall increase in virus output. Importantly, within the memory population, the increased frequency of productively infected T-cells was largely due to a reduction in the rate of apoptosis of these cells, thereby permitting sustained viral output. In contrast, within the naïve population, no such anti-apoptotic influence was detected. Therefore, in addition to supporting active HIV-1 replication, naïve T-cells within lymphoid tissues also serve as inducible reservoirs of viral persistence following *de novo* infection. Recruitment of provirus within these cells to a highly productive state may contribute to the viral loads and progressive immunodeficiency seen in HIV-1 infection *in vivo*.

MeSH-Medline key words: latency, memory, integration, cytokines, replication

INTRODUCTION

Although highly active anti-retroviral therapy (HAART) has successfully inhibited active replication of human immunodeficiency virus type-1 (HIV-1) within in many patients, often reducing viral loads to undetectable levels, it has failed to eradicate virus completely in most patients (1-3). Among the most significant reasons for this failure is the establishment of an inducible viral reservoir within mononuclear cells shortly after initial infection. Seeding of this population is thought to occur concurrently with the burst of viral replication and widespread infection of lymphoid tissues by the virus that characterizes acute HIV-1 disease (4). Due to the low or undetectable level of viral transcription within such cells, this latent proviral population is highly resistant to the therapeutic effects of HAART. Based on an estimated half-life of 6 to 44 months for latently infected cells, it is predicted that complete viral eradication within infected patients receiving HAART would require greater than 60 years (5-7).

Although the mechanism by which latently infected cells arise *in vivo* is not completely understood, it is thought that a transition of the infected host cell from an activated to a quiescent state may be an essential factor in this process. Under normal circumstances, stimulation by appropriate antigen triggers quiescent, naïve T-cells to transition to blasts, reenter the cell cycle, and alter their surface phenotype. A fraction of these dividing cells, now identifiable by memory markers, later return to a quiescent state, characterized by a profound decline in the intracellular levels of host transcription factors. Within cells that are productively infected by HIV-1 but have not yet succumbed

to viral cytopathicity, this return to quiescence and the associated downregulation of transcription factors is thought to impair proviral mRNA expression, thereby inducing post-integration latency. Consistent with this model, the majority of latently infected $CD4^+$ T-cells isolated from HIV-infected patients display a resting, memory phenotype (1, 4, 5). However, latently infected naïve $CD4^+$ T-cells have recently been identified *in vivo* (8). On the basis of *in vitro* evidence, it was formerly thought that productive infection of naïve lymphocytes by HIV-1 was restricted prior to integration, resulting in a pre-integration latency (9-11). Subsequent delivery of an activating signal to these cells may result in the completion of reverse transcription and integration, but such an infection proceeds inefficiently and is often non-productive (12-14). Interestingly, it has recently been shown that the transition of HIV-infected, actively dividing thymocytes to quiescent peripheral T-lymphocytes is sufficient to generate latently infected cells within the SCID-hu (Thy/Liv) mouse model (15). Although this mechanism may contribute to the production of latently infected naïve T-cells in many infected individuals, not all HIV-1 patients exhibit significant thymopoiesis (16-18). Therefore, this process may not account fully for the generation of latently infected naïve T-cells *in vivo*.

By culturing mature human lymphoid tissues and inoculating them with HIV-1 *ex vivo*, we have previously demonstrated that non-dividing, naïve $CD4^+$ T-cells within this microenvironment may be productively infected by HIV-1 *de novo* (19). These results raised the possibility that *de novo* infection may be an alternate mechanism by which latent infection is achieved in mature naïve T-cells. Because these histocultures are

permissive for HIV-1 infection independently of exogenous stimulation (20), they preserve the cytokine milieu and lymphoid cell diversity found *in vivo* (19). Thus, they provide a unique model in which to investigate the generation of mature lymphoid cells harboring latent HIV-1 provirus.

Some models of latency generation invoke a role for soluble cytokines. The cytokines interleukin-2 (IL-2), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) have been found to be increased in the serum and expressed at elevated levels by peripheral blood mononuclear cells isolated from HIV-1-infected patients (21-24). By activating NF-κB and other important HIV-1 transcription factors, these cytokines may enhance virus replication, and are thus postulated to act as important mediators of viral expression and disease progression (25-27). A testable hypothesis is that such cytokines modulate the replication of HIV-1 within latently and/or productively infected cells. We now describe studies employing lymphoid culture techniques and cytokine treatments to investigate the *de novo* formation of an inducible reservoir of viral persistence within mature $CD4^+$ T-cells residing within the lymphoid tissue microenvironment.

METHODS

Preparation of viral stocks. NL4-3 was a gift from Malcom Martin via the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health. Infectious virus stocks were prepared by transfecting 293T cells with proviral DNA as previously described (28).

Culture and infection of human lymphoid tissues ex vivo. Human noninflammatory tonsil tissue removed during tonsillectomy (provided by Mills Peninsula Hospital, Kaiser-San Francisco, South San Francisco and San Rafael, CA) was prepared for lymphoid aggregate culture as previously described (19). Briefly, tonsil tissue was mechanically dispersed and the isolated cells were transferred to 96-well U-bottom plates at a concentration of $1x10^7$ cells per ml, 200μl per well. Cells collected at the bottom of the well and remained undisturbed for the remainder of the culture period. Tissues were inoculated within 24 hours of preparation with HIV-1 at approximately 100 $TCID_{50}$/well, as determined by terminal dilution of the virus stocks in quadruplicate on heterologous phytohemagglutinin-activated PBMC as described previously (20, 29).

Cytokine treatments and assessment of HIV-1 replication. Cultures were treated with the following cytokines alone or in combination: recombinant human (rh) IL-2 (200 IU/ml, a gift of Chiron Corp., Emeryville, CA), rhIL-6 (5 ng/ml, Sigma, St. Louis, MO), or rhTNF-α (2.5 ng/ml, Sigma). Culture media was also supplemented with zidovudine (AZT, 10μM, Sigma) and, as indicated, dexamethasone (10^{-8} M, Sigma). To monitor the

effects of cytokine treatment on HIV-1 replication, the concentration of p24 Gag within the culture media was quantitated by enzyme linked immunosorbent assay (ELISA, NEN Life Sciences, Boston, MA). Results are reported as the mean with standard error of the mean (SEM).

Assessment of CD4$^+$ T-cell infection by anti-p24 staining. Dispersed cells from infected and uninfected lymphoid cultures were immunostained for cell surface markers CD4, CD45RA, and CD62L. Cells were then fixed in 1% paraformaldehyde (PFA) for 10 minutes at room temperature and subsequently permeabilized and immunostained for intracellular CD4 and p24. 50,000 CD4$^+$ lymphocytes were counted and the data were analyzed with FlowJo software (Treestar, San Carlos, CA). To identify memory and naïve subsets the following monoclonal antibodies (mAbs) were used from Beckton Dickinson (BD, San Jose, CA): anti-CD4 (clone SK7, allophycocyanin (APC)-conjugated, 1:20 dilution), anti-CD62L (phycoerythrin (PE)-conjugated, 1:20 dilution). The following mAb from BD Pharmingen (San Jose, CA) was also used: anti-CD45RA (cychrome-conjugated, 1:20 dilution). To identify infected cells, anti-p24 (fluoroscein isothiocyanate (FITC)-conjugated, 1:100 dilution) from Coulter (Miami, FL) was also used. Titration/mixing studies demonstrated that the intracellular p24 staining assay was sensitive to a labeling frequency below 1.0%. Results are reported as the mean with SEM.

Determination of HIV-1 burst size following cytokine treatment. Following 1.5 days of cytokine treatment, the "burst size" of memory and naïve CD4$^+$ T-cells was

measured as previously described (19). Briefly, lymphoid cells were immunostained with a combination of mAbs from BD recognizing CD8 (clone SK1, FITC-conjugated, 1:20 dilution), CD14 (FITC-conjugated, 1:20 dilution), and CD19 (FITC-conjugated, 1:20 dilution). $CD4^+$ T-Cells were purified by anti-FITC magnetic micro-bead separation as per manufacturer instructions (Miltenyi Biotec, Auburn, CA) and then immunostained with mAbs recognizing CD62L (PE-conjugated) and CD45RA (ECD-conjugated, 1:20 dilution, Coulter). Naïve and memory cells were sorted with a FACSVantage (BD) and then lysed. Typically, in excess of $2x10^6$ cells of each phenotype were sorted from each donor used. Reanalysis of sorted populations immediately following collection demonstrated purities in excess of 98%. The p24 content of sorted cells was measured by ELISA.

Assessment of $CD4^+$ T-cell infection by vRNA detection. The frequency of productively infected cells was quantitated by performing ISH to detect HIV-$1_{gag\text{-}pol}$ mRNA transcripts (vRNA) with a cocktail of 134 fluoroscein-conjugated oligonucleotides. Dispersed cells from infected and uninfected lymphoid cultures were immunostained for the cell surface markers CD4 (clone SK7, APC-conjugated, 1:20 dilution, BD) and CD45RA (PE-conjugated, 1:20 dilution, BD Pharmingen). Cells were then fixed and prepared as per manufacturer instructions (ViroTect, Invirion, Chicago, IL). 20,000 $CD4^+$ lymphocytes were counted and the data were analyzed with FlowJo. Results are reported as the mean with SEM.

Flow cytometric analysis of cellular proliferation and apoptosis. Samples were cultured for 1.5 days in standard histoculture media supplemented with cytokines and/or BrdU (50 μM, Sigma). Following this incubation, cells were fixed and permeabilized overnight in a solution of 1% PFA and 0.01% Tween 20. Cells were washed and then treated with DNase (10 mg/ml, Sigma) in phosphate buffered saline (with calcium and magnesium) at 37° C. After washing, cells were immunostained with a combination of mAbs recognizing CD4, CD62L, CD45RA, and BrdU (FITC-conjugated, 1:2.5 dilution, Pharmingen) in 10% fetal bovine serum in PBS for 30 minutes on ice. Titration/mixing studies demonstrated that the BrdU assay was sensitive to a labeling frequency below 0.5%. Samples similarly treated with cytokines but not supplemented with BrdU were harvested and immunostained for 30 minutes with mAbs recognizing CD4, CD62L, CD45RA, and Annexin-V (FITC-conjugated, 1:50 dilution, Caltag, Burlingame, CA). For both studies 50,000 lymphocytes were collected and analyzed by FlowJo. Results are reported as the mean with SEM.

RESULTS

Cytokine treatment enhances HIV-1 replication within lymphoid tissues. We investigated whether treatment of lymphoid tissues *ex vivo* with cytokines could induce HIV-1 replication within infected $CD4^+$ T-cells characterized by otherwise undetectable viral expression. To establish an experimental approach in which viral spread is prevented, we began by infecting human tonsil cultures with the CXCR4-dependent (X4) HIV-1 molecular clone NL4-3 (29-32) in the presence or absence of AZT. Following a 5-day incubation, samples were harvested and immunostained for CD4 and intracellular expression of HIV-1 p24 Gag, a marker of productively infected cells. We found that AZT effectively inhibited greater than 99.9% of HIV-1 replication and spread within these cultures (Figure 1A). In addition, the concentration of soluble p24 within the supernatants from AZT-treated cultures was below the limit of detection by ELISA despite significant replication within untreated samples (data not shown). These findings demonstrate the efficacy of AZT treatment to inhibit viral spread within these tissues.

We next infected lymphoid cultures with NL4-3 and allowed the infections to proceed for 5 days in the absence of AZT. The culture media was then supplemented with AZT at the concentration shown earlier to prevent further viral spread, and replicate samples were incubated with IL-2, IL-6, and/or TNF-α alone or in combination with dexamethasone, which inhibits the activation of NF-κB by these cytokines and has been shown to inhibit HIV-1 replication *in vitro* (27). Following an additional 1.5-day incubation, the culture media was harvested, and the concentration of soluble p24 was

quantitated by ELISA as an indicator of virus output in the absence of recruitment of new target cells via viral spread (Figure 1B). Treatment with IL-2, IL-6, or TNF-α singly had little effect on the output of HIV-1 within these cultures. Nevertheless, dexamethasone significantly reduced the production of virus within these tissues, suggesting that this glucocorticoid effectively reduced the basal level of intracellular transcription factors present in unstimulated tissues. Importantly, treatment with the cocktail of the three cytokines under these conditions resulted in a nearly 200% increase in HIV-1 production. Additional treatment with dexamethasone prevented this augmentation, thereby restricting virus production to a level approximating that observed in untreated cultures. We conclude that the cytokine cocktail efficiently stimulates the production of HIV-1 within lymphoid tissues *ex vivo*, and that glucocorticoid treatment attenuates this simulation. Since the cocktail of three cytokines had the greatest stimulatory effect on HIV-1 production within these cultures, subsequent studies of their mechanism of action focused on this stimulatory condition.

Two major explanations may account for the increased replication of HIV-1 following cytokine treatment. First, a cytokine-dependent increase in the levels of transcription factors within target cells may elevate the transcription of viral RNA, thereby enhancing the production of HIV-1 by cells already actively replicating the virus. That is, the "burst size" of individual, productively infected T-cells may increase in response to cytokines. Second, cytokine-induced increase in levels of transcription factors

within quiescent T-cells may convert latently infected cells to a productive state of viral replication. The following studies tested these mutually non-exclusive alternative models.

Cytokines increase the replication of HIV-1 within productively infected CD4$^+$ T-cells. To determine if cytokines increased the per-cell production of HIV-1 within productively infected T-cells, we again inoculated cultures with NL4-3 and allowed the infection to proceed for 5 days. Culture media was then supplemented with AZT with or without the cytokine cocktail, and with or without dexamethasone. Following an additional 1.5 days of culture, the tissues were harvested and immunostained for memory and naïve T-cell surface markers as well as intracellular expression of p24. We next measured the geometric mean fluorescence intensity of intracellular p24 within productively infected (p24$^+$) cells by flow cytometry. Since this value is proportional to the intracellular concentration of p24 within infected cells, it provides a measure of the average relative concentration of p24 within cells isolated from cytokine-treated and untreated tissues. We found that both memory and naïve cells within tissues incubated with cytokines exhibited a modest increase in the intracellular concentration of p24 compared to cells within untreated tissues (Figure 2A). Not surprisingly, dexamethasone prevented this effect in both cell populations. Indeed, glucocorticoid treatment alone or in the presence of cytokines slightly reduced intracellular p24 concentrations to levels below that seen in untreated tissues.

An alternative analysis was performed in parallel using samples inoculated with NL4-3 and treated with AZT and cytokines as described above. Cultures were harvested

following 1.5 days of cytokine treatment, and then negatively selected using antibodies to CD8 (cytotoxic T-cells), CD14 (macrophages), and CD19 (B-cells) and magnetic bead separation. The enriched $CD4^+$ T-lymphocytes were sorted by FACS into naïve and memory T-cell fractions by using CD45RA/CD62L staining as described previously (19). To determine the proportion of these 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 lysed and the "cell-associated p24" content was quantitated by ELISA; for convenience in comparisons, this value was adjusted by dividing by the average p24 content per HIV-1 virion (approximately 1800 molecules per virion) (33-36) to yield a value representing "virion equivalents per infected cell" (19). Consistent with the earlier results, we found that cytokine treatment modestly increased the intracellular concentration of p24 within both memory and naïve $CD4^+$ T-cells (Figure 2B). This small increase in the "burst size" of productively infected T-cells appears to be insufficient to account for the 3-fold increase in viral output observed earlier (Figure 1B).

Cytokines increase the frequency of productively infected $CD4^+$ T-cells. Since a cytokine-induced increase in the production of HIV-1 by infected T-cells did not adequately explain the increase in viral output we measured, we next determined if an increase in the frequency of productively infected cells might be contributory. As before, following a 5-day infection with NL4-3 we supplemented the culture media with AZT with or without the cytokine cocktail, and with or without dexamethasone. The tissues were harvested 1.5 days later and stained for memory and naïve T-cell surface markers as

well as for intracellular p24. Compared to untreated samples, cytokines increased the frequency of productively infected memory cells 50% and the frequency of productively infected naïve cells nearly 100% (Figure 3A). Predictably, dexamethasone significantly reduced each of these values.

We next sought to confirm that the increase in the frequency of cells expressing detectable levels of viral antigen was correlated with a similar increase in the percentage of $CD4^+$ T-cells expressing viral mRNA transcripts. We utilized a unique flow-based *in situ* hybridization (ISH) technique that employs a cocktail of 134 fluorescently-labeled oligonucleotide probes to identify cells containing *gag* or *pol* RNA transcripts (37, 38). This method has been shown to detect cells containing as few as 10 viral transcripts, and may be combined with traditional immunostaining of samples for detection of cell-surface antigens, thereby providing a highly sensitive and independent method to quantify the frequency of productively infected T-cells within the cultures.

Tonsil tissue from a different donor was inoculated with NL4-3 and incubated with cytokines and AZT as described above. At the end of the treatment, samples were immunostained for surface expression of CD45RA and CD4 and labeled by ISH. As expected, significantly more memory cells were infected compared with naïve cells (Figure 3B). Incubation with cytokines resulted in a greater than 100% increase in the frequency of productively infected memory and naïve $CD4^+$ T-cells compared with untreated samples (Figure 3B), consistent with the previous results. Parallel samples from the same donor tissue were also immunostained to detect intracellular p24 and revealed a

similar level and pattern of infection as detected by ISH (data not shown). We therefore conclude that, even in the absence of viral spread, the cytokine-mediated increase in the proportion of cells that are productively infected is the principal factor contributing to the increased viral replication observed earlier (Figure 1B). Collectively, these results suggest that HIV-1 may silently infect both memory and naïve T-cells *de novo*, and that such cells serve as inducible, cytokine-responsive reservoirs of viral persistence.

IL-2, IL-6, and TNF-α reduce the rate of apoptosis of memory CD4⁺ T-cells. The cellular effects of IL-2, IL-6, and TNF-α may well extend beyond induction of HIV-1 replication, and a significant increase in the proliferation or apoptosis of select T-cell populations may obscure or confound the interpretation that cytokine treatment recruits inactive virus within T-cells to a productive state. We therefore examined the proliferation and apoptosis of untreated and treated cells in these *ex vivo* tissues. To investigate the possibility that cytokines might alter the rate or extent of cellular division, we inoculated tissue with NL4-3 and incubated the samples for 5 days. We subsequently supplemented the media with bromodeoxyuridine (BrdU) together with AZT with or without the cocktail of cytokines. After an additional 1.5-day incubation the samples were immunostained for surface expression of CD4, CD45RA, and CD62L, and intracellular incorporation of BrdU, a marker of cellular division. As previously shown (19), we found that memory cells exhibited a significantly greater rate of proliferation than naïve cells (Figure 4A). Most importantly, no significant change in the proliferative activity of either memory or naïve T-cells was observed in cultures treated with the

cocktail of cytokines compared with untreated cultures. These findings imply that the increased frequency of productively infected T-cells in the presence of cytokines is not a consequence of the selective expansion of productively infected cells relative to uninfected bystanders.

However, the possibility remained that cytokine treatment may selectively reduce the rate of apoptosis of productively infected T-cells, a population of cells sensitive to the cytopathic effects of HIV-1. To test this possibility, we inoculated cultures with NL4-3 and later incubated the samples with AZT with or without the cocktail of cytokines as described above. Following treatment, the samples were again immunostained with antibodies recognizing CD4, CD45RA, CD62L as well with Annexin V to detect exoplasmic phosphatidylserine, a marker of apoptosis. Consistent with the higher rate of HIV-1 infection within the memory cell subset, this population exhibited a significantly higher rate of apoptosis than did naïve cells (Figure 4B). Importantly, the combination of IL-2, IL-6, and TNF-α induced a 3-fold reduction in the frequency of memory $CD4^+$ T-cells staining with Annexin V. However, no significant reduction was observed in the frequency of apoptotic naïve $CD4^+$ T-cells. Consistent with these findings, we also detected an increase in the relative abundance of the memory subset of $CD4^+$ T-cells (data not shown) in the presence of cytokines as well as a decrease in the proportion of naïve cells. These results suggest that cytokines suppress apoptosis among productively infected memory T-cells, thereby extending the half-life of cells that would otherwise have succumbed to the cytopathic effects of the virus. As a result, these $p24^+$ and $vRNA^+$

memory cells may persist within these tissues and thus contribute to the observed increase in the output of HIV-1 (Figure 1B) as well as to the increase in the frequency of productively infected memory cells (Figure 3A and B) following cytokine treatment. We therefore infer that the majority of the increase in virus output from memory cells is due to the persistence of productively infected cells beyond their typical life-span rather than to induction from a latent state of HIV-1 infection. In contrast, since these cytokines caused no detectable change in the rate of apoptosis among naïve cells, we conclude that the increased frequency of productive replication within infected naïve T-cells upon cytokine stimulation is instead due to induction of dormant HIV-1 to actively replicating virus within previously quiescently-infected cells.

DISCUSSION

Although the presence of latently infected T-cells within HIV-infected patients has been well documented (1-3), the mechanism(s) by which they are generated *in vivo* are not well understood. One hypothesis holds that inactivation of an infected host cell and the resulting fall in cellular transcription—as occurs when stimulated effector cells occasionally return to a resting, memory phenotype—is central to the development of quiescent cells harboring transcriptionally silent provirus. Subsequent exposure of such latently infected resting, memory cells to activating stimuli may increase the availability of transcription factors and induce expression of the latent virus (25, 27).

Interestingly, a similar pathway may also underlie the generation of latently infected naïve T-cells, a population recently identified *in vivo* (8). These cells may acquire integrated HIV-1 as thymocytes (39) before entering the circulation and/or secondary lymphoid sites as mature T-cells. Consistent with the inactivation model, subsequent migration from the thymus and diminution in the cellular activation state and availability of transcription factors may generate latently infected peripheral T-cells, as has been demonstrated recently within SCID-hu (Thy/Liv) mice (15). Although latently infected naïve T-cells were not specifically identified in these studies, it is reasonable to speculate that this pathway contributes to the generation of latently infected naïve T-cells in the peripheral circulation *in vivo*. However, since thymopoiesis is extremely limited in many adult patients with HIV disease (16-18), the overall contribution of this pathway may itself be limited.

Although previous *in vitro* studies have shown that these naïve T-cells do not support complete HIV-1 replication (9-11), we have previously demonstrated that within the lymphoid microenvironment naïve $CD4^+$ T-cells support productive infection by X4 HIV-1 (19). We have now shown that infection of these cells may alternatively result in latent viral infection. Furthermore, delivery of the proinflammatory cytokines IL-2, IL-6, and TNF-α efficiently mobilizes this viral reservoir, resulting in a doubling of the frequency of infected naïve $CD4^+$ T-cells that produce virus following stimulation even in the absence of viral spread to new target cells. Although these studies did not distinguish between pre- and post-integration latency, the dramatic increase in the frequency of productively infected cells following cytokine treatment nevertheless suggests that the provirus within these latently infected naïve cells is maintained in a stable, replication-competent state. These findings contrast with earlier *in vitro* reports based on peripheral blood lymphocytes indicating that HIV-1 replication within naïve and resting lymphocytes is blocked at a step in the viral life cycle preceding nuclear import of the preintegration complex, which may be only inefficiently overcome upon subsequent stimulation of these cells (12-14). Such differences underscore the importance of secondary lymphoid organs, and the microenvironment therein, to HIV-1 pathogenesis. We infer that signals delivered to neighboring naïve T-cells by antigen presenting cells and other cell types within lymphoid organs promote the efficient completion of reverse transcription and subsequent stages of the viral replication cycle within these target cells (9, 10, 12-14, 40-44).

The present studies also identified a significant cytokine-dependent increase in the frequency of productively infected memory T-cells in the absence of viral spread. Notably, cytokine stimulation concurrently resulted in a significant reduction in the frequency of apoptotic cells within this population, a well-described effect of IL-2 and other cytokines. Because productively infected cells are among the cells most likely to undergo apoptosis within these tissues, we conclude that the exogenous cytokines artificially supported the continued viability of productively infected memory cells that would otherwise have succumbed to the cytopathic effects of the virus. Therefore, these infected cells persisted in the cultures, and contributed substantially to the production of soluble p24 as well as the observed increase in the representation of $p24^+$ and $vRNA^+$ memory cells in the cultures. These effects make it difficult to determine if a subset of memory cells may have been latently infected by HIV-1 within these cultures. Nevertheless, these results highlight the importance of immune activation within HIV-1-infected patients. Circulation of mononuclear cells through lymphoid tissues and exposure to the locally high concentrations of cytokines therein may play an important role in the pathogenesis of HIV-1 by upregulating viral gene expression and by sustaining the viability of cells that contribute to the local viral load. The proinflammatory milieu of these tissues may contribute substantially both to the establishment of productive infection and to the induction from latency within naïve lymphocytes, and may also mediate the persistence of viral expression within memory T-cells.

Importantly, we have identified a novel pathway for the direct generation of latently infected mature naïve T-cells. Specifically, as our previous studies have revealed that naïve T-cells within lymphoid cultures proliferate only rarely and exhibit a minimal degree of activation, the generation of latently infected naïve cells within this tissue must occur independently of a transition of the host cell from an activated state to an inactivated one. These results implicate a unique, activation-independent pathway for the generation of latently infected T-cells. Such a pathway may contribute significantly to the generation of latently infected naïve T-cells in patients in whom thymopoiesis is compromised. Therefore, within this specific cell population, HIV-1 may proceed along two divergent pathways: productive infection or latency. The specific factors that direct the choice between these divergent *in vivo* fates are not known.

A more complete understanding of the factors governing the outcome of viral infection may yield important clinical insights into the control and eradication of the latent reservoir. The present studies highlight the potentially important role of naïve T-cells within infected individuals as inducible reservoirs of viral persistence. Recruitment of the provirus within these cells to a productive state may contribute to the bursts of viremia frequently observed in patients receiving HAART, to the continued evolution of viral quasiespecies in these patients, and to the profound immunodeficiency characteristic of late-stage HIV-disease.

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REFERENCES

1. Chun, T.W., L. Stuyver, S.B. Mizell, L.A. Ehler, J.A. Mican, M. Baseler, A.L. Lloyd, M.A. Nowak, and A.S. Fauci. 1997. Presence of an inducible HIV-1 latent reservoir during highly active antiretroviral therapy. *Proc. Natl. Acad. Sci. USA*. 94:13193-13197.
2. Finzi, D., M. Hermankova, T. Pierson, L.M. Carruth, C. Buck, R.E. Chaisson, T.C. Quinn, K. Chadwick, J. Margolick, R. Brookmeyer, J. Gallant, M. Markowitz, D.D. Ho, D.D. Richman, and R.F. Siliciano. 1997. Identification of a reservoir for HIV-1 in patients on highly active antiretroviral therapy. *Science*. 278:1295-1300.
3. Wong, J.K., M. Hezareh, H.F. Günthard, D.V. Havlir, C.C. Ignacio, C.A. Spina, and D.D. Richman. 1997. Recovery of replication-competent HIV despite prolonged suppression of plasma viremia. *Science*. 278:1291-1295.
4. Chun, T.W., D. Engel, M.M. Berrey, T. Shea, L. Corey, and A.S. Fauci. 1998. Early establishment of a pool of latently infected, resting CD4(+) T cells during primary HIV-1 infection. *Proc. Natl. Acad. Sci. USA*. 95:8869-8873.
5. Chun, T.W., L. Carruth, D. Finzi, X. Shen, J.A. DiGiuseppe, H. Taylor, M. Hermankova, K. Chadwick, J. Margolick, T.C. Quinn, Y.H. Kuo, R. Brookmeyer, M.A. Zeiger, P. Barditch-Crovo, and R.F. Siliciano. 1997. Quantification of latent tissue reservoirs and total body viral load in HIV-1 infection. *Nature*. 387:183-188.
6. Finzi, D., J. Blankson, J.D. Siliciano, J.B. Margolick, K. Chadwick, T. Pierson, K. Smith, J. Lisziewicz, F. Lori, C. Flexner, T.C. Quinn, R.E. Chaisson, E. Rosenberg,

B. Walker, S. Gange, J. Gallant, and R.F. Siliciano. 1999. Latent infection of CD4+ T cells provides a mechanism for lifelong persistence of HIV-1, even in patients on effective combination therapy. *Nat. Med.* 5:512-517.

7. Zhang, L., B. Ramratnam, K. Tenner-Racz, Y. He, M. Vesanen, S. Lewin, A. Talal, P. Racz, A.S. Perelson, B.T. Korber, M. Markowitz, and D.D. Ho. 1999. Quantifying residual HIV-1 replication in patients receiving combination antiretroviral therapy. *N. Engl. J. Med.* 340:1605-1613.
8. Pierson, T., T.L. Hoffman, J. Blankson, D. Finzi, K. Chadwick, J.B. Margolick, C. Buck, J.D. Siliciano, R.W. Doms, and R.F. Siliciano. 2000. Characterization of chemokine receptor utilization of viruses in the latent reservoir for human immunodeficiency virus type 1. *J.Virol.* 74:7824-7833.
9. Zack, J.A., S.J. Arrigo, S.R. Weitsman, A.S. Go, A. Haislip, and I.S. Chen. 1990. HIV-1 entry into quiescent primary lymphocytes: molecular analysis reveals a labile, latent viral structure. *Cell.* 61:213-222.
10. Stevenson, M., T.L. Stanwick, M.P. Dempsey, and C.A. Lamonica. 1990. HIV-1 replication is controlled at the level of T cell activation and proviral integration. *EMBO J.* 9:1551-1560.
11. Bukrinsky, M.I., N. Sharova, M.P. Dempsey, T.L. Stanwick, A.G. Bukrinskaya, S. Haggerty, and M. Stevenson. 1992. Active nuclear import of human immunodeficiency virus type 1 preintegration complexes. *Proc. Natl. Acad. Sci. USA.* 89:6580-6584.

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

13. Zack, J.A., A.M. Haislip, P. Krogstad, and I.S. Chen. 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.

14. Spina, C.A., J.C. Guatelli, and D.D. Richman. 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.

15. Brooks, D.G., S.G. Kitchen, C.M. Kitchen, D.D. Scripture-Adams, and J.A. Zack. 2001. Generation of HIV latency during thymopoiesis. [Comment In: Nat Med. 2001 Apr;7(4):404-5 UI: 21180037]. *Nat. Med.* 7:459-464.

16. McCune, J.M., R. Loftus, D.K. Schmidt, P. Carroll, D. Webster, L.B. Swor-Yim, I.R. Francis, B.H. Gross, and R.M. Grant. 1998. High prevalence of thymic tissue in adults with human immunodeficiency virus-1 infection. *Clin. Invest.* 101:2301-2308.

17. Jenkins, M., M.B. Hanley, M.B. Moreno, E. Wieder, and J.M. McCune. 1998. Human immunodeficiency virus-1 infection interrupts thymopoiesis and multilineage hematopoiesis in vivo. *Blood.* 91:2672-2678.

18. Smith, K.Y., H. Valdez, A. Landay, J. Spritzler, H.A. Kessler, E. Connick, D. Kuritzkes, B. Gross, I. Francis, J.M. McCune, and M.M. Lederman. 2000. Thymic size and lymphocyte restoration in patients with human immunodeficiency virus

infection after 48 weeks of zidovudine, lamivudine, and ritonavir therapy. *J. Infect. Dis.* 181:141-147.

19. Eckstein, D.A., M.L. Penn, Y.D. Korin, D.D. Scripture-Adams, J.A. Zack, J.F. Kreisberg, M. Roederer, M.P. Sherman, P.S. Chin, and M.A. Goldsmith. 2001. HIV-1 actively replicates in naive CD4+ T-cells residing within human lymphoid tissue. *Immunity*. In press.
20. Glushakova, S., B. Baibakov, J. Zimmerberg, and L.B. Margolis. 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.
21. Bélec, L., D. Meillet, A. Hernvann, G. Grésenguet, and R. Gherardi. 1994. Differential elevation of circulating interleukin-1 beta, tumor necrosis factor alpha, and interleukin-6 in AIDS-associated cachectic states. *Clin. Diagn. Lab. Immunol.* 1:117-120.
22. Roux-Lombard, P., C. Modoux, A. Cruchaud, and J.M. Dayer. 1989. Purified blood monocytes from HIV 1-infected patients produce high levels of TNF alpha and IL-1. *Clin. Immunol. Immunopathol.* 50:374-384.
23. Hober, D., A. Haque, P. Wattre, G. Beaucaire, Y. Mouton, and A. Capron. 1989. Production of tumour necrosis factor-alpha (TNF-alpha) and interleukin-1 (IL-1) in

patients with AIDS. Enhanced level of TNF-alpha is related to a higher cytotoxic activity. *Clin. Exp. Immunol.* 78:329-333.

24. Navikas, V., J. Link, C. Persson, T. Olsson, B. Höjeberg, A. Ljungdahl, H. Link, and B. Wahren. 1995. Increased mRNA expression of IL-6, IL-10, TNF-alpha, and perforin in blood mononuclear cells in human HIV infection. *J. Acquir. Immune. Defic. Syndr. Hum. Retrovirol.* 9:484-489.
25. Poli, G., P. Bressler, A. Kinter, E. Duh, W.C. Timmer, A. Rabson, J.S. Justement, S. Stanley, and A.S. Fauci. 1990. Interleukin 6 induces human immunodeficiency virus expression in infected monocytic cells alone and in synergy with tumor necrosis factor alpha by transcriptional and post-transcriptional mechanisms. *J. Exp. Med.* 172:151-158.
26. Osborn, L., S. Kunkel, and G.J. Nabel. 1989. Tumor necrosis factor alpha and interleukin 1 stimulate the human immunodeficiency virus enhancer by activation of the nuclear factor kappa B. *Proc. Natl. Acad. Sci. USA.* 86:2336-2340.
27. Chun, T.W., D. Engel, S.B. Mizell, L.A. Ehler, and A.S. Fauci. 1998. Induction of HIV-1 replication in latently infected CD4+ T cells using a combination of cytokines. *J. Exp. Med.* 188:83-91.
28. Atchison, R.E., J. Gosling, F.S. Monteclaro, C. Franci, L. Digilio, I.F. Charo, and M.A. Goldsmith. 1996. Multiple extracellular elements of CCR5 and HIV-1 entry: dissociation from response to chemokines. *Science.* 274:1924-1926.

29. Penn, M.L., J.C. Grivel, B. Schramm, M.A. Goldsmith, and L. Margolis. 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.

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

31. Speck, R.F., K. Wehrly, E.J. Platt, R.E. Atchison, I.F. Charo, D. Kabat, B. Chesebro, and M.A. Goldsmith. 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.

32. Schols, D., J.A. Esté, C. Cabrera, and E. De Clercq. 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.

33. Luftig, R., K. Ikuta, M. Bu, and P. Calkins. 1990. Terminal stages of retroviral morphogenesis. *In* Retrovial proteases: Maturation and morphogenesis. L.H. Pearl, editor. Macmillan, London. 141-148.

34. Layne, S.P., M.J. Merges, M. Dembo, J.L. Spouge, S.R. Conley, J.P. Moore, J.L. Raina, H. Renz, H.R. Gelderblom, and P.L. Nara. 1992. Factors underlying spontaneous inactivation and susceptibility to neutralization of human immunodeficiency virus. *Virology*. 189:695-714.

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

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

37. Brodie, S.J., D.A. Lewinsohn, B.K. Patterson, D. Jiyamapa, J. Krieger, L. Corey, P.D. Greenberg, and S.R. Riddell. 1999. In vivo migration and function of transferred HIV-1-specific cytotoxic T cells. *Nat. Med.* 5:34-41.

38. Brodie, S.J., B.K. Patterson, D.A. Lewinsohn, K. Diem, D. Spach, P.D. Greenberg, S.R. Riddell, and L. Corey. 2000. HIV-specific cytotoxic T lymphocytes traffic to lymph nodes and localize at sites of HIV replication and cell death [see comments]. *J. Clin. Invest.* 105:1407-1417.

39. Papiernik, M., Y. Brossard, N. Mulliez, J. Roume, C. Brechot, F. Barin, A. Goudeau, J.F. Bach, C. Griscelli, R. Henrion, and et al. 1992. Thymic abnormalities in fetuses aborted from human immunodeficiency virus type 1 seropositive women. *Pediatrics.* 89:297-301.

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

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

42. Moran, P.A., M.L. Diegel, J.C. Sias, J.A. Ledbetter, and J.M. Zarling. 1993. Regulation of HIV production by blood mononuclear cells from HIV-infected donors: I. Lack of correlation between HIV-1 production and T cell activation. *AIDS Res. Hum. Retroviruses.* 9:455-464.

43. Tsunetsugu-Yokota, Y., K. Akagawa, H. Kimoto, K. Suzuki, M. Iwasaki, S. Yasuda, G. Häusser, C. Hultgren, A. Meyerhans, and T. Takemori. 1995. Monocyte-derived cultured dendritic cells are susceptible to human immunodeficiency virus infection and transmit virus to resting T cells in the process of nominal antigen presentation. *J. Virol.* 69:4544-4547.

44. Frank, I., L. Kacani, H. Stoiber, H. Stössel, M. Spruth, F. Steindl, N. Romani, and M.P. Dierich. 1999. Human immunodeficiency virus type 1 derived from cocultures of immature dendritic cells with autologous T cells carries T-cell-specific molecules on its surface and is highly infectious. *J. Virol.* 73:3449-3454.

FIGURE LEGENDS

FIGURE 1

Replication of HIV-1 within human lymphoid cultures in the presence or absence of cytokines and dexamethasone. (A) Tonsil specimens were inoculated with NL4-3 in the presence or absence of AZT and incubated for 5 days. Tissues were then harvested and immunostained with anti-CD4 and anti-p24 mAbs. (B) Tissues were treated with cytokines and/or dexamethasone (Dex) in the presence of AZT for 1.5 days following an initial 5-day incubation with NL4-3. Culture media was then harvested and the concentration of soluble p24 was quantitated by ELISA. Shown are mean values with SEM (n=3) of a representative experiment from among experiments performed with 3 different donor tissues.

FIGURE 2

Burst size of HIV-1 within productively infected $CD4^+$ T-cells following cytokine treatment. Tissues were treated with cytokines and/or Dex in the presence of AZT for 1.5 days following an initial 5-day incubation with NL4-3. (A) Geometric mean fluorescence of intracellular p24 labeled with FITC-conjugated anti-p24 mAb within memory or naïve $CD4^+$ T-cells following treatment. Shown are mean values with SEM (n=3) of a representative experiment from among experiments performed with 4 different donor tissues. (B) $CD4^+$ T-cells from tonsil tissues were stained with anti-CD45RA and anti-

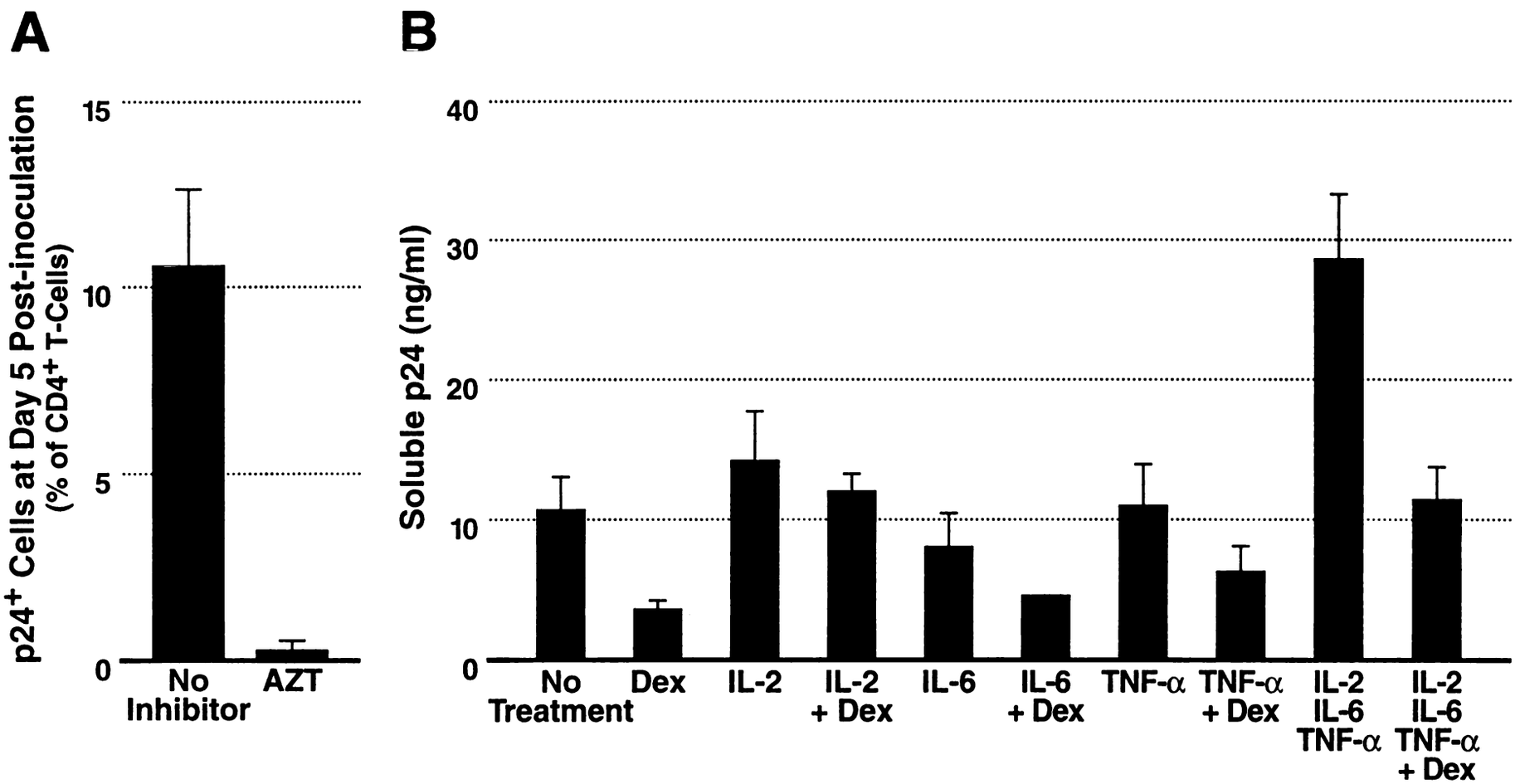
CD62L mAbs and sorted by flow cytometry and lysed. The average p24 content of productively infected memory and naïve T-cells was quantified by ELISA. Shown is a typical experiment from among 3 with indistinguishable results.

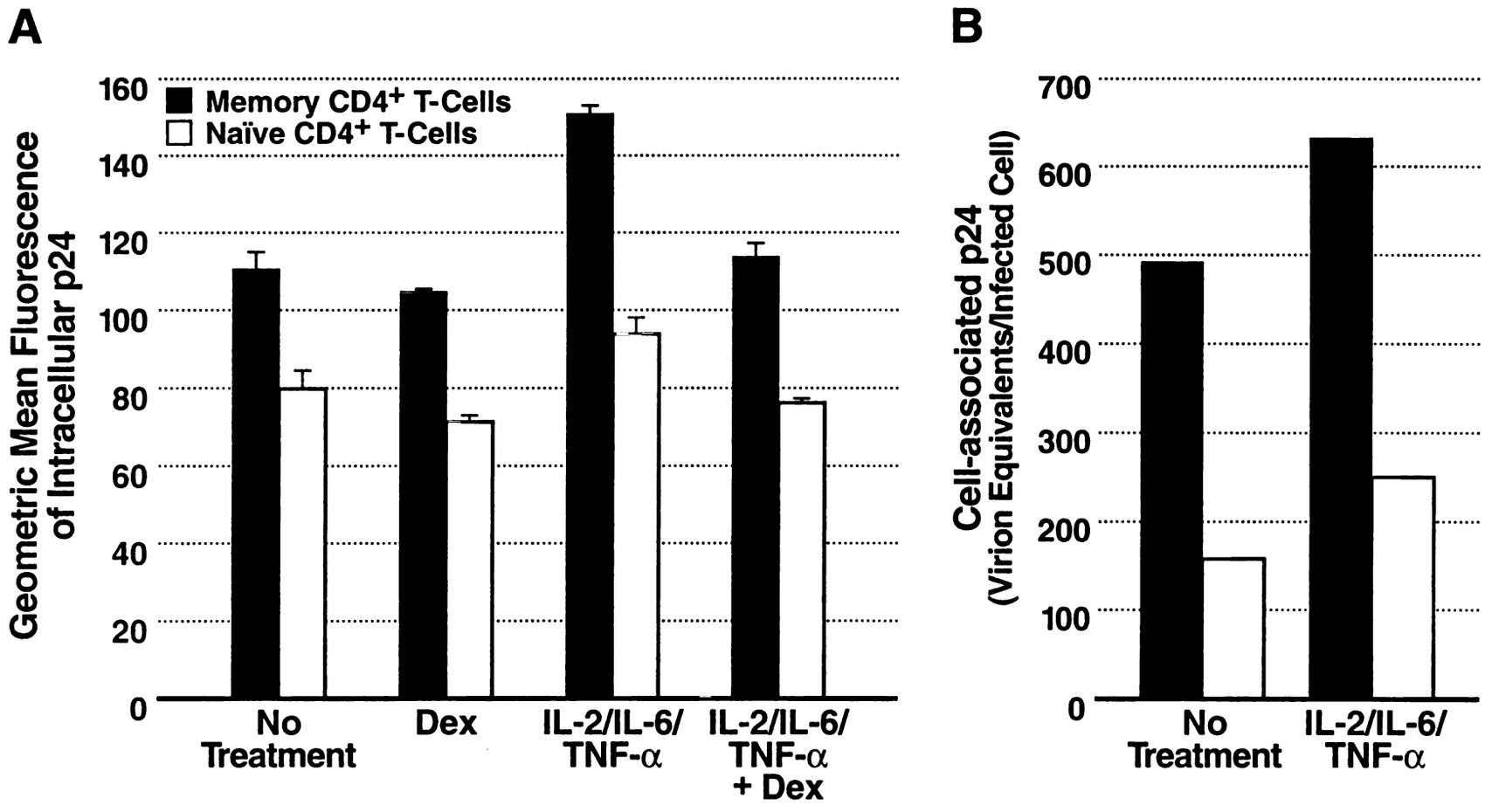
FIGURE 3

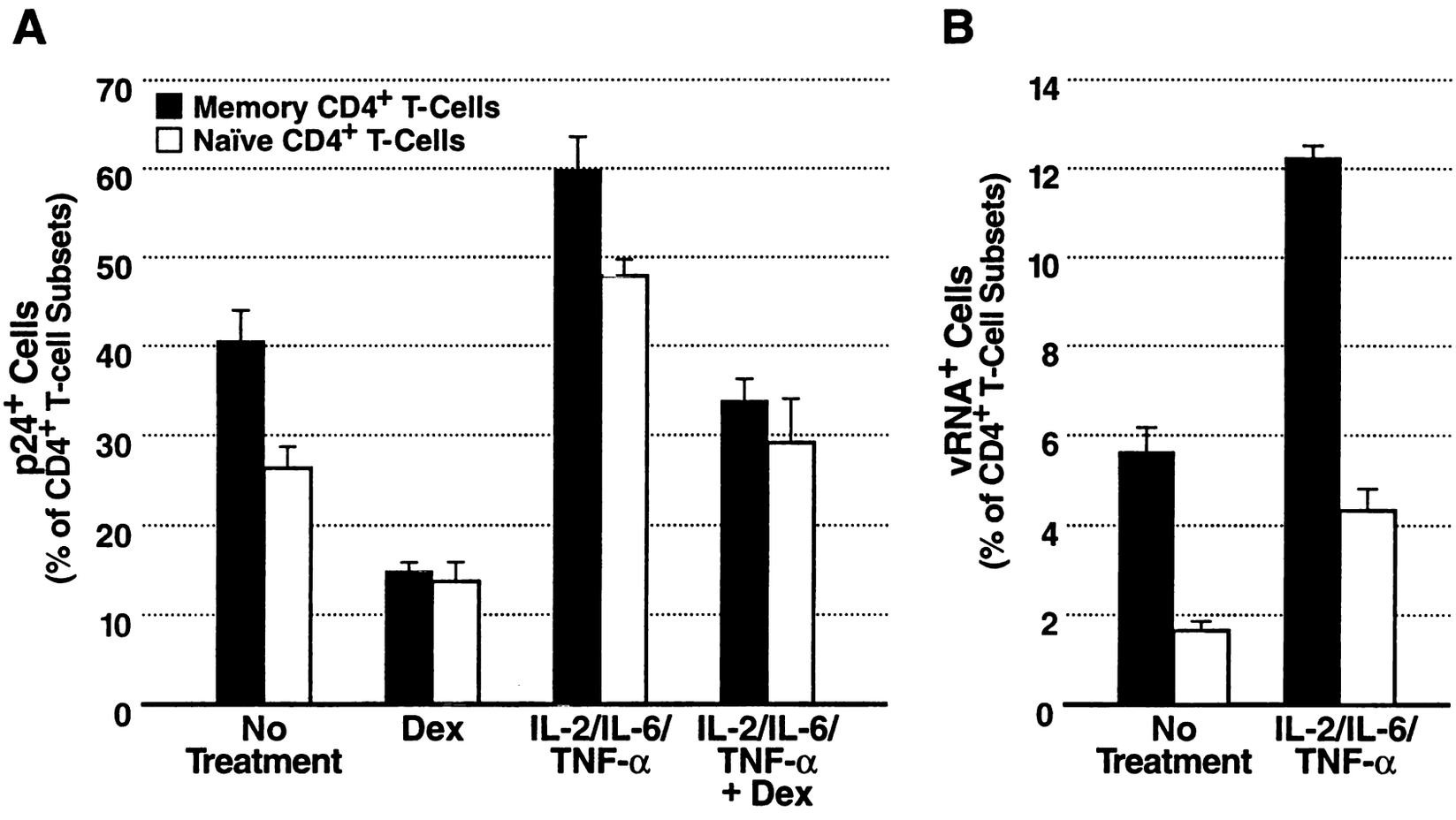
Frequency of productively infected $CD4^+$ T-cells following cytokine treatment. Tissues were treated with cytokines and/or Dex in the presence of AZT for 1.5 days following an initial 5-day incubation with NL4-3. (A) Frequency of memory or naïve $CD4^+$ T-cells expressing detectable quantities of intracellular p24. (B) Frequency of memory or naive $CD4^+$ T-cells expressing detectable quantities of vRNA. Shown are mean values with SEM (n=3) of representative experiments from among experiments performed with 3-4 different donor tissues.

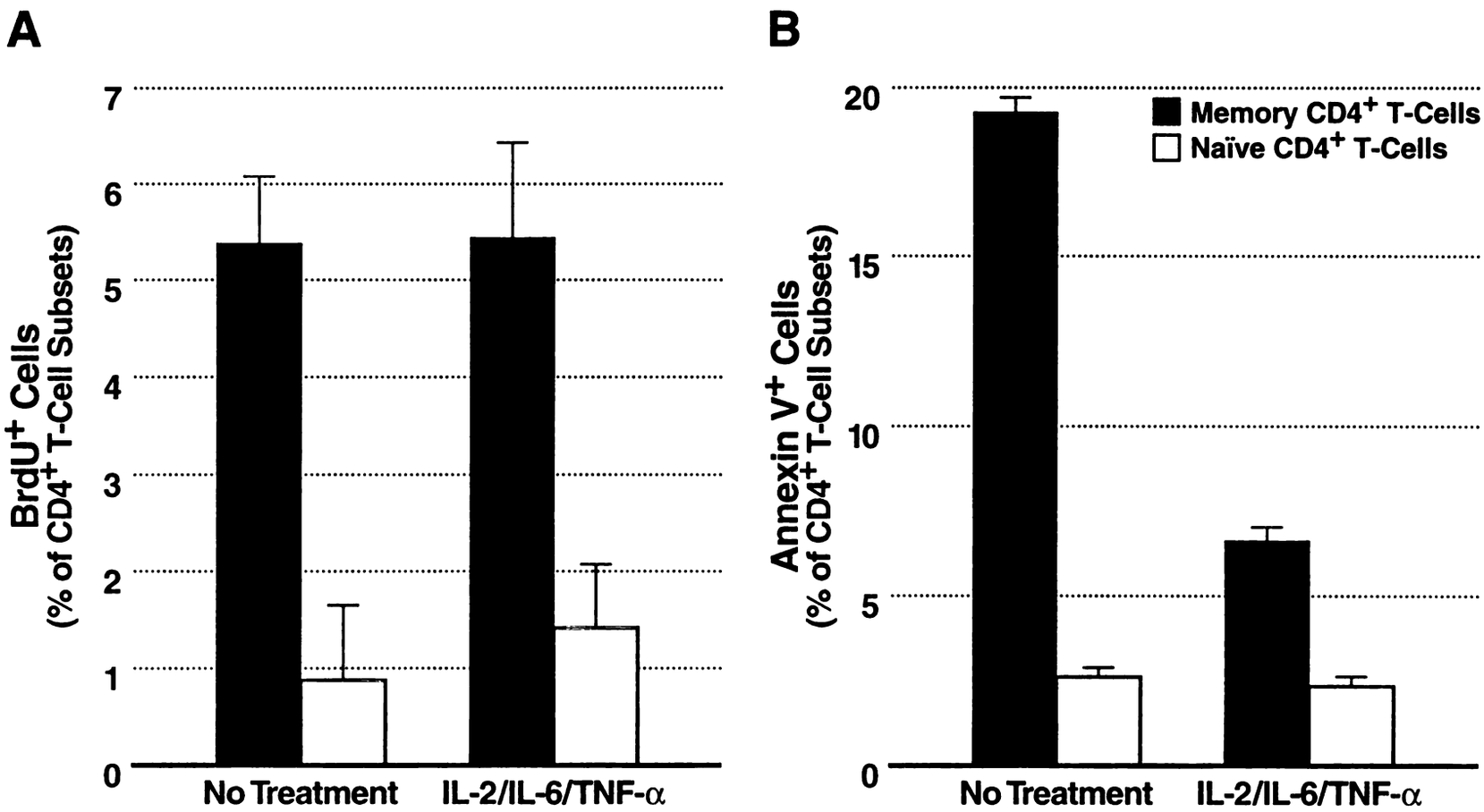
FIGURE 4

Affect of cytokine treatment on the proliferation and apoptosis of $CD4^+$ T-cells. Tissues were treated with cytokines and/or Dex in the presence of AZT for 1.5 days following an initial 5-day incubation with NL4-3. (A) Frequency of memory or naïve $CD4^+$ T-cells that incorporated BrdU during the cytokine incubation. (B) Frequency of memory or naïve $CD4^+$ T-cells staining positive for Annexin V following the cytokine treatment. Shown are mean values with SEM (n=3) of a representative experiment from among experiments performed with 3 different donor tissues


A
p24+ Cells at Day 5 Post-inoculation
(% of CD4+ T-Cells)
15
10
5
0
No Inhibitor
AZT
B
Soluble p24 (ng/ml)
40
30
20
10
0
No Treatment
Dex
IL-2
IL-2 + Dex
IL-6
IL-6 + Dex
TNF-α
TNF-α + Dex
IL-2 IL-6 TNF-α
IL-2 IL-6 TNF-α + Dex


A
Geometric Mean Fluorescence of Intracellular p24
160
140
120
100
80
60
40
20
0
Memory CD4+ T-Cells
Naïve CD4+ T-Cells
No Treatment
Dex
IL-2/IL-6/ TNF-α
IL-2/IL-6/ TNF-α + Dex
B
Cell-associated p24 (Virion Equivalents/Infected Cell)
700
600
500
400
300
200
100
0
No Treatment
IL-2/IL-6/ TNF-α


A
Memory CD4+ T-Cells
Naïve CD4+ T-Cells
p24+ Cells
(% of CD4+ T-cell Subsets)
70
60
50
40
30
20
10
0
No Treatment
Dex
IL-2/IL-6/ TNF-α
IL-2/IL-6/ TNF-α + Dex
B
vRNA+ Cells
(% of CD4+ T-Cell Subsets)
14
12
10
8
6
4
2
0
No Treatment
IL-2/IL-6/ TNF-α


A
BrdU+ Cells
(% of CD4+ T-Cell Subsets)
7
6
5
4
3
2
1
0
No Treatment
IL-2/IL-6/TNF-α
B
Annexin V+ Cells
(% of CD4+ T-Cell Subsets)
20
15
10
5
0
Memory CD4+ T-Cells
Naïve CD4+ T-Cells
No Treatment
IL-2/IL-6/TNF-α

Chapter 6

HIV-1 Enhances Viral Burden by Facilitating Infection of Tissue Macrophages but Not Non-dividing $CD4^+$ T-cells

Prologue

We had previously demonstrated that HIV-1 productively infects non-dividing cells, including naïve T-cells and macrophages, within human lymphoid tissues (Chapters 2 and 3). This distinctive feature of HIV and other lentiviruses is attributed to the capacity of the viral preintegration complex to enter the nucleus of quiescent cells. Several gene products of HIV as well as structural features of the preintegration complex (PIC) itself contribute to this ability. Among these is Vpr, which has been shown to have nucleophilic properties, and contributes to the productive infection of macrophages *in vitro*. Using matched pairs of isogenic viruses that differed only by the presence or absence of a functional *vpr* we investigated the importance of Vpr in mediating infection of non-dividing target cells within lymphoid tissues. Our studies revealed that although Vpr was an essential factor regulating the infection of tissue macrophages, it did not play a role in the infection of non-proliferating T-cells. By selectively blocking the infection of macrophages through inactivation of Vpr, we were also able to estimate the contribution of this viral reservoir to the local viral burden. We found that macrophages accounted for nearly 50% of total viral output within R5-infected tissues. These data highlight the importance of tissue macrophages in local viral burden and further implicate macrophages and Vpr in the evolutionary strategy and pathogenesis of HIV-1.

This work was performed in close collaboration with M. Sherman, with whom I share first authorship. He performed the studies presented in Figures 1 and 4 and

contributed significantly to the other work presented in the manuscript. He also provided critical editing of the manuscript. M. Penn provided significant intellectual insight and advice. P. Chin contributed experimental support and performed the studies presented in Figure 6B. C.M.C. De Noronha generated the Vpr-deficient viral constructs. M. Goldsmith supervised this work and W. Greene provided additional input into experimental interpretation. This chapter is in press at the *Journal of Experimental Medicine* and is reproduced here with permission from The Rockefeller University Press.

HIV-1 Vpr Enhances Viral Burden by Facilitating Infection of Tissue Macrophages but Not Non-dividing CD4$^+$ T-cells

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Abstract

Prior experiments in explants of human lymphoid tissue have demonstrated that human immunodeficiency virus type 1 (HIV-1) productively infects diverse cellular targets including T-cells and tissue macrophages. We sought to determine the specific contribution of macrophages and T-cells to the overall viral burden within lymphoid tissue. To block infection of macrophages selectively while preserving infection of T-cells, we utilized viruses deficient for viral protein R (Vpr) that exhibit profound replication defects in non-dividing cells *in vitro*. We inoculated tonsil histocultures with matched pairs of congenic viruses that differed only by the presence of a wild-type or truncated *vpr* gene. Although these viruses exhibited no reduction in the infection or depletion of T-cells, the ability of the Vpr-deficient R5 virus to infect tissue macrophages was severely impaired compared with matched wild-type R5 virus. Interestingly, the Vpr-deficient R5 virus also exhibited a 50% reduction in overall virus replication compared to its wild-type counterpart despite the fact that macrophages represent a small fraction of the potential targets of HIV-1 infection in these tissues. Collectively, these data highlight the importance of tissue macrophages in local viral burden and further implicate roles for CCR5, macrophages, and Vpr in the life cycle and pathogenesis of HIV-1.

Introduction

Human immunodeficiency virus type 1 (HIV-1) is a member of the lentivirus subfamily of retroviruses. Among the features that distinguish lentiviruses is their productive infection of cells of the monocyte/macrophage lineage. In addition to the *gag*, *pol* and *env* genes found in all retroviruses, the HIV-1 genome contains six additional genes: *tat*, *rev*, *vif*, *vpr*, *vpu,* and *nef*. These genes confer upon HIV-1 a number of unique abilities, including the capacity to infect non-cycling cells. Viral protein R (Vpr) in particular is known to play an important role in facilitating infection of non-dividing tissue macrophages (1-6) as well as inducing G_2 cell-cycle arrest in dividing T-cells (7-11). The *vpr* gene encodes a 96 amino acid, 14-kDa nucleophilic protein that is incorporated into mature virions via an interaction with p6, a proteolytic subunit of the $p55^{gag}$ precursor (12-15). Although *vpr* is frequently deleted during *in vitro* passaging, Vpr is believed to have numerous functions that contribute to the establishment and pathogenesis of HIV-1 infection *in vivo* (8, 16).

Unlike simple retroviruses, HIV-1 does not depend on cellular division and the accompanying breakdown of the nuclear envelope for productive infection (17, 18). It is believed that this special property is due to the concerted and perhaps redundant activities of Matrix (MA), Integrase (IN), the DNA flap and Vpr. Specifically, both MA (19-21) and IN (22-24) bound to the viral genome contain nuclear localization signals that target the preintegration complex (PIC) to the nucleus via interactions with host nuclear import machinery. The central DNA flap, a triple-stranded helix that is common to retroviruses,

may additionally contribute to nuclear targeting through an unknown mechanism (25). Vpr is also highly nucleophilic and utilizes a distinct targeting strategy (1, 3-5, 26, 27). It contains two non-overlapping and unique nuclear localization signals that likely contribute to the nuclear localization of the PIC (28, 29). Previous work has identified Vpr as a contributing factor in the infection of macrophages *in vitro*, which presumably is linked to this nuclear localization function (2, 4, 6).

Vpr also causes G_2 cell-cycle arrest in infected cells cultured *in vitro* (7-11, 30). Expression of Vpr in some cell types by transfection, transduction or productive HIV-1 infection is associated with inactivation of p34Cdc2 kinase, leading to the accumulation of the cells in the G_2 phase of the cell-cycle (9, 11). The biologic significance of this arrest during natural infection is not well understood. However, studies have demonstrated that the HIV-1 LTR is most active in the G_2 phase, implying that G_2 arrest may confer a replicative advantage to viral species encoding a functional Vpr (8, 31, 32). *In vitro* studies have also revealed that prolonged G_2 arrest may induce apoptosis of the infected cell (32-37), although others have not observed this effect (36, 38, 39). Thus, Vpr may variably potentiate or mediate apoptosis, and this function seems to segregate with cell-cycle arrest in mutagenesis studies (36). Based on these studies, it is speculated that Vpr contributes to HIV-mediated immune destruction by promoting depletion of target cells.

To clarify the importance of Vpr to HIV replication and subsequent pathogenesis, we employed a human lymphoid histoculture model. This system is distinguished by its

capacity to support the replication of HIV-1, HIV type 2 (HIV-2) or simian immunodeficiency virus (SIV) without the need for exogenous cell activation or growth factors (40-44). Consequently, this *ex vivo* system preserves the diverse cell types and cellular activation and maturation phenotypes found within lymphoid tissues *in vivo*. Spleen and tonsil histocultures thus represent a valuable model in which to study the cellular tropism and cytopathic potential of these viruses in a physiologically relevant setting. We therefore employed this model to determine in which cell types Vpr plays a role in infection and to establish the contribution of these cells to the viral burden within lymphoid tissue. These studies reveal that while Vpr augments the infection of macrophages, it does not contribute to the productive infection of proliferating or resting T-cells. Furthermore, Vpr-deficient R5 viruses exhibit a significant reduction in the extent of viral replication, emphasizing the importance of tissue macrophages as a permissive reservoir for viral replication *in vivo*. These findings suggest that other host or viral factors may be responsible for the infection of resting lymphocytes and highlight the importance of Vpr and CCR5 coreceptor specificity for HIV-1infection of tissue macrophages.

Materials and Methods

Cloning of Vpr-deficient viruses. The envelope genes determining CXCR4 (X4) or CCR5 (R5) preference were derived from HIV-1 NL4-3 or HIV-1 BaL. The region extending from the 5' EcoR1 restriction endonuclease site located within the Vpr gene to the NheI site distal to the V3 region within the envelope gene were cloned from BaL and introduced into the NL4-3 proviral clone thereby creating the 107 strain (45). These sequences are sufficient to confer CCR5-tropism to 107. The Vpr^- proviral clones were generated by introducing the AgeI to EcoRI (within *vpr*) fragment from the pNL4-3-derived partial proviral clone p210–19 (46) (obtained from the NIH AIDS Research and Reference Reagent Program) into the AgeI and EcoRI sites of the respective proviruses. This cloning strategy preserved the *pol* and *vif* sequence identity between NL4-3 and 107, truncated the Vpr product by introducing a 115-nucleotide deletion, and also introduced two stop codons after the 21st amino acid of Vpr and downstream of the Vif stop codon in an otherwise isogenic virus.

Preparation of viral stocks. Plasmids encoding NL4-3 or 107 were transfected into 293T cells using calcium phosphate precipitation and 48 hours later the resultant supernatants were centrifuged at 5000 RPM for 10 minutes to remove cell debris and then aliquoted for subsequent infection of histocultures. Virus stocks were normalized for infectivity by determining the $TCID_{50}$ on PHA-stimulated peripheral blood mononuclear cells (PBMC) obtained from at least two separate donors as previously described (40, 41).

Western blot of Vpr. For hemagglutenin-Vpr (HA-Vpr) constructs, equal number of 293T cells were transfected using calcium phosphate and then harvested, pelleted, and lysed directly with SDS loading buffer. Similar transfection efficiency was confirmed by cotransfection with pEGFP (Clontech). Western blot was then performed with anti-HA monoclonal antibody (HA.11, Covance). For Vpr within HIV-1 virions, 300 ng of virus in 1 ml of media was concentrated by ultra-centrifugation at 40,000g at 4° C for 1 hour. The precipitate was resuspended in loading buffer containing SDS and beta-mercaptoethanol and subjected to standard PAGE and blotted with antibodies to p24 (Coulter) or Vpr (28).

Culture and infection of human lymphoid tissues ex vivo. Human noninflammatory tonsil tissue removed during tonsillectomy (provided by San Francisco General Hospital, Kaiser-San Francisco and San Rafael, CA) were cut into 2–3 mm blocks and placed into culture as described (40). Tissues were inoculated within 24 hours of preparation with viruses at approximately 50 $TCID_{50}$/tissue block. Following one- or two-week infections, cells were mechanically isolated from infected and uninfected tissue and analyzed by flow cytometry (see below). No significant differences in the *ex vivo* behavior and HIV-1 or SIV permissivity of spleen or tonsil tissue have been observed.

Assessment of $CD4^+$ T-cell infection by flow cytometry. At the indicated time points post-inoculation (at peak infection, usually one-week post-inoculation), dispersed cells from infected and uninfected lymphoid histocultures were immunostained for cell surface markers CD4, CD62L, and CD45RA as described previously (47). Cells were

then fixed in 1% paraformaldehyde and subsequently permeabilized and immunostained for intracellular CD4 and p24. 50,000 $CD4^+$ lymphocytes were counted and the data were analyzed with CellQuest software (Becton Dickinson, San Jose, CA). To identify naïve and memory subsets the following monoclonal antibodies (mAbs) were used from Beckton Dickinson (BD): anti-CD4 (clone SK7, allophycocyanin conjugated, 1:20 dilution), and anti-CD62L (phycoerythrin, 1:20 dilution). The following antibody from BD Pharmingen (San Jose, CA) was also used: anti-CD45RA (cychrome conjugated, 1:20 dilution). To identify infected cells, anti-p24 (fluoroscein isothiocyanate, 1:100 dilution) from Coulter (Miami, FL) was also used. Results are reported as the mean with standard error of the mean (SEM).

Flow cytometric analysis of cellular proliferation. After culturing for 6 days in standard histoculture media supplemented with BrdU (50 μM, Sigma, St. Louis, MO), lymphoid histocultures were stained for BrdU incorporation as described previously. Briefly, following this incubation, cells were dispersed and fixed and permeabilized overnight. Cells were washed, treated with DNase, and then immunostained with a combination of mAbs recognizing CD4 (BD, clone SK3, allophycocyanin conjugated, 1:40 dilution), CD45RA (BD Pharmingen, cychrome conjugated, 1:2.5 dilution), CD62L (BD, phycoerythrin conjugated, 1:20 dilution), and BrdU (BD Pharmingen, fluoroscein isothiocyanate conjugated, 1:2.5 dilution). 50,000 $CD4^+$ lymphocytes were then collected and analyzed by CellQuest.

Assessment of DNA content by flow cytometry. Cell-cycle analysis for HA-Vpr constructs was performed by cotransfecting pEGFP (Clonetech) and HA-Vpr DNA constructs into 293T cells in a 1:8 molar ratio to identify plasmid-expressing cells. 36 hours later cells were trypsinized, fixed in 2% formaldehyde for 30 minutes, washed, and treated with 0.1mg/ml Ribonuclease (RNase) A (Sigma) and 10mg/ml propidium iodide in PBS for 30 minutes. Cellular DNA content in the transfected (GFP^+) and untransfected (GFP^-) cells was assessed using a FACScan flow cytometer. For analysis of infected and uninfected lymphoid histocultures, cells were dispersed from the tissue at the indicated time points post-inoculation (at peak infection, usually one-week post-inoculation), and immunostained for the cell surface marker CD4. Cells were then fixed in 1% paraformaldehyde and subsequently permeabilized and immunostained for intracellular CD4 and p24. Samples were then incubated for 30 minutes in a solution of 0.01mM To-Pro-3 iodide (Molecular Probes) and 0.1mg/ml RNase A. 200,000 lymphocytes were counted and the data were analyzed with CellQuest and FlowJo (Tree Star, San Carlos, CA). The following mAb was used from BD: anti-CD4 (clone SK7, phycoerythrin conjugated, 1:20 dilution). To identify infected cells, anti-p24 was also used.

Assessment of $CD4^+$ T-cell depletion by flow cytometry. At the indicated time points (usually two-weeks post-inoculation), dispersed cells from infected and uninfected lymphoid histocultures were immunostained for cell surface markers CD3, CD4, CD8, and CCR5 as described previously (40, 41). 5,000–20,000 $CD3^+$ lymphocytes were counted and the data were analyzed with CellQuest and FlowJo. $CD4^+$ T-cell depletion

was expressed as the ratio of $CD4^+$ to $CD8^+$ T-cells in infected relative to uninfected tissues as described previously (40, 41). The following mAbs were used from BD: anti-CD3 (clone SK7, allophycocyanin conjugated, 1:80 dilution), anti-CD4 (clone SK3, fluoroscein isothiocyanate conjugated, 1:20 dilution), and anti-CD8 (clone SK1, phycoerythrin conjugated, 1:20 dilution). The following antibody from BD Pharmingen was also used: anti-CCR5 (allophycocyanin conjugated, 1:20 dilution). Cell numbers in infected tissues were normalized to those obtained for uninfected control tissues. Results are reported as the mean with standard error of the mean (SEM).

Assessment of macrophage infection by flow cytometry. At the indicated time points (usually two-weeks post-inoculation), dispersed cells from infected and uninfected lymphoid histocultures were immunostained for cell surface markers CD3 and CD14 as described previously (43). Cells were then fixed in 1% paraformaldehyde and subsequently permeabilized and immunostained for intracellular CD68 and p24. 50,000–100,000 lymphocytes were counted and the data were analyzed with CellQuest. Results are reported as the mean with standard error of the mean (SEM).

Assessment of viral replication. At the indicated time points, a sample of culture media was withdrawn from infected tissue, diluted 1:1000 with diluent, and stored at –20° Celsius. The histoculture media was then replaced. At the end of the experiment, samples were thawed, and the concentration of HIV-1 p24 in the media was measured by enzyme-linked immunosorbent assay (ELISA) from NEN Life Science Products (Boston, MA).

Results

Construction of Vpr-deficient viruses. To examine the tropism and replication of Vpr-deficient viruses in lymphoid tissue, we studied the matched pair of viruses, NL*4*-3 and 107. NL*4*-3 is a molecularly cloned strain of HIV-1 that exhibits strict preference for the coreceptor CXCR4 and does not replicate in macrophages (43, 45). 107 is a recombinant virus derived from NL4-3 that shares the same genetic backbone but incorporates coreceptor-determining envelope sequences cloned from BaL, a CCR5-dependant primary HIV-1 isolate, as well as additional upstream sequences (45). These cloned sequences from BaL extend through the 3' end of *vpr* and, as a result of this substitution, some minor differences exist in the amino acid sequences of Vpr derived from NL*4*-3 and 107 (Fig. 1A). However, the genomes of NL*4*-3 and 107 are isogenic upstream of *vpr*. To assess the functionality of these proteins we transiently transfected 293T cells with expression vectors encoding HA-tagged NL*4*-3- or 107-derived versions of Vpr. Western-blot analysis with anti-HA mAb of whole-cell lysates from these transfectants revealed slightly lower expression of 107 Vpr compared with NL*4*-3 Vpr (Fig. 1B). Because mutations or deletions in the 3' end of *vpr* can alter expression levels of Vpr (48), we infer that the sequence differences between the NL*4*-3 and 107 *vpr* genes may result in stability differences between the two Vpr proteins leading to differential expression. We next transfected 293T cells with empty HA vector, NL4-3-derived, or 107-derived HA-Vpr expression vectors and stained the cells for intracellular DNA-content 36 hours later. Cell-cycle analysis by flow cytometry revealed that both NL4-3-

small size, low granularity, and low proliferative activity "lymphocytes." The second population, characterized by cells of greater size, increased granularity, and significant proliferative activity, was termed "blasts." Although lymphocytes exhibit limited proliferation, our earlier studies have directly demonstrated productive infection of non-dividing cells within this subset (47). These results confirm that the virally infected T-lymphocytes do not represent a subpopulation of dividing T-cells and establish that HIV-1 can productively infect non-dividing lymphocytes *de novo*. To determine if Vpr contributed to the infection of these non-dividing cells, we inoculated histoculture tissue with equivalent $TCID_{50}$ of wild-type or Vpr-deficient strains of NL4-3 or107. Following a 7-day incubation, cells were harvested from the tissue and immunostained for intracellular p24, a marker of productive HIV infection. Examination of $CD4^+$ T-cells by FACS revealed significant infection of both lymphocytes and blasts by the wild-type and Vpr-deficient variants of NL4-3 and 107 (Fig. 2). As observed previously, NL4-3 infection resulted in higher levels of productive infection than did 107 due to a broad distribution of CXCR4 on target cells and limited expression of CCR5. Most importantly, within a given X4 (Fig. 2A) or R5 (Fig. 2B) proviral backbone, wild-type and Vpr-deficient strains equivalently infected the lymphocyte pool, suggesting that Vpr is not an essential viral factor for the productive infection of non-dividing or dividing T-cells in such tissue contexts. This finding was unexpected given the putative role of Vpr in the nuclear targeting of the PIC in non-dividing cells such as tissue macrophages. Wild-type and Vpr-deficient viruses likewise equivalently infected the blast pool (Fig. 2A,B). We

and 107-Vpr induced significant G_2 cell-cycle arrest, thereby establishing the functionality of both NL4-3- and 107-Vpr *in vitro* (Fig. 1C).

For the present studies, we wished to eliminate Vpr expression in NL4-3 and 107 viral clones for comparisons to their respective parental viruses. We introduced a deletion and two premature stop codons into the nucleotide sequence of *vpr* in both the NL4-3WT and 107WT proviral contexts, thereby generating NL4-3ΔVpr and 107ΔVpr, respectively (Fig. 1D). Because NL4-3 and 107 have identical sequences upstream of the EcoR1 site utilized to delete Vpr, this cloning strategy preserved the isogenicity between NL4-3 and 107 including *vif* and *pol*. Neither of these Vpr-deficient viruses produced detectable quantities of the truncated Vpr product (data not shown). Consistent with this observation, immunoblot analysis of wild-type and mutant virions established the presence of Vpr in NL4-3WT and 107WT virions but not in NL4-3ΔVpr and 107ΔVpr virions, confirming that Vpr was not expressed in the deletion mutants (Fig. 1E). We detected lower levels of Vpr within 107WT virions compared with NL4-3WT virions, consistent with the inferred stability differences between these different versions of the proteins (Fig. 1B). Comparing wild-type and Vpr-deficient versions of NL4-3 or 107 we subsequently examined the cellular tropism and replication profiles of these viruses within human lymphoid tissues *ex vivo*.

Vpr is not required for productive infection of resting T-cells. On the basis of morphologic characteristics we have previously identified two distinct lymphocyte populations within lymphoid histocultures (47). We termed one population of cells with

note that Vpr has been shown to have a greater influence on HIV-1 infectivity at low titers, and only a small impact at high multiplicity of infection (m.o.i.) (22). Importantly, the experiments reported here were carried out at a m.o.i. below $2x10^{-4}$, a very low inoculum, which likely accounts for the 5-day delay in the appearance of detectable intracellular and soluble p24 following inoculation. Experiments performed with one-half the virus inoculum used above also revealed no differences in the infection of $CD4^+$ T-lymphocytes and blasts by wild-type or Vpr-deficient viruses under these conditions (data not shown).

We previously demonstrated by pulse-chase analysis that naïve and memory T-cells in these tissues exhibited significantly different proliferative activity (47). We therefore hypothesized that while Vpr may not be necessary for infection of T-cells in general, it may nonetheless be a critical factor for infection of the non-proliferating, naïve T-cell subset, a population typically accounting for less than 20% of the total $CD4^+$ T-lymphocyte population. To measure the cumulative fraction of cycling T-cells during a typical HIV infection, we cultured tonsil tissue in medium containing BrdU for 7 days. FACS analysis of samples harvested after labeling revealed that while greater than 16% of memory $CD4^+$ T-lymphocytes incorporated BrdU during this period, only 2.3% of naïve $CD4^+$ T-lymphocytes did so (Fig. 3A). This pattern of enhanced labeling of memory cells compared with naïve cells was preserved in the blast compartment as well, although the blast subsets exhibited greater proliferative activity overall than did their lymphocyte counterparts.

We next compared the capacity of wild-type and Vpr-deficient viruses to productively infect each of these subsets. Tonsil histocultures were inoculated with equivalent titers of X4 or R5 wild-type or Vpr-deficient viruses, and following a 7-day incubation, cells were immunostained for maturation markers and intracellular p24. Stratification of T-cells into memory and naïve subsets revealed no statistically significant differences between cultures infected with wild-type or Vpr-deficient NL4-3 (Fig. 3B) or 107 (Fig. 3C) variants. Most importantly, this analysis demonstrated equivalent infection of the non-dividing, naïve T-lymphocyte population by the wild-type and Vpr-deficient viruses. This finding substantiates our earlier conclusion that Vpr does not contribute to lymphocyte infection in general, and specifically indicates that Vpr function is dispensable for productive infection of the non-proliferating subset of T-cells.

Vpr does not trigger cell-cycle arrest in infected lymphocytes ex vivo. We next sought to determine if Vpr induces G_2 cell-cycle arrest in these tissues. Tonsil histocultures were inoculated with equivalent titers of NL4-3WT or NL4-3ΔVpr virus, and following a 6-day incubation, the tonsil tissue was harvested and stained for intracellular p24 and DNA content. Samples were then analyzed by flow cytometry; we gated on $CD4^+$ T-cells and examined them for intracellular p24 expression. Analysis of the DNA content of $p24^+$ T-cells in the samples inoculated with wild-type or Vpr-deficient virus revealed a similar percentage of cells in the G_2 phase of the cell-cycle irrespective of the presence or absence of Vpr (Fig. 4A,C). Interestingly, in samples inoculated with either wild-type or Vpr-deficient virus the percentage of cells in G_2 was modestly greater in the fraction of

productively-infected T-cells ($p24^+$) (Fig. 4A,C) compared with uninfected T-cells in the same tissue (Fig. 4B,D). Similar results were observed over several different infections in lymphoid tissue, suggesting that there is no detectable Vpr-dependent G_2 cell-cycle arrest and that other viral factors may play a key role in this phenomenon.

Vpr does not contribute to HIV-1-mediated depletion of T-cells in lymphoid histoculture. To determine directly if equivalent infectivity correlated with equivalent cytopathicity, we measured virus-induced depletion of $CD4^+$ T-cells following infection with HIV-1. We began by subsetting T-cells based on expression of the HIV-1 coreceptor CCR5. As expected from previous studies, NL4-3WT extensively depleted both $CCR5^+$ and $CCR5^-$ subpopulations of $CD4^+$ T-lymphocytes (Fig. 5A). Likewise, NL4-3ΔVpr also markedly depleted both subpopulations in a manner indistinguishable from its parental virus. In contrast to the extensive depletion by NL4-3, the cytopathic effects of 107WT were limited to the $CCR5^+$ subset of $CD4^+$ T-lymphocytes, as has been observed previously (41, 43). Again, the Vpr-deficient 107 strain exhibited equivalent cytopathic activity compared with wild-type 107. These findings demonstrate that Vpr is not essential for the cytopathic effects of HIV-1 on $CD4^+$ T-cells within lymphoid tissues.

To determine further whether the state of T-cell maturation influences the susceptibility of these cells to depletion by Vpr-deficient viruses, we measured the loss of naïve and memory $CD4^+$ lymphocytes following infections with HIV-1. Both NL4-3WT and NL4-3ΔVpr induced severe depletion of naïve and memory $CD4^+$ lymphocytes (Fig. 5B), although the cytopathic effects of NL4-3ΔVpr were slightly reduced compared with

that of NL4-3WT, consistent with our earlier finding of slightly reduced infectivity of NL4-3ΔVpr. We also measured depletion in tissue that had been inoculated with 107WT or 107ΔVpr. As is characteristic of all R5 viruses, overall depletion was not severe. Nevertheless, the degree of depletion of the memory and naïve populations was equivalent for both 107 variants (Fig. 5C). Thus, Vpr does not contribute to the cytopathic activity of either HIV-1 R5 or X4 variants in T-lymphocytes.

Vpr contributes substantively to the productive infection of tissue macrophages as well as to the viral burden in lymphoid tissue. In addition to infecting activated T-cells, R5 viruses also productively infect cells of the monocyte/macrophage lineage. We therefore examined the putative role of Vpr in mediating the infection of non-dividing lymphoid tissue macrophages. We inoculated tonsil histocultures with equivalent titers of 107WT or 107ΔVpr. Following incubation, the tissue was dispersed and cells were immunostained for specific macrophage (CD14 and CD68) and T-cell (CD3) markers as well as intracellular p24. Because macrophages associate tightly with B- and T-cells in lymphoid tissues, macrophages free of complexed T-cells ($CD14^+$ or $CD68^+$ and $CD3^-$) were identified by flow cytometry and analyzed for productive infection. In contrast to the behavior observed earlier in T-cells, 107ΔVpr exhibited a greater than 4-fold reduction in infectivity in lymphoid tissue macrophages compared to 107WT (Fig. 6A). A similar examination of the same tissue inoculated with NL4-3WT and NL4-3ΔVpr revealed little detectable infection within macrophages by either virus (data not shown).

These findings imply that Vpr contributes significantly to the productive infection of tissue macrophages by R5 strains.

Interestingly, within the genomes of HIV-2 and SIV the arrest and nuclear import functions of HIV-1 Vpr are divided between two related gene products, Vpr and viral protein X (Vpx) (49). Cell-cycle arrest is mediated by Vpr in both HIV-2 and SIV, whereas nuclear localization is mediated by Vpx in both species. The significance of this functional division is not well understood; however, the evolutionary conservation of both functions implies their importance in the viral life cycle. To determine if the macrophage-specific defect we observed in 107ΔVpr was specifically attributable to the nuclear entry activity of Vpr, we inoculated tonsil samples with SIV PBj6.6WT, PBj6.6ΔVpx or PBj6.6ΔVpr. Cells were later dispersed and stained for macrophage-lineage markers and intracellular p27. FACS analysis revealed a greater than 6-fold reduction in the number of productively infected macrophages in tissue that had been inoculated with PBj6.6ΔVpx, compared to tissue infected with wild-type virus (Fig. 6B), consistent with a role for Vpx in nuclear targeting. In contrast, tissue infected with PBj6.6ΔVpr showed no significant decrease in the frequency of infected macrophages (Fig. 6B). Indeed, across several experiments the frequency was either unchanged or modestly increased compared with PBj6.6WT. Therefore, these results implicate the nuclear targeting function of HIV-1 Vpr in the infection of macrophages by R5 HIV-1.

Finally, we measured the extent and kinetics of viral replication of wild-type and Vpr-deficient viruses in lymphoid histocultures. Tissue was inoculated with equal titers

of NL4-3WT or NL4-3ΔVpr (Fig. 7A) as well as 107WT or 107ΔVpr (Fig. 7B). Supernatants were collected serially throughout 18-day incubations, and the concentrations of p24 in the media were assessed by ELISA. As expected from their strict T-cell tropism and the earlier findings of similar infectivity, NL4-3WT and NL4-3ΔVpr exhibited similar replication profiles (Fig. 7A). Both the overall growth kinetics as well as the total virus production were similar. In contrast, a substantially different pattern of replication was found for 107WT compared with 107ΔVpr. 107ΔVpr exhibited an approximately 50% reduction in viral replication compared to 107WT (Fig. 7B). Based on the selective effects of Vpr we observed earlier in macrophages rather than T-cells, we infer that the differential viral output in tissues infected with 107ΔVpr compared with 107WT is the result of decreased macrophage infection by Vpr-deficient viruses. Thus, these results reveal the significant contribution of tissue macrophages to the viral burden within human lymphoid tissues. Consistent with this conclusion, quantitation of soluble p27 in the culture media of the SIV-infected tissues revealed an 8-fold reduction in viral output from tissues inoculated with the Vpx-deficient strain of PBj6.6 compared to the wild-type and Vpr-deficient counterparts (data not shown). Thus, the Vpx-deficient SIV strain behaved similarly to the Vpr-deficient R5 HIV-1 strain. Overall, these results suggest that the major role of Vpr in HIV-1 pathogenesis is to facilitate infection of macrophages, a population that may also contribute substantially to the viral burden in infected individuals.

Discussion

Peripheral blood mononuclear cells in culture typically require exogenous stimulation in order to support productive infection by either wild-type or Vpr-deficient viruses. This dependency on activation appears to relate to the nuclear membrane breakdown associated with activation-induced cellular division, which evidently abrogates the need for Vpr to facilitate nuclear import of the PIC. In contrast, T-cells within the lymphoid tissue microenvironment are permissive for productive HIV-1 infection independent of exogenous stimulation or cytokines. Moreover, our previous studies have revealed no absolute restriction to HIV-1 infection, replication, or depletion of T-cells within these cultures on the basis of target cell maturation or proliferation (47). In particular, these studies demonstrated *de novo* infection and depletion of resting, naïve lymphocytes, which may explain the observation of productively infected resting T-cells *in vivo* (50-52). Because these cells divide very infrequently (Fig. 3), infection must proceed independently of nuclear membrane dissolution. Multiple determinants of nuclear import contribute to this unique property of HIV. The apparent redundancy of mechanisms highlights the importance of nuclear entry in the HIV-1 life cycle. However, it is possible that individual import signals may play a more or less dominant role in particular cellular contexts. Thus, the multiple nuclear import signals may not be redundant after all. We therefore examined the importance of one such nuclear import signal, Vpr, in mediating nuclear import in diverse primary cell types including non-dividing T-cells and macrophages.

Surprisingly, these studies revealed equivalent infection of resting, naïve T-cells within histocultures inoculated by matched wild-type or Vpr-deficient HIV-1 strains despite differential infection of macrophages. This finding suggests that other host or viral factors contribute to the nuclear import of the PIC in this subset of non-dividing T-lymphocytes. At least two additional viral gene products may be responsible. We speculate that the weak nuclear localization signals (NLS) within MA (19-21) may function relatively efficiently within T-cells, which would reduce the dependence on Vpr-mediated translocation mechanisms. In addition, multiple NLS have also been identified within viral IN (22-24) that may also be relatively effective in T-cells. In particular, a novel NLS within IN has recently been shown to contribute significantly to the infection of dividing as well as growth-arrested cells (24). Finally the nucleophilic properties of the central DNA flap may also play a more significant role in the nuclear import of the PIC within infected T-cells (25). Thus, the individual nuclear import signals within the PIC may each be optimized for a specific primary cell type.

The significance of other Vpr functions in viral expansion is less clear. For example, our analysis of infected lymphoid cultures failed to detect specific G_2 cell-cycle arrest in samples inoculated with wild-type HIV-1 compared to Vpr-deficient virus, suggesting that Vpr-dependent arrest is not manifest in this tissue context. These observations are also supported by equivalent virus replication of X4 viruses irrespective of the presence of Vpr. Alternatively, it is possible that cells arrested in G_2 by Vpr may be rapidly and selectively eliminated within lymphoid histocultures thereby preventing their

detection. However, these studies did reveal a modest accumulation of $p24^+$ cells in the G_2 phase of the cell cycle in HIV-1-infected cultures independent of Vpr status. This finding implies that selective elimination of Vpr-arrested cells is not a likely feature of this system and suggests that viral factors in addition to Vpr may contribute to arrest in this tissue context. Indeed, a previous study has demonstrated that envelope glycoprotein may induce arrest in cultured cells (53). The individual contributions of these two pathways remain to be determined.

We also detected equivalent depletion of T-cells by wild-type and Vpr-deficient HIV-1 variants. Although earlier studies have reported a pro-apoptotic role for Vpr, our findings suggest that Vpr is not required for such effects in lymphoid tissue. Indeed, mutagenesis studies have revealed that the apoptotic function ascribed to Vpr seems to segregate with the cell-cycle arrest activity (36), and we have found that neither activity is evident in these histocultures. We conclude that neither cell-cycle arrest nor apoptosis induced by Vpr contribute significantly to virus-induced T-cell death in this context.

The identical replication kinetics of wild-type and Vpr-deficient X4 viruses within lymphoid tissue is consistent with our earlier conclusions regarding X4 HIV-1 infection and depletion. However, we note that a slight trend towards reduced replication by Vpr-deficient strains was evident from these studies, which may correlate with the observed trend toward slightly reduced infectivity and depletion by Vpr-deficient X4 virus. Although few of these differences reached statistical significance, these patterns were

observed in multiple tissue specimens, which may indicate a small contribution of Vpr to the fitness and replication of X4 HIV-1 strains *in vivo*.

In contrast to the behavior of cultured peripheral blood T-cells (2, 4, 54), monocyte-derived macrophages matured *in vitro* depend substantially on intact Vpr for productive infection (2, 4, 6, 55). However, this effect is evident only at very low multiplicity of infection and only after weeks of *in vitro* culture. Indeed, some investigators have observed no effect of Vpr on infection of cultured monocyte-derived macrophages (56). In addition, an important issue that remains unanswered by these studies is the importance of Vpr for infection of macrophages residing within lymphoid tissues. Lymphoid organs are the principal sites of HIV-1 replication *in vivo* and account for greater than 90% of the viral load during HIV-1 infection (57), particularly during the acute and asymptomatic phases of disease when viruses selectively utilizing CCR5 predominate. CCR5 is expressed on most tissue macrophages, dendritic cells, and a subset of activated T-cells. The selection of CCR5-dependent viruses during acute infection suggests that macrophage infection may be a key factor in the establishment of HIV-1 infection as well as the subsequent development of functional immunodeficiency. We therefore examined the role of Vpr in the establishment of HIV-1 infection in tissue-resident macrophages rather than artificially-matured blood-derived cells. Our results revealed a substantial defect in the productive infection of macrophages by Vpr-deficient viruses, which stands in sharp contrast to its lack of effect in T-cells. This macrophage-restricted phenotype was also apparent in tissues inoculated with Vpx-deficient, but not

Vpr-deficient, strains of SIV. These observations confirm the specific importance of nuclear import in the life cycle of HIV-1 and SIV within macrophages. Furthermore, these findings suggest that the nuclear import signals within the viral PIC may be cell type-specific and may not be completely redundant in all cellular environments. Indeed, each NLS within the PIC may be conserved so as to preserve the ability of HIV-1 to productively infect distinct cell types. We conclude that Vpr contributes substantially to the replication of R5 HIV-1 within macrophages but does not play a significant role in T-cells.

Recent *in vitro* work has demonstrated that HIV-1 Nef expression within macrophages induces production of the soluble CC-chemokines macrophage inflammatory protein-1α and -1β and other unidentified soluble factors (58). These chemokines may recruit and partially activate neighboring resting lymphocytes and thereby render them permissive for infection by HIV-1, which would assign infection of macrophages an important role in establishing infection in resting lymphocytes. Selective impairment of macrophage infection, as is seen with the Vpr-deficient viruses tested here, would be expected to interrupt this *trans*-activating function. However, this effect was not evident in the histoculture system. Specifically, the frequency of infected resting, naïve T-cells by 107ΔVpr or NL4-3ΔVpr was identical to that of their wild-type counterparts. Therefore, experimental conditions or viral factors other than, or in combination with, Nef may play key roles in the ability of HIV-1 to infect resting, naïve lymphocytes in a lymphoid tissue microenvironment.

Finally, macrophages may contribute substantially to plasma viral load during late infection, particularly when $CD4^+$ T-cells have been extensively depleted (57, 59, 60). However, the contribution of macrophages to viral load during other disease stages has not been well characterized but is proposed to be minimal based on the viral and cellular kinetics observed during acute antiviral treatment (61-64). Based on the assumption that HAART completely suppresses virus replication, such studies suggest that plasma viremia declines with a half-life of approximately 1 day, implying that the cells releasing virus into the plasma must have a similar half-life. This conclusion implicates T-cells as the principle source of plasma viremia rather than infected tissue macrophages, which are believed to have a half-life of nearly 2 weeks (61-64). However, more recent studies have generated evidence of other persistent viral reservoirs that may contribute to plasma viremia (65-68). The studies reported herein address this controversy directly by examining the replication kinetics of viruses that fail to infect macrophages productively while their capacity to infect and replicate within lymphocytes is preserved. Thus, any difference in viral output may be interpreted as the contribution of macrophage infection. The results reveal a significant reduction in the concentration of soluble p24 in cultures infected with 107ΔVpr as compared to wild-type virus. This finding is correlated with a greater than 4-fold reduction in the frequency of infected macrophages and preserved numbers of infected T-cells in these same samples. We therefore conclude that the differential output between cultures infected with wild-type and Vpr-deficient R5 viruses is attributable to the output of the productively infected macrophage pool.

This conclusion implies that within lymphoid organs infected with R5 viruses the contribution to cell-free virus by infected macrophages is equal to that of all infected $CCR5^+$ $CD4^+$ T-cells within the particular tissue—a population that significantly outnumbers tissue macrophages by at least 10-fold. Therefore, on a per-cell basis, we infer that productively infected macrophages cumulatively release at least 10-fold more p24 than do productively infected T-cells within the same tissue over the 2-week time interval of these experiments. Thus, it appears that HIV replication within macrophages contributes significantly to tissue viral burden and may account for a substantial fraction of plasma viremia to the extent that plasma virus derives from tissue sources. However, it remains a theoretical possibility that T-cells infected by macrophage-derived virus or in the proximity of infected macrophages may produce more p24 than cells infected by T-cell-derived or cell-free virus. Nevertheless, our conclusions agree well with recent work demonstrating significant and sustained plasma viremia in SHIV-infected macaques following virus-induced depletion of $CD4^+$ T-cells, in which the authors concluded that the viremia was supported by viral replication in productively infected macrophages (60). Our results extend this observation by demonstrating the significant contribution of human lymphoid macrophages to HIV-1 viral burden even when T-cells are present.

Given the close physical association between macrophages and T-cells within lymphoid organs, it is possible that the majority of virus produced by macrophages may be transmitted to neighboring lymphoid cells by means of cell-to-cell spread. Virus produced by macrophages and transmitted in this manner may not contribute

substantially to the cell-free virus detected in the plasma. As a consequence, the dynamics of viral decline within lymphoid tissues may differ from that of the plasma. Although few in number, productively infected lymph node macrophages have in the past been thought to play a large role in viral burden (57, 59). Our findings underscore the potential importance of this relatively small cellular reservoir of virus. Spread of R5 virus directly from macrophages to T-cells might contribute substantially to HIV-induced immune pathogenesis during the early and asymptomatic stages of HIV-induced disease, as well as later stages of disease when T-cells have been depleted. Tissue macrophages may therefore represent a clinically important cellular reservoir of virus, as macrophage-derived virus would be expected to contribute substantially to the progressive attrition of T-cells that characterizes all stages of HIV disease.

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Figure Legends

Figure 1. NL4-3- and 107-derived Vpr are functional and incorporated into mature virions. (A) HIV-1 Vpr from NL4-3 is identical to the BaL-derived, macrophage-tropic 107 variant except at residues 78 and 83–85. (B and C) DNA encoding HA-tagged NL4-3 Vpr or 107 Vpr and GFP was transfected into 293T cells. (B) Cells were lysed 36 hours later, and expression of Vpr was determined by western blot with anti-HA mAb. (C) Cells were harvested 36 hours later, and labeled with propidium iodide. DNA content was measured in GFP^+ cells by flow cytometry. (D) Cloning strategy for truncation of *vpr* within both NL4-3 and 107 proviral constructs. Note that the sequences of *vif*, *pol*, and *tat* are unaffected by these mutations within *vpr*. The shaded box identifies the deleted region within *vpr*. (E) DNA encoding each of the HIV-1 recombinants was transfected into 293T cells and supernatants were collected forty-eight hours later. Virus production was normalized for p24 concentration, and equal amounts of virus were concentrated for each strain by ultra-centrifugation. The viral pellet was then subjected to Western blot analysis. Shown are representative experiments.

Figure 2. HIV-1 infection of $CD4^+$ T-lymphocytes and blasts. (A) Productively infected cell subsets in tonsil tissue following a 7-day infection with NL4-3WT (open bars) or NL4-3ΔVpr (closed bars) as measured by intracellular p24 expression detected by FACS. (B) Productively infected cell subsets in tonsil tissue following a 7-day infection with 107WT (open bars) or 107ΔVpr (closed bars). Presented are mean values with SEM

(n=3) from a representative experiment.

Figure 3. BrdU incorporation and HIV-1 infection of naïve and memory $CD4^+$ T-cell subsets. (A) Tissue samples were cultured continuously in medium containing BrdU for 7 days. Cells were isolated, immunostained for CD4, CD62L, CD45RA and BrdU and analyzed by FACS. Shown are mean values with SEM (n=3) from a representative experiment. (B and C) Tonsil histocultures were inoculated with equivalent titers of HIV-1, incubated for 7 days and harvested. Cells were dispersed, immunostained for p24, CD62L, CD45RA and CD4, and analyzed by FACS. T-cells were also stratified based on forward and side scatter properties defining lymphocyte and blast morphologies. (B) Productively infected subsets in tissue inoculated with NL4-3WT or NL4-3ΔVpr as measured by intracellular p24 expression. (C) Productively infected subsets in tissue inoculated with 107WT or 107ΔVpr. Shown are mean values with SEM (n=3) from a representative experiment.

Figure 4. Cell-cycle analysis of $p24^+$ or $p24^-$ $CD4^+$ T-cells. (A) Tonsil specimens were inoculated with equivalent titers of HIV-1 NL4-3WT (A and B) or NL4-3ΔVpr (C and D). Following a 7-day incubation, samples were dispersed, stained for intracellular p24, CD4 and To-Pro-3 iodide and analyzed by FACS. DNA content is presented for cells stratified into $p24^+$ (A and C) and $p24^-$ (B and D) fractions. Shown is a representative experiment.

Figure 5. Depletion of $CD4^+$ T-cells by HIV-1 in lymphoid histoculture. (A) Following a 13-day infection, cells were isolated from histocultures and immunostained for surface expression of CD4, CD3, CD8 and CCR5. The mean $CD4^+$ subset/$CD8^+$ ratio relative to uninfected control tissue for $CCR5^-$ T-cells (open bars) and $CCR5^+$ T-cells (closed bars) was then calculated. Shown are mean values with SEM (n=3) from a representative experiment. (B and C) Depletion of naïve and memory $CD4^+$ T-cell subsets by HIV-1. Following a 12-day infection with NL4-3WT and NL4-3ΔVpr (B) or 107WT and 107ΔVpr (C) cells were dispersed from histocultures and immunostained for CD4, CD62L, CD45RA and CD8. The mean $CD4^+$ subset/$CD8^+$ ratio relative to uninfected control tissue for memory T-cells (open bars) and naïve T-cells (closed bars) was then calculated. Shown are mean values with SEM (n=3) from a representative experiment.

Figure 6. Flow cytometric analysis of HIV-1 and SIV infection of macrophages in lymphoid tissue. Following a 9-day incubation with 107WT or 107ΔVpr (A) or PBj6.6WT, PBj6.6ΔVpr or PBj6.6Δvpx (B), cells were dispersed and immunostained for surface expression of CD3 and CD14 as well as intracellular CD68 and p24 or p27. Shown are mean values with SEM (n=3) from a representative experiment.

Figure 7. Kinetic analysis of HIV-1 replication in lymphoid histoculture. Tissue was inoculated with NL4-3WT or NL4-3ΔVpr (A) or 107WT or 107ΔVpr (B). Culture

medium was sampled at the indicated time points and HIV-1 p24 concentration was measured by ELISA. Shown are mean values with SEM (n=3) from a representative experiment.

References

1. Bukrinsky, M.I., N. Sharova, M.P. Dempsey, T.L. Stanwick, A.G. Bukrinskaya, S. Haggerty, and M. Stevenson. 1992. Active nuclear import of human immunodeficiency virus type 1 preintegration complexes. *Proc. Natl. Acad. Sci. USA* 89:6580-6584.

2. Balliet, J.W., D.L. Kolson, G. Eiger, F.M. Kim, K.A. McGann, A. Srinivasan, and R. Collman. 1994. Distinct effects in primary macrophages and lymphocytes of the human immunodeficiency virus type 1 accessory genes vpr, vpu, and nef: mutational analysis of a primary HIV-1 isolate. *Virology* 200:623-631.

3. Heinzinger, N.K., M.I. Bukinsky, S.A. Haggerty, A.M. Ragland, V. Kewalramani, M.A. Lee, H.E. Gendelman, L. Ratner, M. Stevenson, and M. Emerman. 1994. The Vpr protein of human immunodeficiency virus type 1 influences nuclear localization of viral nucleic acids in nondividing host cells. *Proc. Natl. Acad. Sci. USA* 91:7311-7315.

4. Connor, R.I., B.K. Chen, S. Choe, and N.R. Landau. 1995. Vpr is required for efficient replication of human immunodeficiency virus type-1 in mononuclear phagocytes. *Virology* 206:935-944.

5. Popov, S., M. Rexach, G. Zybarth, N. Reiling, M.A. Lee, L. Ratner, C.M. Lane, M.S. Moore, G. Blobel, and M. Bukrinsky. 1998. Viral protein R regulates nuclear import of the HIV-1 pre-integration complex. *EMBO J.* 17:909-917.

6. Vodicka, M.A., D.M. Koepp, P.A. Silver, and M. Emerman. 1998. HIV-1 Vpr interacts with the nuclear transport pathway to promote macrophage infection. *Genes Dev.* 12:175-185.

7. Bartz, S.R., M.E. Rogel, and M. Emerman. 1996. Human Immunodeficiency Virus Type 1 Cell Cycle Control: Vpr Is Cytostatic and Mediates G2 Accumulation by a Mechanism Which Differs from DNA Damage Checkpoint Control. *J. Virol.* 70:2324-2331.

8. Goh, W.C., M.E. Rogel, C.M. Kinsey, S.F. Michael, P.N. Fultz, M.A. Nowak, B.H. Hahn, and M. Emerman. 1998. HIV-1 Vpr increases viral expression by manipulation of the cell cycle: a mechanism for selection of Vpr in vivo. *Nat. Med.* 4:65-71.

9. He, J., S. Choe, R. Walker, P. Di Marzio, D.O. Morgan, and N.R. Landau. 1995. Human immunodeficiency virus type 1 viral protein R (Vpr) arrests cells in the G2 phase of the cell cycle by inhibiting p34cdc2 activity. *J. Virol.* 69:6705-6711.

10. Jowett, J.B., V. Planelles, B. Poon, N.P. Shah, M.L. Chen, and I.S. Chen. 1995. The human immunodeficiency virus type 1 vpr gene arrests infected T cells in the G2 + M phase of the cell cycle. *J. Virol.* 69:6304-6313.

11. Re, F., D. Braaten, E.K. Franke, and J. Luban. 1995. Human immunodeficiency virus type 1 Vpr arrests the cell cycle in G2 by inhibiting the activation of p34cdc2-cyclin B. *J. Virol.* 69:6859-6864.

12. Cohen, E.A., E.F. Terwilliger, Y. Jalinoos, J. Proulx, J.G. Sodroski, and W.A. Haseltine. 1990. Identification of HIV-1 vpr product and function. *J. Acquir. Immune Defic. Syndr.* 3:11-18.

13. Cohen, E.A., G. Dehni, J.G. Sodroski, and W.A. Haseltine. 1990. Human immunodeficiency virus vpr product is a virion-associated regulatory protein. *J. Virol.* 64:3097-3099.

14. Paxton, W., R.I. Connor, and N.R. Landau. 1993. Incorporation of Vpr into human immunodeficiency virus type 1 virions: requirement for the p6 region of gag and mutational analysis. *J. Virol.* 67:7229-7237.

15. Yuan, X., Z. Matsuda, M. Matsuda, M. Essex, and T.H. Lee. 1990. Human immunodeficiency virus vpr gene encodes a virion-associated protein. *Aids Res. Hum. Retroviruses* 6:1265-1271.

16. Yedavalli, V.R., C. Chappey, and N. Ahmad. 1998. Maintenance of an intact human immunodeficiency virus type 1 vpr gene following mother-to-infant transmission. *J. Virol.* 72:6937-6943.

17. Lewis, P.F., and M. Emerman. 1994. Passage through mitosis is required for oncoretroviruses but not for the human immunodeficiency virus. *J. Virol.* 68:510-516.

18. Humphries, E.H., and H.M. Temin. 1974. Requirement for cell division for initiation of transcription of Rous sarcoma virus RNA. *J. Virol.* 14:531-546.

19. Bukrinsky, M.I., S. Haggerty, M.P. Dempsey, N. Sharova, A. Adzhubel, L. Spitz, P. Lewis, D. Goldfarb, M. Emerman, and M. Stevenson. 1993. A nuclear localization signal within HIV-1 matrix protein that governs infection of non-dividing cells. *Nature* 365:666-669.

20. Gallay, P., V. Stitt, C. Mundy, M. Oettinger, and D. Trono. 1996. Role of the karyopherin pathway in human immunodeficiency virus type 1 nuclear import. *J. Virol.* 70:1027-1032.

21. von Schwedler, U., R.S. Kornbluth, and D. Trono. 1994. The nuclear localization signal of the matrix protein of human immunodeficiency virus type 1 allows the establishment of infection in macrophages and quiescent T lymphocytes. *Proc. Natl. Acad. Sci. USA* 91:6992-6996.

22. Gallay, P., T. Hope, D. Chin, and D. Trono. 1997. HIV-1 infection of nondividing cells through the recognition of integrase by the importin/karyopherin pathway. *Proc. Natl. Acad. Sci. USA* 94:9825-9830.

23. Petit, C., O. Schwartz, and F. Mammano. 2000. The karyophilic properties of human immunodeficiency virus type 1 integrase are not required for nuclear import of proviral DNA. *J. Virol.* 74:7119-7126.

24. Bouyac-Bertoia, M., J.D. Dvorin, R.A. Fouchier, Y. Jenkins, B.E. Meyer, L.I. Wu, M. Emerman, and M.H. Malim. 2001. HIV-1 infection requires a functional integrase NLS. *Mol. Cell* 7:1025-1035.

25. Zennou, V., C. Petit, D. Guetard, U. Nerhbass, L. Montagnier, and P. Charneau. 2000. HIV-1 genome nuclear import is mediated by a central DNA flap. *Cell* 101:173-185.

26. Miller, M.D., C.M. Farnet, and F.D. Bushman. 1997. Human immunodeficiency virus type 1 preintegration complexes: studies of organization and composition. *J. Virol.* 71:5382-5390.

27. Popov, S., M. Rexach, L. Ratner, G. Blobel, and M. Bukrinsky. 1998. Viral protein R regulates docking of the HIV-1 preintegration complex to the nuclear pore complex. *J. Biol. Chem.* 273:13347-13352.

28. Jenkins, Y., M. McEntee, K. Weis, and W.C. Greene. 1998. Characterization of HIV-1 vpr nuclear import: analysis of signals and pathways. *J. Cell Biol.* 143:875-885.

29. Sherman, M.P., C.M. de Noronha, M.I. Heusch, S. Greene, and W.C. Greene. 2001. Nucleocytoplasmic shuttling by human immunodeficiency virus type 1 Vpr. *J. Virol.* 75:1522-1532.

30. Hrimech, M., X.J. Yao, F. Bachand, N. Rougeau, and E.A. Cohen. 1999. Human immunodeficiency virus type 1 (HIV-1) Vpr functions as an immediate-early protein during HIV-1 infection. *J. Virol.* 73:4101-4109.

31. Forget, J., X.J. Yao, J. Mercier, and E.A. Cohen. 1998. Human immunodeficiency virus type 1 vpr protein transactivation function: mechanism and identification of domains involved. *J. Mol. Biol.* 284:915-923.

32. Yao, X.J., A.J. Mouland, R.A. Subbramanian, J. Forget, N. Rougeau, D. Bergeron, and E.A. Cohen. 1998. Vpr stimulates viral expression and induces cell killing in human immunodeficiency virus type 1-infected dividing Jurkat T cells. *J. Virol.* 72:4686-4693.

33. Stewart, S.A., B. Poon, J.B. Jowett, and I.S. Chen. 1997. Human immunodeficiency virus type 1 Vpr induces apoptosis following cell cycle arrest. *J. Virol.* 71:5579-5592.

34. Stewart, S.A., B. Poon, J.B. Jowett, Y. Xie, and I.S. Chen. 1999. Lentiviral delivery of HIV-1 Vpr protein induces apoptosis in transformed cells. *Proc. Natl. Acad. Sci. USA* 96:12039-12043.

35. Stewart, S.A., B. Poon, J.Y. Song, and I.S. Chen. 2000. Human immunodeficiency virus type 1 vpr induces apoptosis through caspase activation. *J. Virol.* 74:3105-3111.

36. Conti, L., P. Matarrese, B. Varano, M.C. Gauzzi, A. Sato, W. Malorni, F. Belardelli, and S. Gessani. 2000. Dual role of the HIV-1 vpr protein in the modulation of the apoptotic response of T cells. *J. Immunol.* 165:3293-3300.

37. Poon, B., K. Grovit-Ferbas, S.A. Stewart, and I.S.Y. Chen. 1998. Cell cycle arrest by Vpr in HIV-1 virions and insensitivity to antiretroviral agents. *Science* 281:266-269.

38. Ayyavoo, V., A. Mahboubi, S. Mahalingam, R. Ramalingam, S. Kudchodkar, W.V. Williams, D.R. Green, and D.B. Weiner. 1997. HIV-1 Vpr suppresses immune activation and apoptosis through regulation of nuclear factor kappa B. *Nat. Med.* 3:1117-1123.

39. Conti, L., G. Rainaldi, P. Matarrese, B. Varano, R. Rivabene, S. Columba, A. Sato, F. Belardelli, W. Malorni, and S. Gessani. 1998. The HIV-1 vpr protein acts as a negative regulator of apoptosis in a human lymphoblastoid T cell line: possible implications for the pathogenesis of AIDS. *J. Exp. Med.* 187:403-413.

40. Glushakova, S., B. Baibakov, J. Zimmerberg, and L.B. Margolis. 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.

41. Penn, M.L., J.C. Grivel, B. Schramm, M.A. Goldsmith, and L. Margolis. 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.

42. Schramm, B., M.L. Penn, E.H. Palacios, R.M. Grant, F. Kirchhoff, and M.A. Goldsmith. 2000. Cytopathicity of human immunodeficiency virus type 2 (HIV-2) in human lymphoid tissue is coreceptor dependent and comparable to that of HIV-1. *J. Virol.* 74:9594-9600.

43. Grivel, J.C., M.L. Penn, D.A. Eckstein, B. Schramm, R.F. Speck, N.W. Abbey, B. Herndier, L. Margolis, and M.A. Goldsmith. 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.

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

45. Toohey, K., K. Wehrly, J. Nishio, S. Perryman, and B. Chesebro. 1995. Human immunodeficiency virus envelope V1 and V2 regions influence replication efficiency in macrophages by affecting virus spread. *Virology* 213:70-79.

46. Gibbs, J.S., D.A. Regier, and R.C. Desrosiers. 1994. Construction and in vitro properties of HIV-1 mutants with deletions in "nonessential" genes. *Aids Res. Hum. Retroviruses* 10:343-350.

47. Eckstein, D.A., M.L. Penn, Y.D. Korin, D.D. Scripture-Adams, J.A. Zack, J.F. Kreisberg, M. Roederer, M.P. Sherman, P.S. Chin, and M.A. Goldsmith. 2001. HIV-1 actively replicates in naive CD4+ T-cells residing within human lymphoid tissue. *Immunity* In press.

48. Yao, X.J., R.A. Subbramanian, N. Rougeau, F. Boisvert, D. Bergeron, and E.A. Cohen. 1995. Mutagenic analysis of human immunodeficiency virus type 1 Vpr: role of a predicted N-terminal alpha-helical structure in Vpr nuclear localization and virion incorporation. *J. Virol.* 69:7032-7044.

49. Fletcher, T.M., 3rd, B. Brichacek, N. Sharova, M.A. Newman, G. Stivahtis, P.M. Sharp, M. Emerman, B.H. Hahn, and M. Stevenson. 1996. Nuclear import and cell

cycle arrest functions of the HIV-1 Vpr protein are encoded by two separate genes in HIV-2/SIV(SM). *EMBO J.* 15:6155-6165.

50. Zhang, Z., T. Schuler, M. Zupancic, S. Wietgrefe, K.A. Staskus, K.A. Reimann, T.A. Reinhart, M. Rogan, W. Cavert, C.J. Miller, R.S. Veazey, D. Notermans, S. Little, S.A. Danner, D.D. Richman, D. Havlir, J. Wong, H.L. Jordan, T.W. Schacker, P. Racz, K. Tenner-Racz, N.L. Letvin, S. Wolinsky, and A.T. Haase. 1999. Sexual transmission and propagation of SIV and HIV in resting and activated CD4+ T cells. *Science* 286:1353-1357.

51. Ostrowski, M.A., T.W. Chun, S.J. Justement, I. Motola, M.A. Spinelli, J. Adelsberger, L.A. Ehler, S.B. Mizell, C.W. Hallahan, and A.S. Fauci. 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.

52. Blaak, H., A.B. van't Wout, M. Brouwer, B. Hooibrink, E. Hovenkamp, and H. Schuitemaker. 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.

53. Kolesnitchenko, V., L.M. Wahl, H. Tian, I. Sunila, Y. Tani, D.P. Hartmann, J. Cossman, M. Raffeld, J. Orenstein, L.E. Samelson, and et al. 1995. Human immunodeficiency virus 1 envelope-initiated G2-phase programmed cell death. *Proc. Natl. Acad. Sci. USA* 92:11889-11893.

54. Dedera, D., W. Hu, N. Vander Heyden, and L. Ratner. 1989. Viral protein R of human immunodeficiency virus types 1 and 2 is dispensable for replication and cytopathogenicity in lymphoid cells. *J. Virol.* 63:3205-3208.

55. Hattori, N., F. Michaels, K. Fargnoli, L. Marcon, R.C. Gallo, and G. Franchini. 1990. The human immunodeficiency virus type 2 vpr gene is essential for productive infection of human macrophages. *Proc. Natl. Acad. Sci. USA* 87:8080-8084.

56. Kootstra, N.A., and H. Schuitemaker. 1999. Phenotype of HIV-1 lacking a functional nuclear localization signal in matrix protein of gag and Vpr is comparable to wild-type HIV-1 in primary macrophages. *Virology* 253:170-180.

57. Pantaleo, G., C. Graziosi, L. Butini, P.A. Pizzo, S.M. Schnittman, D.P. Kotler, and A.S. Fauci. 1991. Lymphoid organs function as major reservoirs for human immunodeficiency virus. *Proc. Natl. Acad. Sci. USA* 88:9838-9842.

58. Swingler, S., A. Mann, J. Jacqué, B. Brichacek, V.G. Sasseville, K. Williams, A.A. Lackner, E.N. Janoff, R. Wang, D. Fisher, and M. Stevenson. 1999. HIV-1 Nef mediates lymphocyte chemotaxis and activation by infected macrophages. *Nat. Med.* 5:997-103.

59. Hockett, R.D., J.M. Kilby, C.A. Derdeyn, M.S. Saag, M. Sillers, K. Squires, S. Chiz, M.A. Nowak, G.M. Shaw, and R.P. Bucy. 1999. Constant mean viral copy number per infected cell in tissues regardless of high, low, or undetectable plasma HIV RNA. *J. Exp. Med.* 189:1545-1554.

60. Igarashi, T., C.R. Brown, Y. Endo, A. Buckler-White, R. Plishka, N. Bischogberger, V. Hirsch, and M.A. Martin. 2001. Macrophages are the principal reservoir and sustain high virus loads in rhesus macaques after the depletion of CD4+ T cells by a highly pathogenic simian immunodeficiency virus/HIV type 1 chimera (SHIV): Implications for HIV-1 infections of humans. *Proc. Natl. Acad. Sci. USA* 98:658-663.

61. Ho, D.D., A.U. Neumann, A.S. Perelson, W. Chen, J.M. Leonard, and M. Markowitz. 1995. Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. *Nature* 373:123-126.

62. Wei, X., S.K. Ghosh, M.E. Taylor, V.A. Johnson, E.A. Emini, P. Deutsch, J.D. Lifson, S. Bonhoeffer, M.A. Nowak, B.H. Hahn, and et al. 1995. Viral dynamics in human immunodeficiency virus type 1 infection. *Nature* 373:117-122.

63. Perelson, A.S., A.U. Neumann, M. Markowitz, J.M. Leonard, and D.D. Ho. 1996. HIV-1 dynamics in vivo: virion clearance rate, infected cell life-span, and viral generation time. *Science* 271:1582-1586.

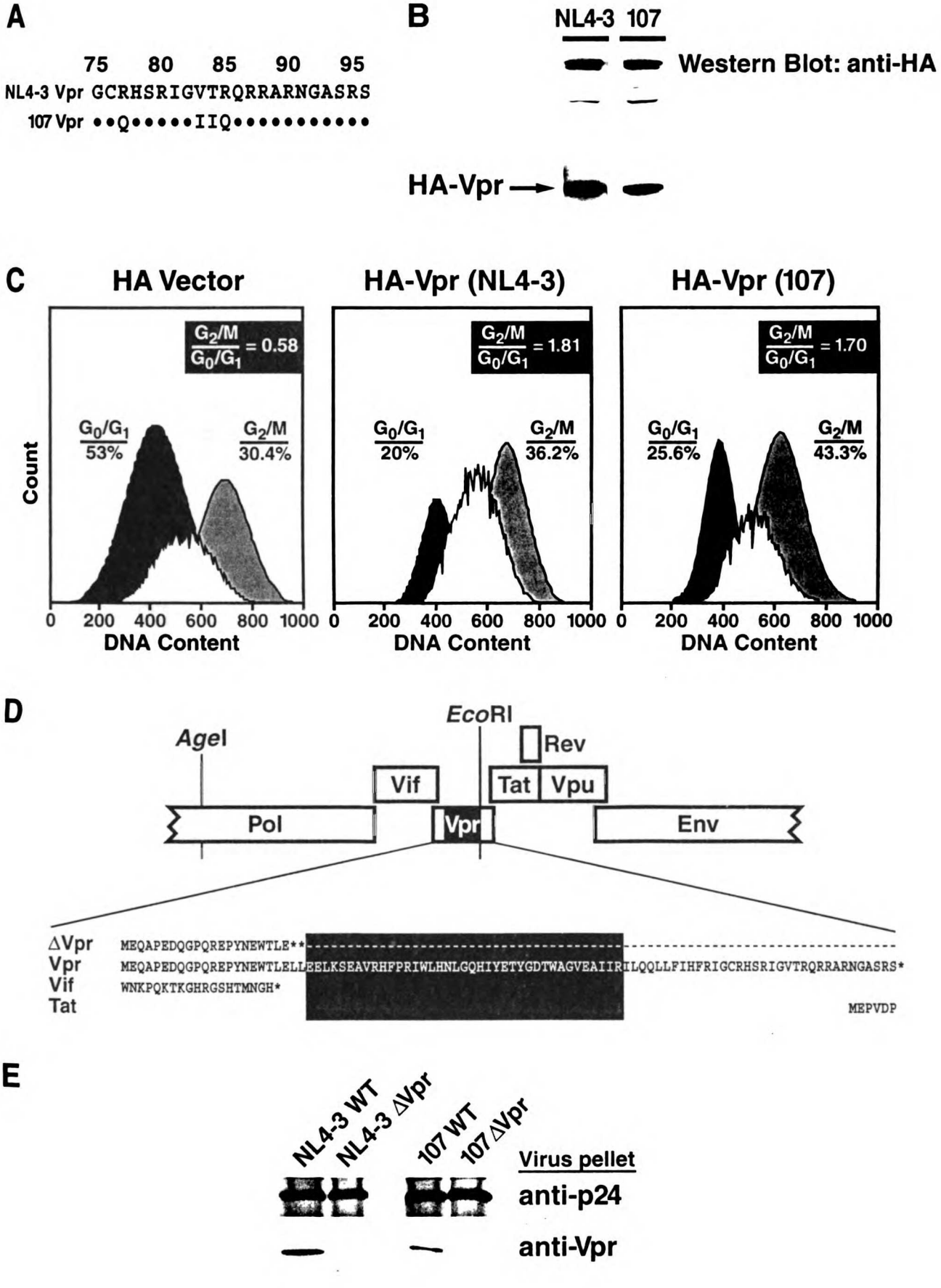
64. Perelson, A.S., P. Essunger, Y. Cao, M. Vesanen, A. Hurley, K. Saksela, M. Markowitz, and D.D. Ho. 1997. Decay characteristics of HIV-1-infected compartments during combination therapy. *Nature* 387:188-191.

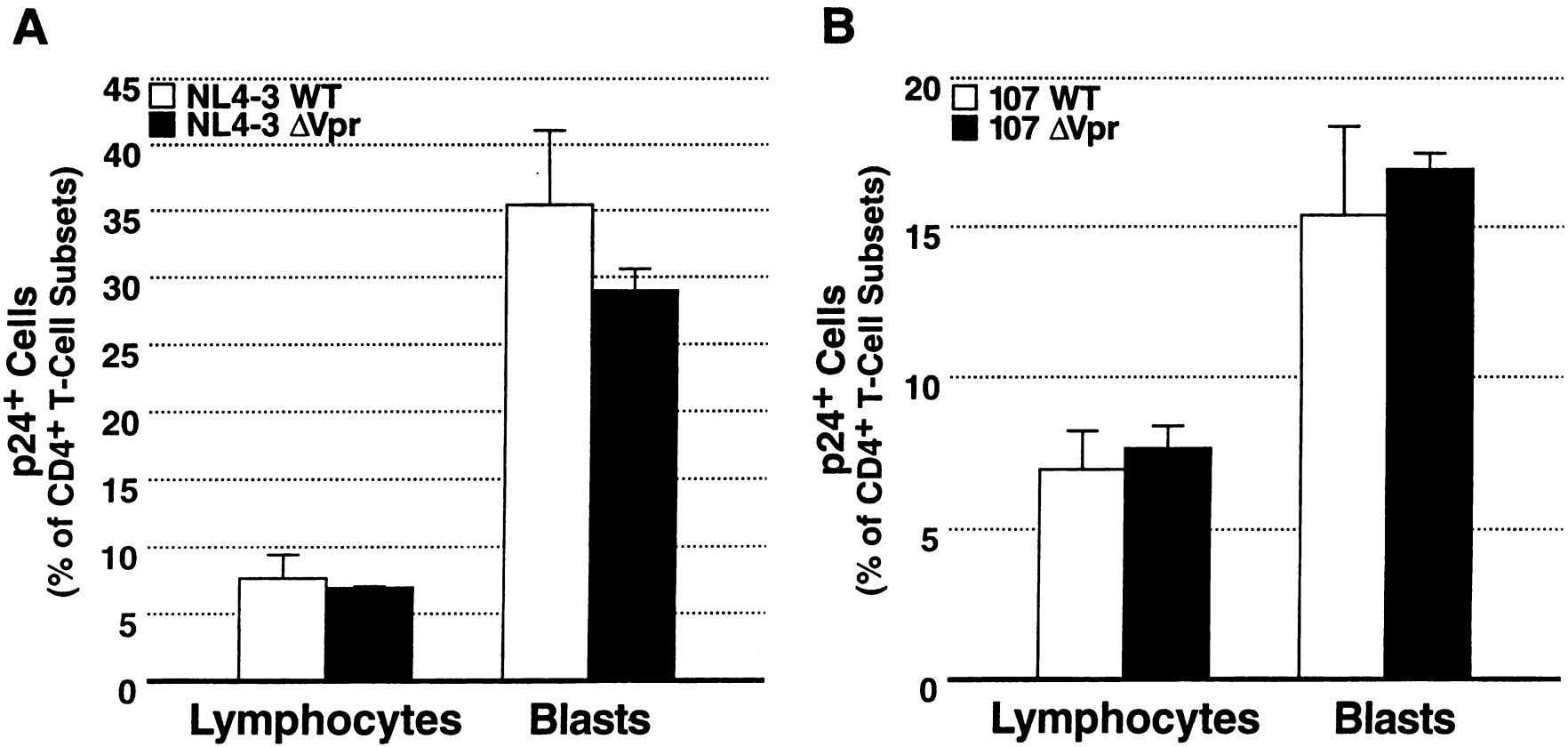
65. Wong, J.K., M. Hezareh, H.F. Günthard, D.V. Havlir, C.C. Ignacio, C.A. Spina, and D.D. Richman. 1997. Recovery of replication-competent HIV despite prolonged suppression of plasma viremia. *Science* 278:1291-1295.

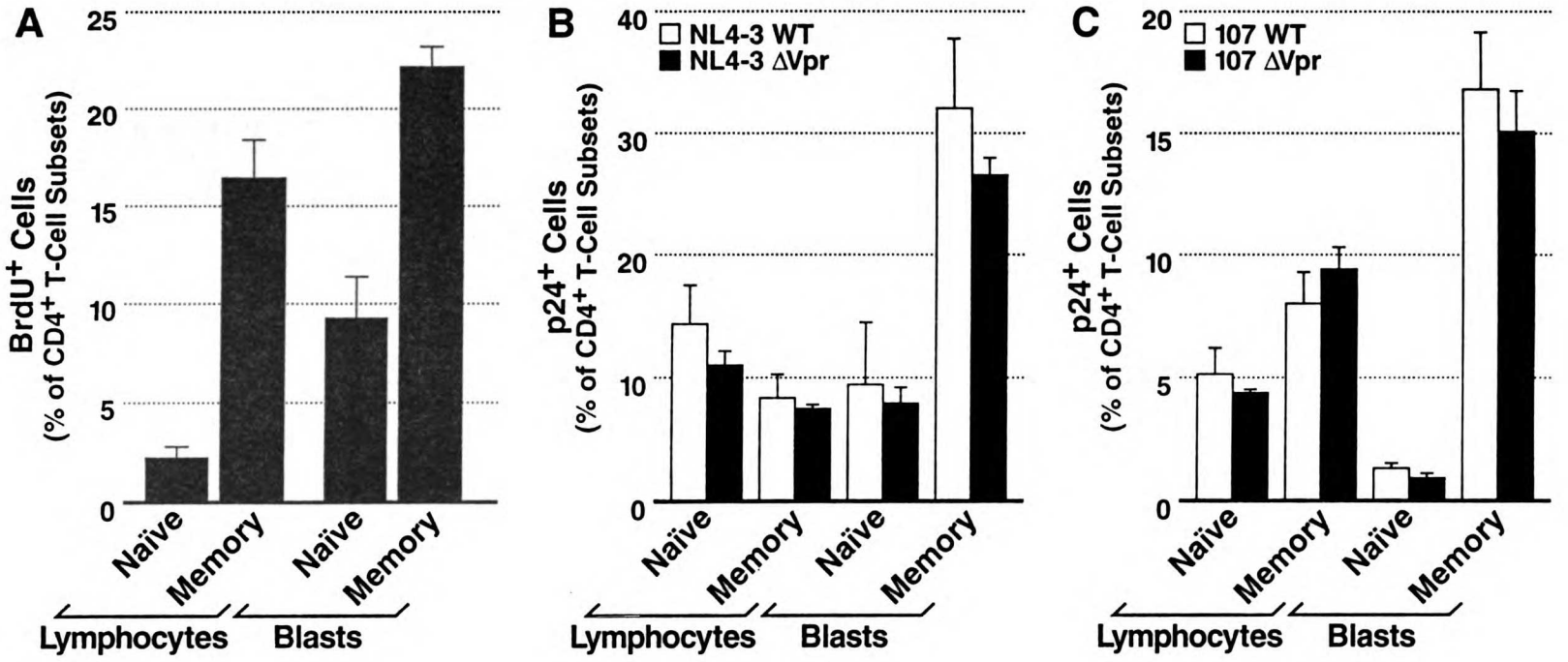
66. Finzi, D., M. Hermankova, T. Pierson, L.M. Carruth, C. Buck, R.E. Chaisson, T.C. Quinn, K. Chadwick, J. Margolick, R. Brookmeyer, J. Gallant, M. Markowitz, D.D. Ho, D.D. Richman, and R.F. Siliciano. 1997. Identification of a reservoir for HIV-1 in patients on highly active antiretroviral therapy. *Science* 278:1295-1300.

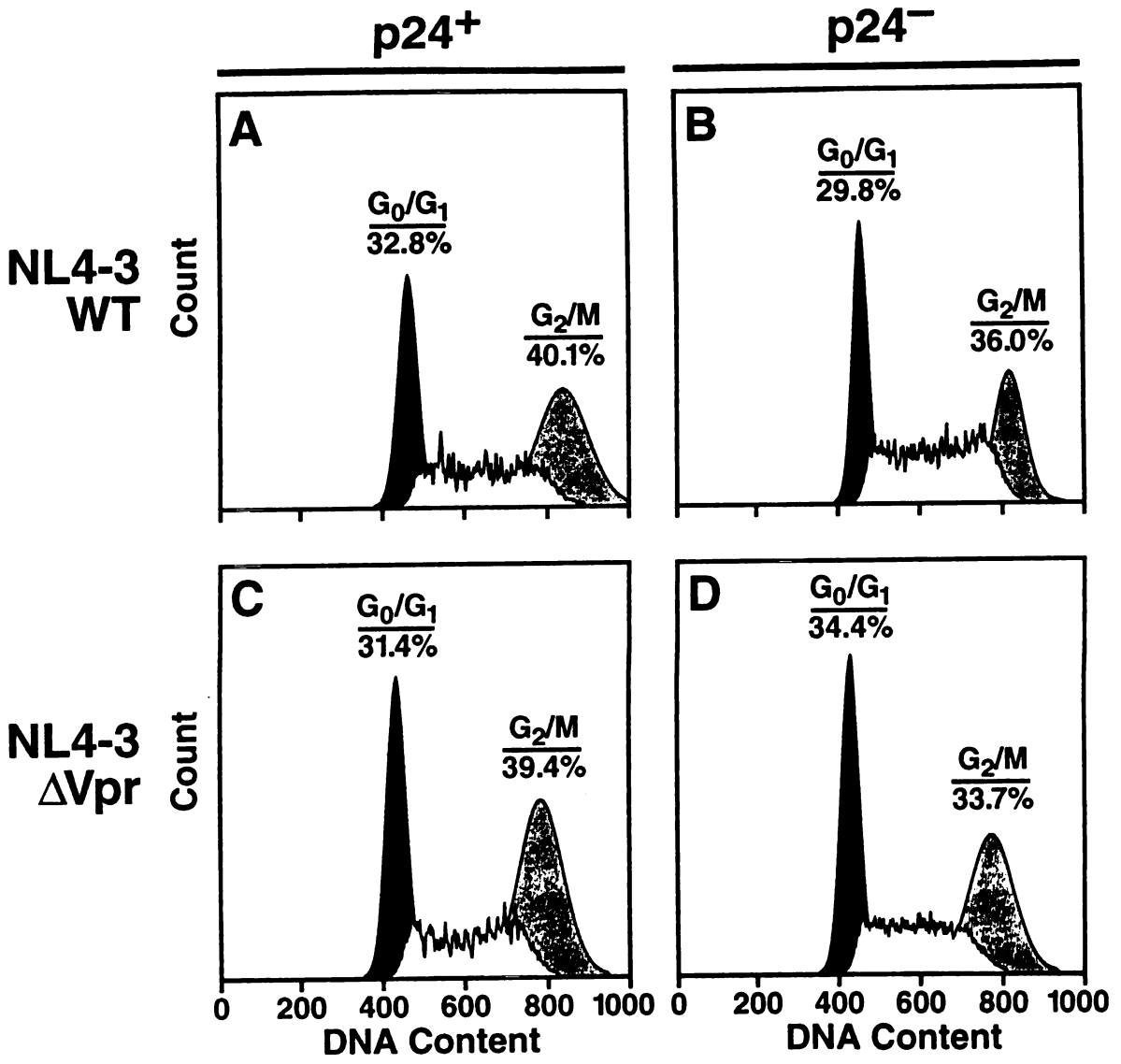
67. Chun, T.W., L. Stuyver, S.B. Mizell, L.A. Ehler, J.A. Mican, M. Baseler, A.L. Lloyd, M.A. Nowak, and A.S. Fauci. 1997. Presence of an inducible HIV-1 latent reservoir during highly active antiretroviral therapy. *Proc. Natl. Acad. Sci. USA* 94:13193-13197.

68. Chun, T.W., R.T. Davey, Jr., M. Ostrowski, J. Shawn Justement, D. Engel, J.I. Mullins, and A.S. Fauci. 2000. Relationship between pre-existing viral reservoirs and the re-emergence of plasma viremia after discontinuation of highly active anti-retroviral therapy. *Nat. Med.* 6:757-761.


A
75 80 85 90 95
NL4-3 Vpr GCRHSRIGVTRQRRARNGASRS
107 Vpr ••Q•••••IIQ••••••••••••
B
NL4-3 107
Western Blot: anti-HA
HA-Vpr
C
HA Vector
HA-Vpr (NL4-3)
HA-Vpr (107)
G2/M / G0/G1 = 0.58
G2/M / G0/G1 = 1.81
G2/M / G0/G1 = 1.70
G0/G1 53%
G2/M 30.4%
G0/G1 20%
G2/M 36.2%
G0/G1 25.6%
G2/M 43.3%
Count
0 200 400 600 800 1000
DNA Content
D
AgeI
EcoRI
Rev
Vif
Tat
Vpu
Pol
Vpr
Env
ΔVpr MEQAPEDQGPQREPYNEWTLE**
Vpr MEQAPEDQGPQREPYNEWTLELLEELKSEAVRHFPRIWLHNLGQHIYETYGDTWAGVEAIIRILQQLLFIHFRIGCRHSRIGVTRQRRARNGASRS*
Vif WNKPQKTKGHRGSHTMNGH*
Tat MEPVDP
E
NL4-3 WT
NL4-3 ΔVpr
107 WT
107 ΔVpr
Virus pellet
anti-p24
anti-Vpr


A
45
40
35
30
25
20
15
10
5
0
NL4-3 WT
NL4-3 ΔVpr
p24+ Cells
(% of CD4+ T-Cell Subsets)
Lymphocytes
Blasts
B
20
15
10
5
0
107 WT
107 ΔVpr
p24+ Cells
(% of CD4+ T-Cell Subsets)
Lymphocytes
Blasts


A
BrdU+ Cells
(% of CD4+ T-Cell Subsets)
25
20
15
10
5
0
Naïve
Memory
Naïve
Memory
Lymphocytes
Blasts
B
p24+ Cells
(% of CD4+ T-Cell Subsets)
40
30
20
10
0
NL4-3 WT
NL4-3 ΔVpr
Naïve
Memory
Naïve
Memory
Lymphocytes
Blasts
C
p24+ Cells
(% of CD4+ T-Cell Subsets)
20
15
10
5
0
107 WT
107 ΔVpr
Naïve
Memory
Naïve
Memory
Lymphocytes
Blasts


p24+
p24−
A
B
C
D
NL4-3
WT
NL4-3
ΔVpr
Count
Count
G0/G1
32.8%
G2/M
40.1%
G0/G1
29.8%
G2/M
36.0%
G0/G1
31.4%
G2/M
39.4%
G0/G1
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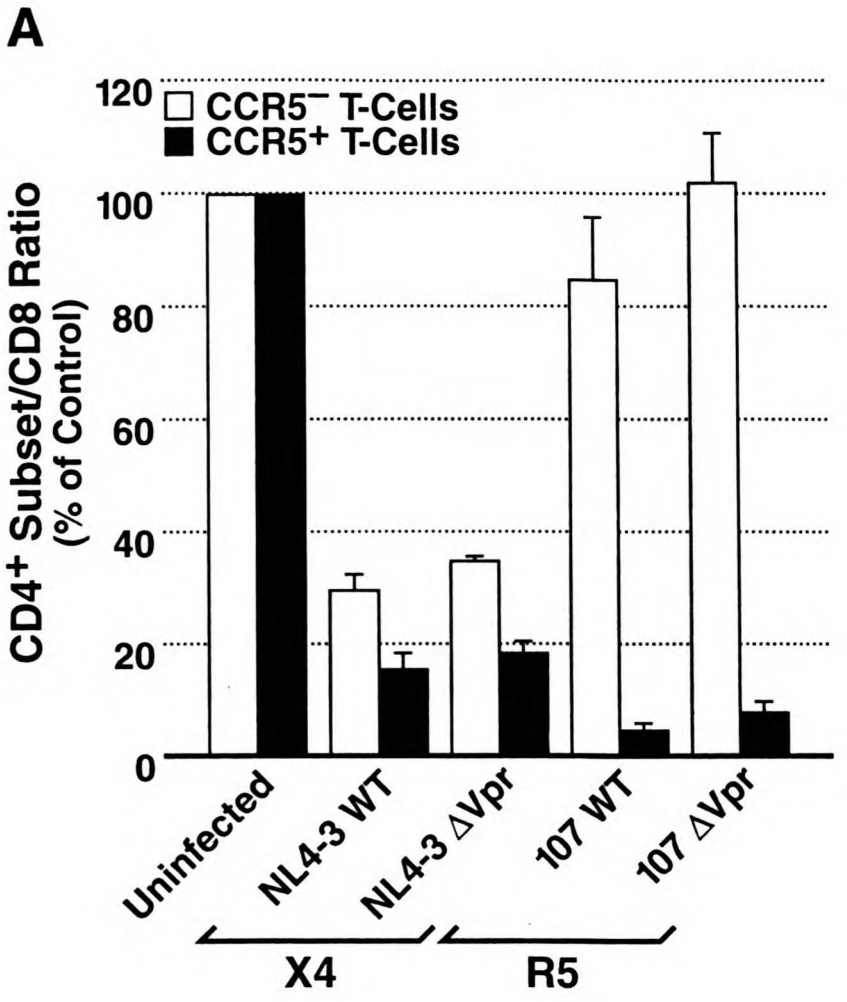
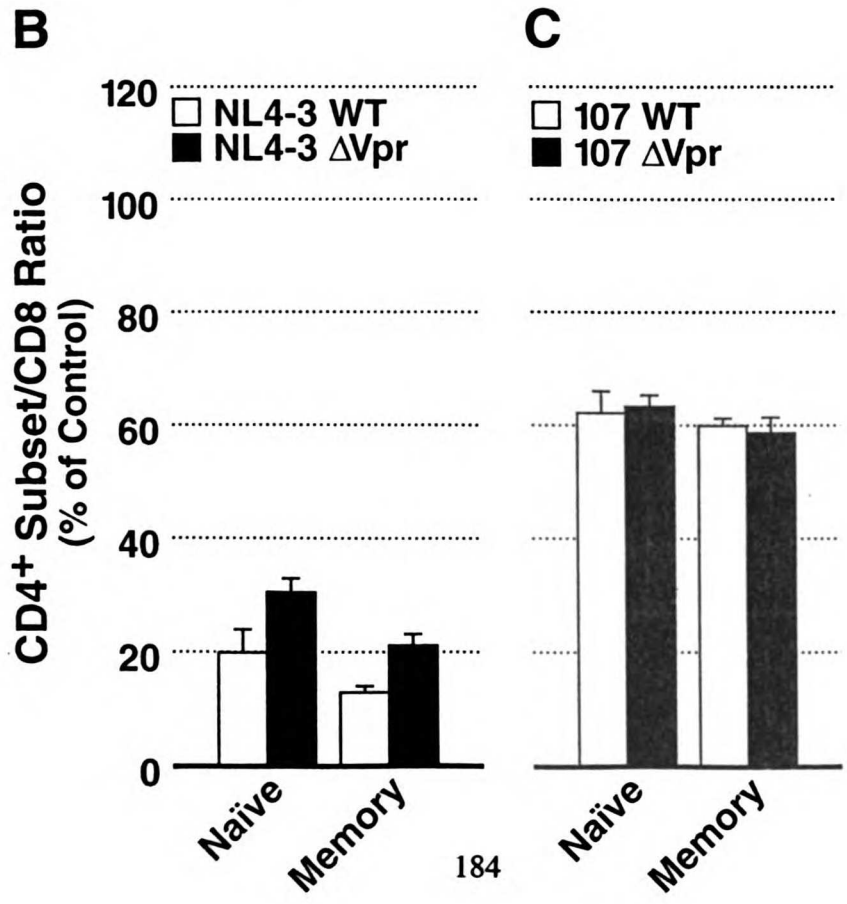
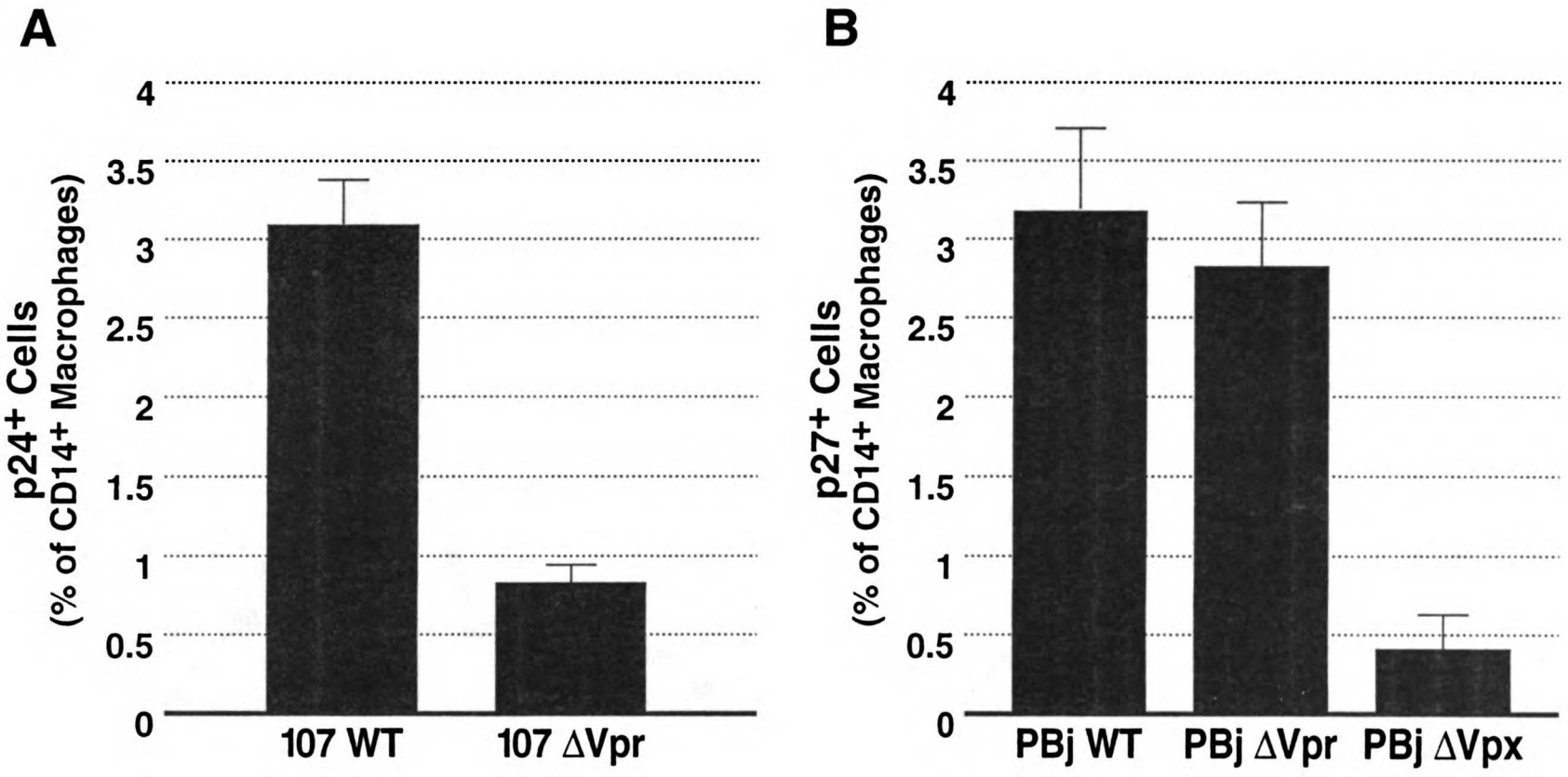
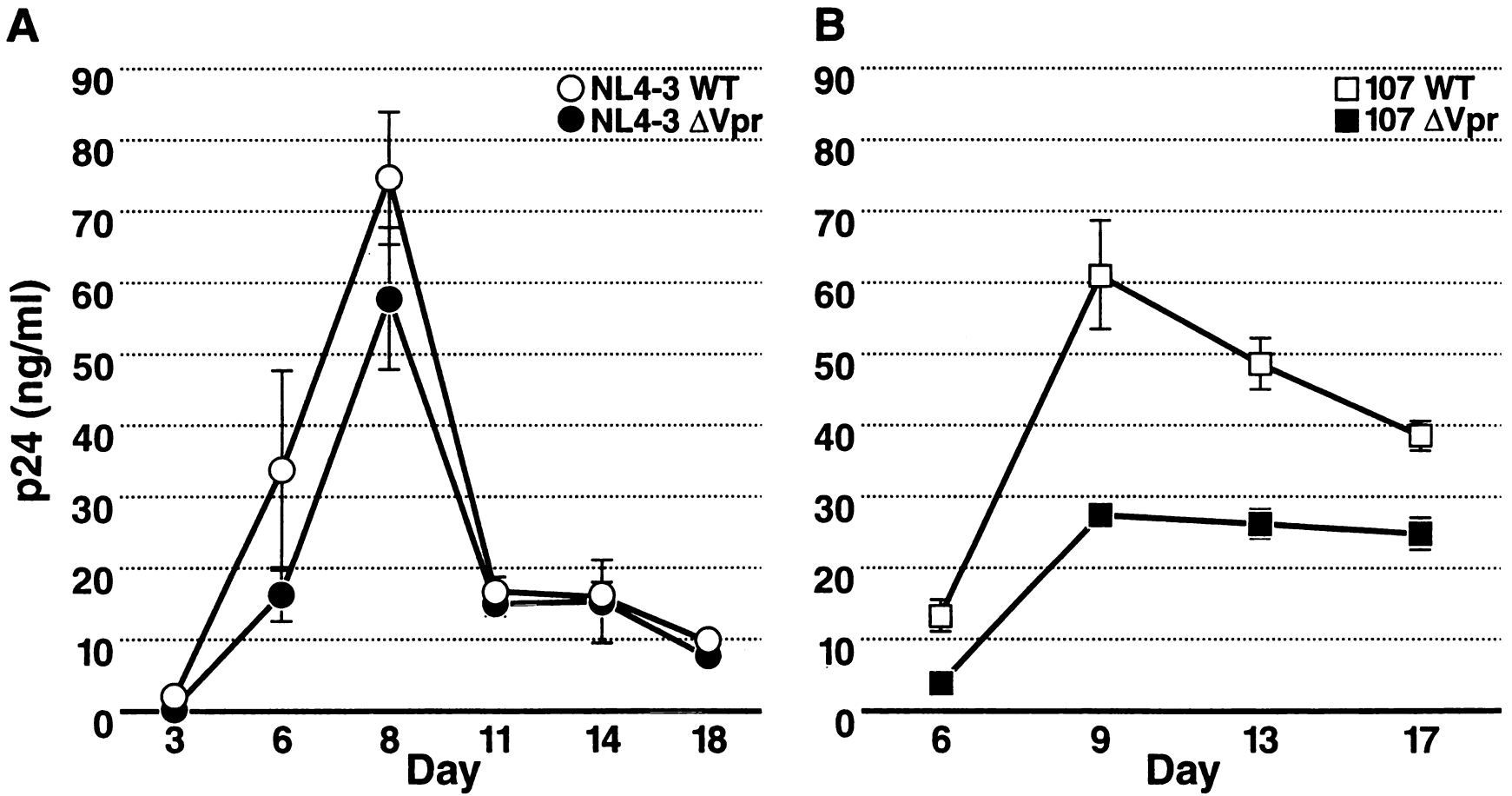
800
1000
DNA Content


A
120
100
80
60
40
20
0
CCR5⁻ T-Cells
CCR5+ T-Cells
CD4+ Subset/CD8 Ratio
(% of Control)
Uninfected
NL4-3 WT
NL4-3 ΔVpr
107 WT
107 ΔVpr
X4
R5


B
C
120
100
80
60
40
20
0
NL4-3 WT
NL4-3 ΔVpr
107 WT
107 ΔVpr
CD4+ Subset/CD8 Ratio
(% of Control)
Naïve
Memory
Naïve
Memory




A
B
NL4-3 WT
NL4-3 ΔVpr
107 WT
107 ΔVpr
p24 (ng/ml)
0
10
20
30
40
50
60
70
80
90
3
6
8
11
14
18
Day
6
9
13
17
Day

Chapter 7

Conclusions

I. IMPLICATIONS

The goal of these studies was to identify the host and viral factors that regulate cellular tropism and viral replication. It is well established that the expression of the coreceptors CXCR4 and CCR5 are a dominant determinant of cell tropism. However, many factors apart from CD4 and coreceptor expression contribute to the efficiency of viral replication. It has previously been shown that in some experimental settings many cells expressing appropriate coreceptors are nevertheless refractory to infection. In particular, resting, naïve cells *in vitro* permit viral entry, but do not complete reverse transcription. Therefore, viral replication is blocked. However, we have demonstrated that within the lymphoid microenvironment many of these cells support the complete life cycle of HIV-1. Interestingly, although both memory and naïve T-cells may be productively infected by HIV-1, we have found quantitative differences in the viral output ("burst size") of these cells. That is, replication of HIV-1 within memory and naïve T-cells results in the release of approximately 6-fold more virus compared to naïve T-cells on a per-cell basis. Therefore, cell permissivity maybe thought of in relativistic terms. Although we may define populations as "permissive" or "non-permissive," a more informative approach may be to consider the average productive capacity of different cells. While cells demonstrating small burst sizes are still sensitive to the cytopathic effects of the virus, the destruction of the immune system results not just from the direct loss of these cells, but may also relate to the systemic effects of HIV-1 replication (e.g.,

bystander killing). A cataloguing and an appreciation of the relative "burst sizes" of different permissive cell types may provide an insight into the mechanisms underlying HIV-mediated immune destruction. Therefore, understanding the factors that modulate proviral transcription and virion assembly and release is an important step towards the continued development of effective anti-retroviral therapies and treatments.

We have shown that replication and T-cell destruction by X4 HIV-1 contributes to the activation of uninfected bystander T-cells. We and others have also demonstrated that activated and/or proliferating cells are the most suitable hosts for HIV replication. Therefore, it appears that X4 replication manipulates the cellular microenvironment within lymphoid organs to create an environment more suitable for viral spread. This may lead to an acceleration of viral infection and replication, and may contribute to a significant increase in viral load. Importantly, this surprising activity is a feature exclusively of X4 viruses. Therefore, the emergence of X4 viruses during late-stage disease may catalyze the production and output of new virions from the newly infected T-cells. That is, X4 viruses may feed-forward to potentiate their own replication. This pathway may underlie the rapid and dramatic increases in the viral loads and severe declines in $CD4^+$ T-cell counts of patients with AIDS. Therefore, pharmocologic inhibition of viral entry through CXCR4, which may prevent the specific emergence of X4 viruses, may be an effective therapeutic strategy to control HIV-1 *in vivo*. Although such treatments would undoubtedly be of little benefit to patients during acute and asymptomatic disease, it may nevertheless delay progression of HIV-disease to AIDS (8,

15). These results highlight the importance of continued pharmaceutical development of coreceptor inhibitors.

We have also shown that infection of naïve T-cells by X4 viruses may result in the latent infection of the host cell. The establishment of this reservoir contributes significantly to the persistence of virus within infected individuals. Therefore control or prevention of X4 viremia may also be an effective means of eradicating the virus within infected individuals. Although it was formerly thought that generation of these cells required a transition of activated cells to an inactive state, our findings indicate that latency may result as a direct outcome of viral entry and reverse transcription of the provirus. That is, latency within mature T-cells may arise independently of prior active viral gene expression and independently of a cellular transition to quiescence. Such a transition within infected cells occurs only rarely, and is not believed to have been exploited as an essential strategy in the pathogenesis of HIV-1. However, our results suggest that latency may result independently of such an uncommon event. Therefore, latent infection must not be thought of as an abortive outcome of viral infection, but rather as an integral part of the viral life cycle. However, the host and viral factors that influence the "choice'" between productive and latent infection have not been identified.

In addition to latently infected T-cells, macrophages may also provide a long-lived reservoir for HIV *in vivo*. Although R5 viruses are selectively transmitted and predominate during much of clinical disease, the role of macrophages in these processes has often been questioned. Some investigators posit that the infection of these cells may

occur as a "byproduct" of the infection of $CCR5^+$ T-cells. It is suggested by some that the importance of the aberrant infection is minimal. However, investigation of the functions of Vpr revealed that tissue macrophages contributed approximately 50% of the total R5 viral output of the experimental lymphoid tissues. This surprising conclusion contradicts the findings of Ho and others that implicated T-cells as the predominant source of viremia. The basis for these different conclusions may lie in the initial assumptions inherent in their mathematical models. Alternatively, the difference may be related to the possible compartmentalization of the viral loads in the peripheral circulation and within lymphoid organs. Nevertheless, it is clear that tissue macrophages are a substantial source of replicating virus within tissue. The spread of this virus to neighboring permissive T-cells undoubtedly contributes to the pathogenesis of HIV *in vivo*. Furthermore, because of the longevity of these tissue macrophages, they may serve as resilient and stable reservoirs for HIV replication during all disease stages *in vivo*. Collectively, this suggests a prominent role of macrophages as an important element in the evolutionary strategy and cytopathicity of HIV-1.

We have demonstrated that Vpr contributes substantially to the infection of macrophages, but does not play a role in the infection of T-cells regardless of the cellular state of activation, proliferation, or maturation. We infer that other viral factors must mediate the infection of these cells. This finding suggests that HIV-1 may have evolved distinct and possibly redundant mechanisms of nuclear targeting of the PIC. Each of these putative factors, including Vpr, may serve as suitable drug targets for therapeutic

treatments. Thus, many potential targets may exist to inhibit the spread and replication of HIV-1 *in vivo*. However, there is only limited evidence that a single viral factor may play a dominant role in nuclear entry of the PIC within all cell types. Therefore, effective inhibition of this step in the viral life cycle would depend on the development of multiple different pharmaceutical agents. Thus, the efficacy of each drug alone would be limited, and the cost of their collective development may be prohibitive.

Our findings also highlight the importance of continued anti-retroviral drug development. We have shown that quiescent cells (including resting T-cells and tissue macrophages) support active viral gene expression. Thus, these cells contribute substantially to the local viral burden and may play an important role in the spread of HIV-1. Many of these cells are also sensitive to the cytopathic effects of the virus. Therefore, replication of HIV within these host cells likely ultimately contributes to immune dysfunction and collapse. Unfortunately, many of the therapeutic agents in use today exhibit limited penetration into these cells and/or are inefficiently activated within these quiescent populations (2, 5, 10, 18). Thus, significant viral reservoirs may persist within infected individuals receiving HAART. This conclusion is supported by the finding that viral replication and evolution continue within these patients despite reductions in viral loads to levels below detection (3, 4, 6, 20). This continued viral production may contribute to the development of drug-resistant viruses and may also contribute to continued immune destruction in infected individuals in whom viral replication appears to be controlled. We have also shown that the infection of many of

these non-dividing cells may result in a latent infection. This low-level replication of HIV may continually "reseed" this important reservoir. Therefore, it is imperative that research and development of new anti-retroviral drugs be directed at the control of virus within these resistant cell populations. One possibility discussed above is the generation of CXCR4 antagonists. Such agents would not depend on biochemical modification within infected or susceptible cells (as is necessary for many reverse transcriptase inhibitors and other drugs in use today), and therefore may be effective at inhibiting the establishment of these important reservoirs. Without such clinical treatments, the eradication of virus from infected individuals may remain an impossible goal.

Finally, we have demonstrated that HIV-1 can productively infect resting, non-proliferating cells. This suggests that HIV-1-based vectors may be suitable candidates for experimental gene delivery vehicles. However, although we have shown that quiescent cells (including those in G0 and G1a) are permissive for infection, we have also found that the consequence of this infection is qualitatively different than that of activated cells. That is, we have shown that the viral productive capacity of these resting cells is substantially lower than that of activated cells. Therefore, although we may indeed be technically capable of delivering genes to terminally differentiated cells via HIV-1 derived vectors, expression of these genes in these cells may be limited. Exchange of the endogenous HIV-1 LTR with other, more "potent" promoters may effectively improve gene expression. However, deletion of viral genes from the HIV-1 genome, which is necessary to "carry" the therapeutic genes of interest, may inadvertently interfere with or

ablate the unique ability of HIV-1 to infect non-dividing cells—ironically, the very property that makes HIV-1 an attractive therapeutic tool. Before informed and rational decisions may be made to engineer HIV-1-based vectors, a comprehensive examination of the viral factors that contribute to the infection of divergent cell types must be undertaken. Furthermore, we do not fully understand the mechanisms underlying and contributing to HIV-1 pathogenesis, and until these pathways are completely understood, HIV-1 is, of course, unsuitable for gene delivery. Although there is hope that HIV-1 might ultimately be used for clinical therapies, the need for much more scientific investigation remains.

II. LIMITATIONS OF SCOPE OF PRESENT WORK BASED ON *EX VIVO* MODEL SYSTEM

Although many different and complimentary experimental techniques were utilized in the investigations included in this dissertation, the work described relies exclusively on the study and manipulation of mature lymphoid tissues *ex vivo*. As we have demonstrated, significant differences exist between mature lymphocytes present within peripheral lymphoid organs and those isolated from peripheral circulation. We infer that important differences may also characterize the cells and microenvironments of other lymphoid organs, including thymic tissue and gut and mucosal-associated lymphoid tissue, and the present work did not address these. These conclusions highlight the need to study all relevant tissues that harbor HIV and contribute to viral pathogenesis *in vivo*.

In addition, although we based our work on a single model system, a study of HIV behavior at the cellular and tissue levels, investigations of HIV at the molecular and organismic levels are also of great importance. Such investigations are complimentary to the work described here, and are essential to our understanding of HIV biology.

III. SPECIFIC EXPERIMENTAL LIMITATIONS OF THE LYMPHOID CULTURE TECHNIQUES

Although the HLH and HLAC models employed in these studies represent valuable tools to investigate the behavior and pathogenesis of HIV, they nevertheless suffer from some experimental limitations. In particular, lymphoid tissues *in vivo* are dynamic organs with significant lymphocyte traffic through the tissue. Thus, they are part of a systemic immune system. Excision of these tissues removes these linkages, and isolates the tissues from the systemic affects and regulation normally imposed *in vivo*. Therefore, these tissues *ex vivo* are static systems that do not recapitulate the full behavior of lymphocytes and organs *in vivo*. The importance of these features absent from HLH and HLAC to the biology of HIV has not been assessed.

HIV infection results in significant immune responses *in vivo*. Although HLH has been shown to respond to recall antigens (tetanus toxoid), no evidence has been found for the formation of novel immune responses within these tissues *in vitro*. It is believed that the initial immune responses to HIV *in vivo* contribute to the early immune control of viral replication and are responsible for the significant decline in viremia following acute

infection. However, these responses may also contribute to the profound and widespread immune dysfunction (involving cells in addition to $CD4^+$ T-lymphocytes) observed in infected individuals. These responses may include the capture of virions by the reticuloendothelial system thereby altering dendritic and other cells, the generation of activated HIV-specific effector cells that then become permissive for HIV infection and replication, and the production of envelope-specific antibodies that may catalyze the shedding of the viral envelope. However, the importance of these responses to the dynamics of HIV within lymphoid tissues and the progression of disease *in vivo* is not known, and because HLH and HLAC may not generate these responses, they are not sufficient models in which to study the full spectrum of HIV pathogenesis.

Finally, these tissues are viable *in vitro* for only 2–3 weeks, whereas HIV infection of individuals may persist for a decade or more. As such, HLH and HLAC serve as effective models of acute viral infection. However, they may not fully reproduce the characteristics of chronically-infected immune tissues. Consequently, we may employ these models to study the initial behavior of HIV upon entering formerly uninfected tissues in the absence of antiviral immune responses, but they may be inadequate to investigate the complete set of viral dynamics during asymptomatic infection and AIDS. Nevertheless, our study of the activating potential of X4 viruses within tissues that had been extensively depleted of $CD4^+$ T-cells by the virus suggests that these tissues may recapitulate select aspects of progressive HIV disease. Collectively, these limitations highlight the importance of exploiting existing and complementary models of HIV

pathogenesis and also underscore the importance of developing small animal models of HIV infection that recapitulate a broader range of virus-host interactions that characterizes HIV disease *in vivo*.

IV. FUTURE DIRECTIONS

Perhaps the most striking result of these studies is the dramatic difference between the behavior of mature lymphocytes in different organ systems. We have shown that non-dividing, naïve T-lymphocytes are permissive for HIV-1 infection when present within lymphoid tissues, despite the fact that these cells isolated from peripheral blood are refractory to infection. In addition, we have also observed that tissue lymphocytes isolated from the organ culture become resistant to productive infection. However, the factors underlying these phenomena have not been determined. Identification of the endogenous elements capable of transforming resistant cells to permissive targets is a vital step in understanding the mechanism of viral spread and may prove to be helpful in combating HIV replication and disease progression *in vivo*.

Furthermore, it is also essential that we identify the viral factors that contribute to the infection of resting T-cells within these model systems and *in vivo*. Studies have demonstrated reduced or delayed replication by Nef-deficient viruses, consistent with the putative role of this factor in the spread of new virions (7, 9, 11, 13). However, virion-associated Nef is also postulated to contribute to the activation of newly infected host cells (1, 12, 14, 16, 17, 19). Recent findings have also demonstrated that fully reverse-

transcribed but unintegrated provirus present within the nuclei of infected cells may be transcribed into mRNA and translated into proteins by host machinery (21). Although not all viral proteins may be made by this pathway, it permits significant expression of Nef, Tat, and Rev. As with virion-associated protein, Nef produced in this manner may also contribute to the activation of host cells. The importance of this function in the infection of the diverse T-cell subsets identified in this dissertation has not been investigated. We speculate that Nef may play a critical role in the productive infection of naïve and/or non-activated T-cells. Stimulation of these cells by Nef to an activation state above an undetermined threshold may be an essential (but not sufficient) step in the life cycle of HIV in these cells. Studies comparing the replication and tropism of matched wild-type and *nef*-deleted HIV variants are necessary to investigate this possibility.

Our studies also demonstrated that Vpr contributes significantly to the ability of HIV to infect non-dividing macrophages. However, Vpr does not play a role in the productive infection of non-dividing T-cells. This suggests that other viral factors may be responsible for this function alone or in combination with Vpr. Other investigators have identified nucleophilic sequences and properties within both matrix (MA) and integrase (Int). We speculate that these signals may play a dominant role in mediating infection of non-cycling T-cells. Because these proteins are crucial for viral replication, they may not be deleted from the virus without incapacitating replication. However, many of the relevant signal sequences have now been identified within these proteins. Thus, we may design and construct viruses incorporating limited deletions or amino acid substitutions

within these sequences thereby generating virus strains deficient for these signals. Some of this work has already been accomplished. Subsequent investigation of the behavior and tropism of these viruses within human lymphoid tissues *ex vivo* are necessary to determine the role of each of these signals. Identification of a dominant viral protein, amino acid residue(s), or factor may provide therapeutic targets for drug discovery.

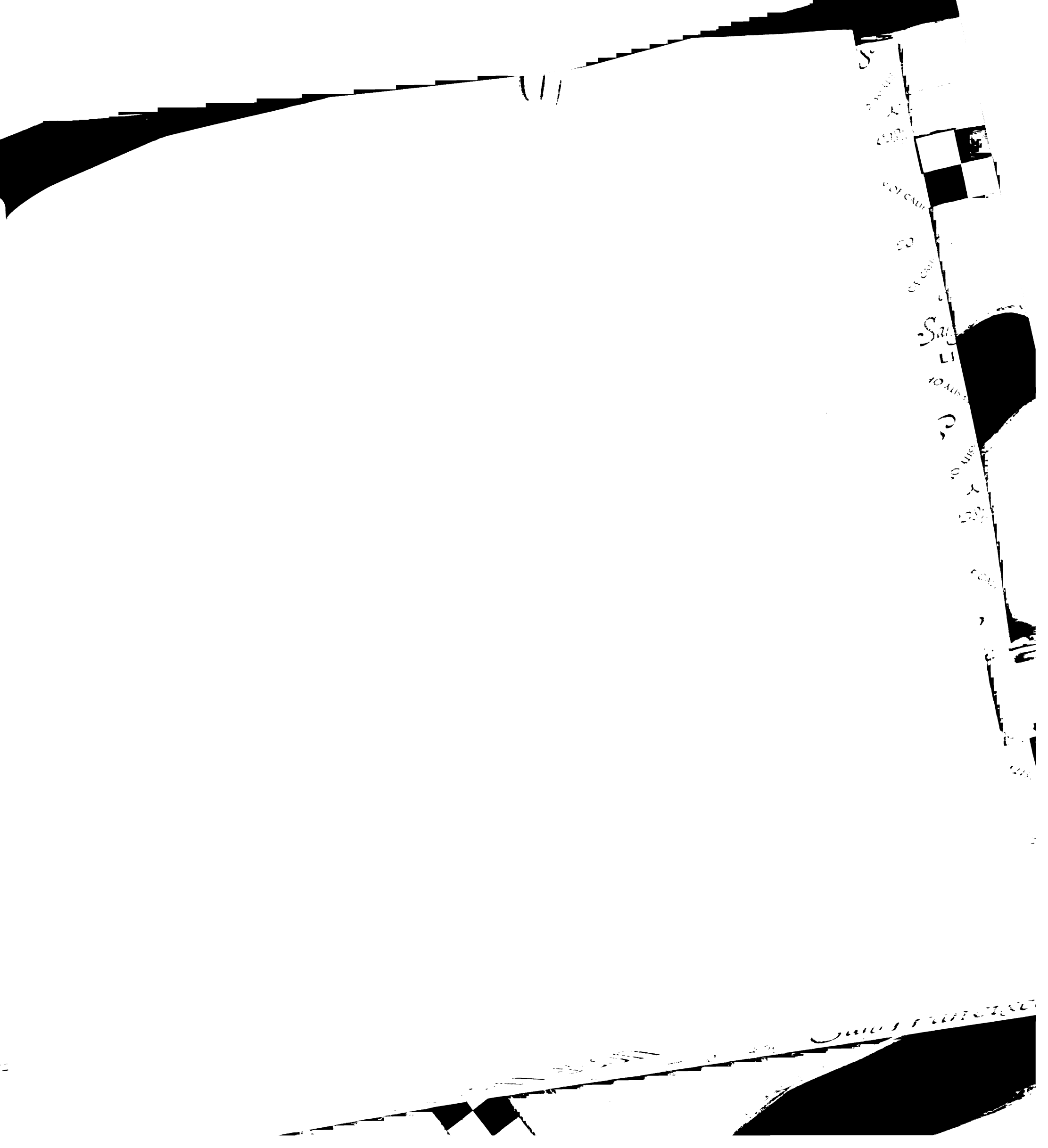
The same investigations of Vpr function also revealed that tissue macrophages contribute substantially to local viral burden. However, despite the fact that only $CCR5^+$ T-cells contributed to viral output in tissues inoculated with Vpr-deficient strains of R5 HIV-1, virus replicated within these tissues to a level approximately 50% of that seen in tissues inoculated with wild-type strains of X4 HIV-1. Interestingly, the number of host cells supporting X4 virus replication (the number of $CXCR4^+$ T-cells) is approximately 5 times greater than the number of host cells supporting R5 virus replication (the number of $CCR5^+$ T-cells). This suggests that the productive capacity of virus within the $CCR5^+$ subset of T-cells is much greater than the productive capacity of $CXCR4^+$ $CCR5^-$ T-cells. Rough estimates suggest that the burst size of $CCR5^+$ T-cells may exceed that of $CXCR4^+$ $CCR5^-$ T-cells by at least a factor of 4. If these estimates are correct, this finding may have significant implications for the dominance of R5 viruses during transmission, acute, and asymptomatic stages of HIV disease. We have begun to apply our techniques of measuring viral burst sizes to quantitate the productive capacity of HIV-infected $CCR5^+$ and $CCR5^-$ T-cells and also to compare the replication of representative X4 and R5 strains of HIV-1 within each of these host cells.

Our studies have also revealed that a substantial fraction of naïve $CD4^+$ T-cells are latently infected by X4 HIV-1. Such inducible reservoirs of viral replication pose a substantial challenge to the elimination of virus within infected individuals. Defining the host and viral factors that direct HIV along the mutually exclusive pathways leading to productive or latent infection of the host cell is an important step in controlling, flushing, or eradicating this reservoir. Studies aimed at identifying phenotypic differences (such as surface expression of activation markers) between productively infected and latently infected naïve T-cells may be accomplished within HLH and HLAC models. Furthermore, it is also important to identify endogenous signals that regulate the induction of viral expression within latently infected T-cells *in vivo*. Our studies concentrated on the cytokines IL-2, IL-6, and TNF-α, which are found in abundance within lymphoid tissues. However other signals, such as those delivered by antigen presenting cells, or those delivered upon encounter of the latently infected cell with its responsive antigen, may also be important. Fractionation of the lymphoid subsets into cellular subsets may be an effective way to identify cells that contribute to this process.

Finally, we discovered evidence for trans-activation of bystander T-cells within lymphoid tissues inoculated with X4 strains of HIV-1 but not R5 strains. This activation was not restricted to the $CD4^+$ T-cell subset, and was not inhibited by the CXCR4 antagonist AMD3100. These findings imply, but do not prove, that the effect is mediated by factors other than the viral envelope. Since the viruses in these experiments were otherwise isogenic, we suspect that bystander activation is due to non-viral factors. We

have inferred but not proven that activation is instead due to the release of proinflammatory mediators following the massive T-depletion and cell death associated with X4 infections. Identification of these factors through supernatant transfers and fractionation may be revealing. Furthermore, significant controversy surrounds the mechanism of T-cell loss *in vivo*. Numerous reports have demonstrated that a substantial fraction of apoptotic cells within the lymphoid tissues of infected individuals is not productively infected by HIV. The investigators have posited that HIV-1 may induce cytopathic effects within bystander cells. Although we have not observed this phenomenon directly, our results may be related to bystander killing. Specifically, we have shown that activated and proliferating cells within HLH and HLAC exhibit the highest rates of apoptosis. Therefore, activation of cells by HIV may set them on a course ultimately resulting in cell death. We are now carefully investigating the dynamics of cell death within these tissues and directly correlating the findings with the infection of T-cell subsets. These studies are utilizing multiple strains of HIV-1, including molecular clones and primary isolates, as well as various markers of T-cell apoptosis and necrosis. Understanding these associations and pathways may illuminate important therapeutic strategies that may help preserve T-cell diversity and numbers *in vivo*.

Collectively, these proposed experiments build on the understanding of the host and cellular factors regulating T-cell infection that have been identified by the studies within this dissertation. Ultimately, the goal of this work is to understand the mechanisms of HIV-1 pathogenesis and the genesis and evolution of HIV-1-mediated



immunodeficiency. We have demonstrated that nearly all T-cells are permissive hosts for viral replication, that macrophages contribute substantially to viral burden, and that HIV-1 has evolved specialized features permitting infection of these diverse targets. Hopefully, these insights will provide a foundation for future discoveries that will advance our scientific understanding of HIV-1 biology and improve our therapeutic approaches to HIV disease.

REFERENCES

1. **Alexander, L. N., and C. M. Wilcox.** 1997. A prospective trial of thalidomide for the treatment of HIV-associated idiopathic esophageal ulcers. Aids Research and Human Retroviruses. **13:**301-4.

2. **Aquaro, S., C. F. Perno, E. Balestra, J. Balzarini, A. Cenci, M. Francesconi, S. Panti, F. Serra, N. Villani, and R. Caliò.** 1997. Inhibition of replication of HIV in primary monocyte/macrophages by different antiviral drugs and comparative efficacy in lymphocytes. J. Leukoc. Biol. **62:**138-43.

3. **Chun, T.-W., R. T. Davey Jr., M. Ostrowski, J. S. Justement, D. Engel, J. I. Mullins, and A. S. Fauci.** 2000. Relationship between pre-existing viral reservoirs and the re-emergence of plasma viremia after discontinuation of highly active anti-retroviral therapy. Nat. Med. **6:**757-761.

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

5. **Davis, C., A. Heredia, N. Le, J. K. Dominique, and R. R. Redfield.** 2001. Differential human immunodeficiency virus-suppressive activity of reverse transcription inhibitors in resting and activated peripheral blood lymphocytes: implications for therapy. J. Hum. Virol. **4:**113-122.

6. **Finzi, D., M. Hermankova, T. Pierson, L. M. Carruth, C. Buck, R. E. Chaisson, T. C. Quinn, K. Chadwick, J. Margolick, R. Brookmeyer, J. Gallant, M. Markowitz, D. D. Ho, D. D. Richman, and R. F. Siliciano.** 1997. Identification of a reservoir for HIV-1 in patients on highly active antiretroviral therapy. Science. **278:**1295-1300.

7. **Glushakova, S., J. C. Grivel, K. Suryanarayana, P. Meylan, J. D. Lifson, R. Desrosiers, and L. Margolis.** 1999. Nef enhances human immunodeficiency virus replication and responsiveness to interleukin-2 in human lymphoid tissue ex vivo. J. Virol. **73:**3968-74.

8. **Kreisberg, J. F., D. Kwa, B. Schramm, V. Trautner, R. Connor, H. Schuitemaker, J. I. Mullins, A. B. van't Wout, and M. G. Goldsmith.** 2001. Cytopathicity of Human Immunodeficiency Virus Type 1 Primary Isolates Depends on Coreceptor Usage and Not Patient Disease Status. J. Virol. **75:**8842-8847.

9. **Miller, M. D., M. T. Warmerdam, I. Gaston, W. C. Greene, and M. B. Feinberg.** 1994. The human immunodeficiency virus-1 nef gene product: a positive factor for viral infection and replication in primary lymphocytes and macrophages. J. Exp. Med. **179:**101-13.

10. **Perno, C. F., F. M. Newcomb, D. A. Davis, S. Aquaro, R. W. Humphrey, R. Caliò, and R. Yarchoan.** 1998. Relative potency of protease inhibitors in monocytes/macrophages acutely and chronically infected with human immunodeficiency virus. J. Infect. Dis. **178:**413-22.

11. **Petit, C., F. Buseyne, C. Boccaccio, J. P. Abastado, J. M. Heard, and O. Schwartz.** 2001. Nef is required for efficient HIV-1 replication in cocultures of dendritic cells and lymphocytes. Virology. **286:**225-36.

12. **Rhee, S. S., and J. W. Marsh.** 1994. HIV-1 Nef activity in murine T cells. CD4 modulation and positive enhancement. J. Immunol. **152:**5128-34.

13. **Ross, T. M., A. E. Oran, and B. R. Cullen.** 1999. Inhibition of HIV-1 progeny virion release by cell-surface CD4 is relieved by expression of the viral Nef protein. Curr. Biol. **9:**613-21.

14. **Schrager, J. A., and J. W. Marsh.** 1999. HIV-1 Nef increases T cell activation in a stimulus-dependent manner. Proc. Natl. Acad. Sci. USA. **96:**8167-72.

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

16. **Simmons, A., V. Aluvihare, and A. McMichael.** 2001. Nef triggers a transcriptional program in T cells imitating single-signal T cell activation and inducing HIV virulence mediators. Immunity. **14:**763-77.

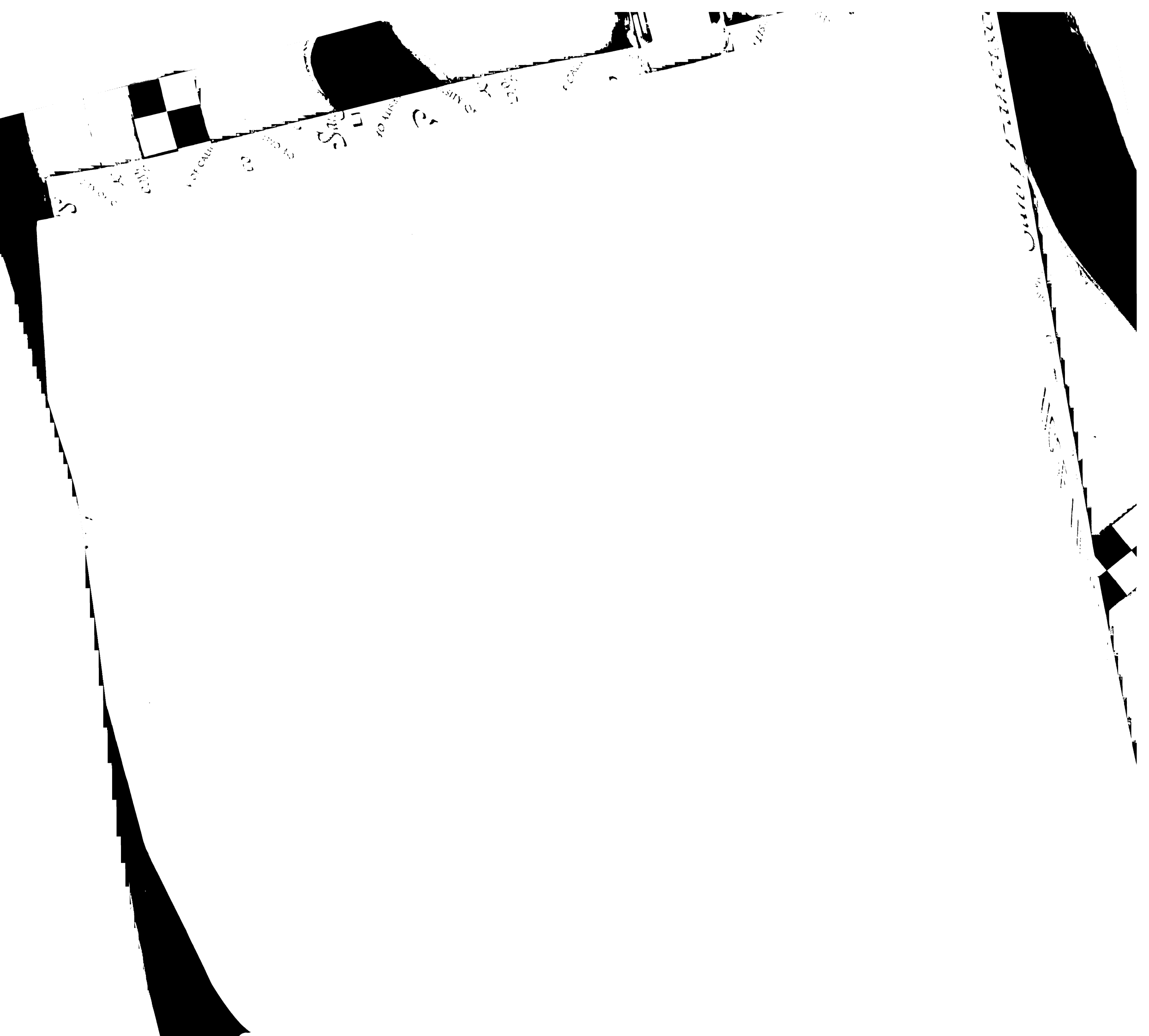
17. **Swingler, S., A. Mann, J. Jacqué, B. Brichacek, V. G. Sasseville, K. Williams, A. A. Lackner, E. N. Janoff, R. Wang, D. Fisher, and M. Stevenson.** 1999. HIV-1 Nef mediates lymphocyte chemotaxis and activation by infected macrophages. Nat. Med. **5:**997-1003.

18. **van't Wout, A. B., L. J. Ran, M. D. de Jong, M. Bakker, R. van Leeuwen, D. W. Notermans, A. E. Loeliger, F. de Wolf, S. A. Danner, P. Reiss, C. A. Boucher, J. M. Lange, and H. Schuitemaker.** 1997. Selective inhibition of syncytium-inducing and nonsyncytium-inducing HIV-1 variants in individuals receiving didanosine or zidovudine, respectively. J. Clin. Invest. **100:**2325-32.

19. **Wang, J. K., E. Kiyokawa, E. Verdin, and D. Trono.** 2000. The Nef protein of HIV-1 associates with rafts and primes T cells for activation. Proc. Natl. Acad. Sci. USA. **97:**394-9.

20. **Wong, J. K., M. Hezareh, H. F. Günthard, D. V. Havlir, C. C. Ignacio, C. A. Spina, and D. D. Richman.** 1997. Recovery of replication-competent HIV despite prolonged suppression of plasma viremia. Science. **278:**1291-5.

21. **Wu, Y., and J. W. Marsh.** 2001. Selective transcription and modulation of resting T cell activity by preintegrated HIV DNA. Science. **293:**1503-6.



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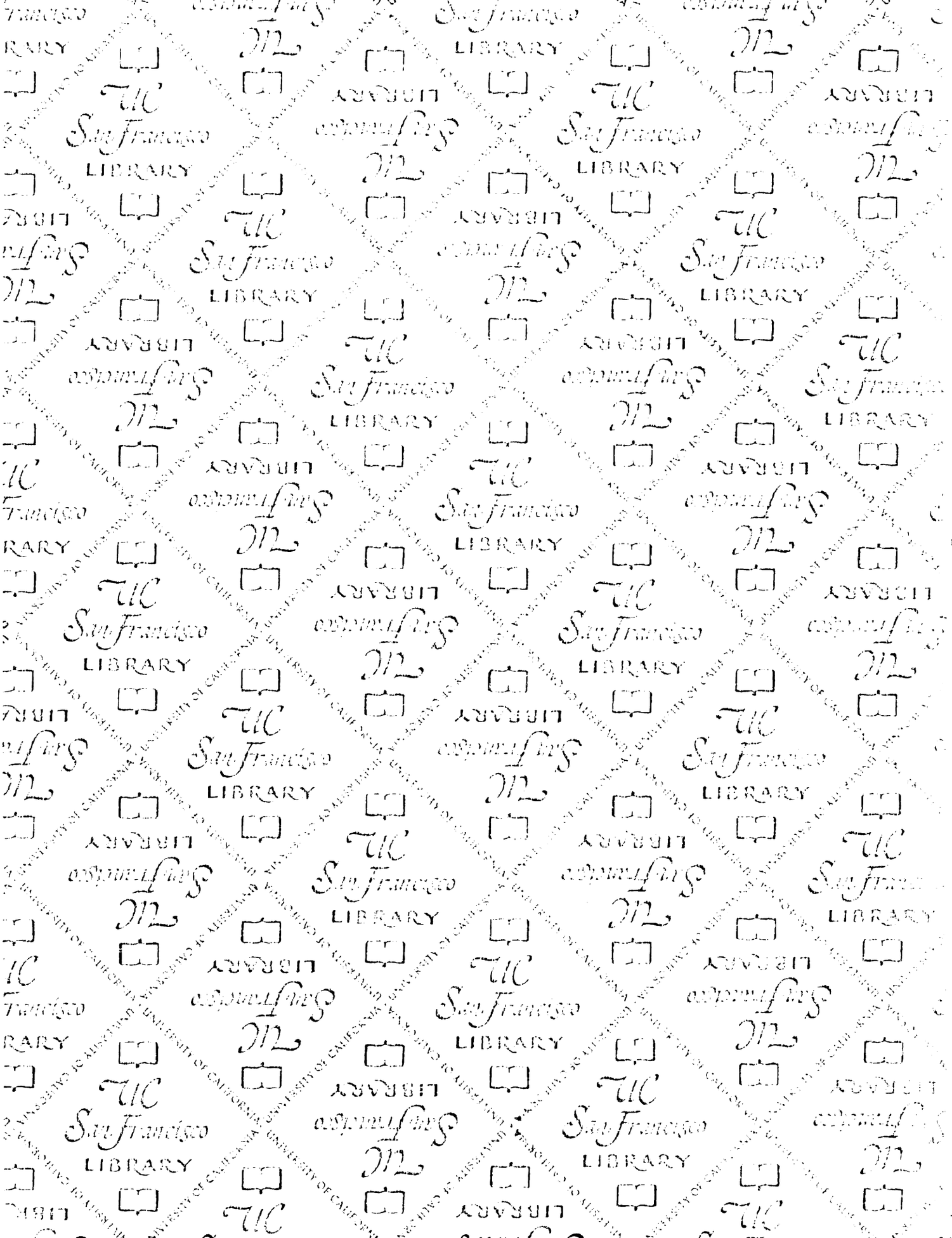
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